

Expression of Elafin in Fallopian Tubes of Ectopic Pregnancies Is Reduced

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Abstract: Elafin is a natural antimicrobial molecule member of the antileukoproteinase (Trappin) family that is normally expressed in the mucosa of human fallopian tubes and neutrophils. Ectopic pregnancy is a condition in which neutrophil influx is present. Current data on elafin expression on fallopian tubes with ectopic pregnancy do not differentiate the expression of elafin in these 2 compartments. The objective of this study was to analyze the protein expression of elafin on epithelial mucosa of fallopian tubes with and without ectopic pregnancy using immunohistochemical analysis. Tissue sections of ectopic pregnancies (n = 10) and normal tubes (n = 10) were analyzed for the intensity of the staining with 3,3'-diaminobenzidine using ImageJ software. Statistical analysis was performed using unpaired *t* test and analysis of covariance. Elafin expression (mean ± SD) in the mucosa of fallopian tubes was 73.3 ± 19.7 (control) versus 48.9 ± 17.8 (ectopic pregnancy) (*P* = 0.009). The immunoeexpression of elafin is reduced in tubal epithelium of ectopic pregnancies, compared with nonectopic pregnancy tubes.

Key Words: elafin, fallopian tube, ectopic pregnancy, immunohistochemistry, ImageJ

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Elafin is a serine protease inhibitor, also known as elastase specific inhibitor, Trappin-2, or skin-derived antileukoprotease.^{1,2} This protein has a low molecular weight, and it belongs to the family of whey acidic protein proteins.³ This secreted protein is produced by epithelial cells and cells of the immune system.⁴ Elafin has been shown to inhibit neutrophil elastase and has an antimicrobial action.⁵ In endometrium, the expression of elafin occurs only during menses⁶; in the mucosa of fal-

lopian tubes, however, its expression is constant independently of the phase of menstrual cycle.⁷

Recently, our group has published that women with hydrosalpinx have a reduced expression of elafin either in the endometrium⁸ or in the fallopian tubes.⁹ Damaged fallopian tubes due to previous infection have a higher risk for tubal pregnancy.¹⁰ Tubal pregnancy is a condition that affects 1% to 2% of all pregnancies.¹¹ The current literature evaluating the relationship between elafin and ectopic pregnancy is scant. After a PUBMED search (Pubmed search: ectopic pregnancy AND elafin, no limits, from 1966 until December 2013, search performed on December 23, 2013), only 4 articles were identified.^{7,12–14} Of these, 2 were review articles.^{13,14} King et al⁷ demonstrated that elafin mRNA and protein expression were upregulated in tubal pregnancy. However, their results must be interpreted with caution. Messenger RNA analyses were made in the whole tissue from ectopic pregnancy, and as this condition has a neutrophil infiltration, it is likely that these high levels of elafin are due to neutrophil infiltrate, and not due to the mucosa of the fallopian tube; the increased expression of elafin during the menstrual period is mainly related to the neutrophils.⁶ In addition, King et al⁷ reported that elafin expression was not increased in OE-E6/E7 oviductal cell line infected with *Chlamydia trachomatis* after 48 hours. Another type of fallopian tube injury, hydrosalpinx, in which neutrophil infiltrate is not present, elafin protein expression is reduced in the mucosa of fallopian tubes.⁹ It is noteworthy that mRNA levels in fallopian tubes with hydrosalpinx do not correlate with protein expression.⁹ Therefore, as ectopic pregnancy is a process that takes >48 hours, we hypothesize that elafin levels in the fallopian tube with ectopic pregnancy would be low. To verify this hypothesis, the protein expression of elafin on epithelial mucosa of fallopian tubes with and without ectopic pregnancy was analyzed by immunohistochemistry using ImageJ software. The importance of this study is justified by a new input in the pathophysiology of ectopic pregnancy and by the potential use of elafin as an adjuvant treatment, as others have reported in heart conditions,¹⁵ or as a prognostic factor.¹⁶

MATERIAL AND METHODS

In this case-control study, formalin-fixed paraffin-embedded tissues were obtained from the archives of the Pathology Service of Hospital de Clínicas de Porto

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Alegre, from Jan 1, 2010 and December 31, 2012, until the desired sample size was reached. An expert pathologist confirmed the diagnosis of ectopic pregnancy and the normality of the tubes of all stained tissues. Tissue samples were obtained from total hysterectomy specimens for benign conditions (leiomyoma and heavy menses). Cases with cancer, unsatisfactory specimen in paraffin blocks, and acute salpingitis were excluded. Patient data, such as age and parity, were obtained from patients' electronic records.

Immunohistochemistry

Immunohistochemical staining was performed as previously reported, using the primary antibody against elafin (mAb TRAB2F — HM2063-0603, Hycult Biotechnology, Udem, The Netherlands) diluted at 1:10.⁹ Briefly, paraffin sections were deparaffinized, rehydrated, and rinsed with phosphate-buffered saline solution (PBS). Slides were incubated in citrate buffer at pH 6 and heated in a microwave for 21 minutes at maximum power. After antigen retrieval with citrate buffer heated in a microwave, each slide was washed with distilled water, followed by PBS. Endogenous peroxidase was blocked with 5% H₂O₂ in distilled water, and nonspecific sites were blocked with 5% powdered skim milk in PBS. After rinsing the slides with distilled water and PBS, slides were incubated with primary antibody against elafin, diluted in PBS 1:10 for 1 hour at 22°C in a humid chamber. After rinsing with PBS, slides were incubated with a secondary antibody (LSAB2; Dako, Glostrup, Denmark). Detection of the primary antibody was performed using the Strepto ABC, LSAB2 system (Dako), according to the manufacturers' instructions, using diaminobenzidine (DAB) as a chromogen. Negative external controls were obtained by using a nonspecific primary antibody (MAB1435- EMD; Millipore Co., MA), of the same class, in the same concentration as the primary antibody. Human tonsil tissue, a known elafin-positive specimen, was used as an extra positive external control. Stained sections were analyzed under optical microscope (Olympus BX51 microscope; Olympus Optical Co., Tokyo, Japan) connected to a digital color camera/Q-Color 5 (Olympus). When necessary, multiple pictures were taken from the whole slide to perform immunohistochemical analysis. Images were obtained with a ×4 objective UPLanFI (resolution: 2.75 μm), at a size of 2560 × 1920 pixels (resolution: 1 mm = 590 pixels), under standard conditions.

ImageJ Analyses

Image analysis was performed by 1 of the authors (M.G.). To reduce bias, each slide was coded and blindly analyzed with a specific image analysis software (ImageJ v1.43j; National Institutes of Health, Bethesda, MD, available at <http://rsbweb.nih.gov/ij/>). To confirm normal distribution of elafin along the extension of the fallopian tube, the extension from the ampulla until the isthmic portion of the tube was analyzed. Areas of interest consisted in a representative transversal section of the epithelial mucosa of the normal fallopian tube. The region of

interest (ROI) in cases of ectopic pregnancy was the epithelial mucosa of the fallopian tube, with or without the presence trophoblastic tissue, a sign of ectopic pregnancy. Only the epithelial mucosa was selected for image analysis. The muscle layer and the complex of the ectopic pregnancy inside the tube and the serosa of the fallopian tube were not analyzed. After selecting the epithelial mucosal area inside the lumen of the fallopian tube, either from normal tubes or from ectopic pregnancy, the selected image (ROI) was submitted to the analytical procedure. The analytical procedure, named "color deconvolution," is written as a built-in "plugin" for ImageJ using the hematoxylin and DAB built-in vector.¹⁷ Analysis was performed as previously described.⁹ Briefly, from the 3 images obtained from "color deconvolution," the picture with hematoxylin was selected and converted into a binary picture (image → adjust → threshold). This binary picture was converted into a "selected area" (edit → selection → create selection). This selected area was added and saved as the ROI (ROI manager). The picture with DAB intensity was selected, and the "selected area" saved as an ROI file was overlaid on the image. The software measured the mean DAB immunostaining intensity and the total area from the ROI (Fig. 1). When multiple pictures were taken from the same specimen, the average intensity of DAB was calculated. The final DAB intensity was calculated according to the formula: $f = 255 - i$, where f = final DAB intensity, i = mean DAB intensity obtained from the software. The final DAB intensity varied from 0 (white, no expression) to 255 (dark brown, highest expression).

Ethics, Sample Size Calculation, and Statistical Analysis

Ethical approval for this study was obtained from Comissão Científica e o Comitê de Ética em Pesquisa do Hospital de Clínicas de Porto Alegre, under protocol 13-0107. Sample size for immunohistochemical analysis was calculated according to the formula: $n = [(z\alpha + z\beta)^2 \times 2(s)^2] / (d)^2$, as described in the literature,¹⁸ using the following parameters: an alpha error ($z\alpha$) = 0.05, power ($z\beta$) = 0.8, an estimate SD of elafin of 18, thus variance $(s)^2 = 324$, and a difference (d) of 25 points in a scale ranging from 0 to 255 (ie, a difference of 10%). The variance of elafin was obtained from a pilot study with 18 cases of normal tubes. These figures yielded a sample size of at least 8 cases in each group.

GraphPad Prism version 6 for Macintosh (GraphPad Software Inc., San Diego, CA) was used for statistical analysis, using the unpaired Student t test with Welch correction to compare the expression of elafin (final DAB intensity) in the tubal epithelium of the 2 groups, if data had a Gaussian distribution and different SDs. Gaussian distribution was calculated with the D'Agostino & Pearson omnibus normality test. Association between categorical variables was tested with the Fisher exact test. The Mann-Whitney nonparametric test was used for discrete variables. Analysis of covariance (ANCOVA) (SPSS - IBM SPSS Statistics for Windows,

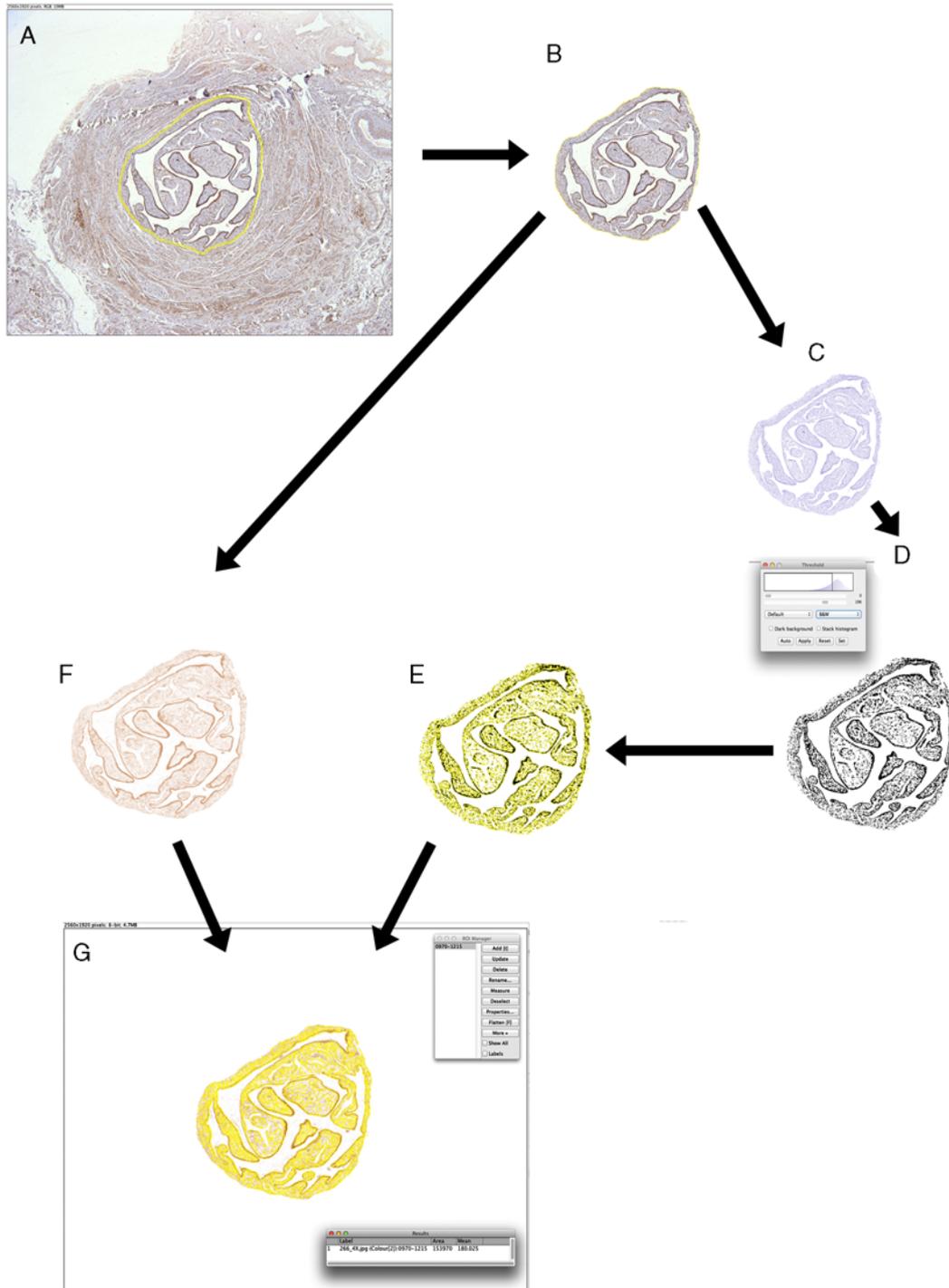


FIGURE 1. Representation of ImageJ use for elafin expression in a selected area of the fallopian tube. A, Segment of the fallopian tube to be analyzed. The luminal area is selected (yellow line). B, The luminal area is isolated. C, “Color deconvolution” split the original image into 3 panels; (C) hematoxylin only staining, (F) diaminobenzidine (DAB) staining and background (not shown). D, Hematoxylin is converted into a binary picture. E, The binary picture is converted into a “mask” (yellow). G, The “mask” obtained from (E) is overlaid on panel with DAB staining (F). G, The software calculates the area and the DAB staining intensity.

Version 19.0. Armonk, NY: IBM Corp.) was used to correct the difference of age between groups. A $P < 0.05$ was considered significant.

RESULTS

Twenty samples were obtained (10 ectopic pregnancies; 10 controls). Demographics of the sampled population

TABLE 1. Demographics of the Sample

Characteristics	Normal Tubes (n = 10)	Ectopic Pregnancy (n = 10)	P
Age (mean ± SD) (y)	39 ± 7.7	30.3 ± 3.7	0.006*
Menarche (y) (mean ± SD) (y)	13.4 ± 1.3	12.5 ± 1.2	0.1*
Skin color: White/black	6/4	9/1	0.3†
Pregnancies [median (range)]	0.5 (0-4)	1 (0-3)	0.07‡

*Student *t* test with Welch correction.

†Fisher exact test.

‡Mann-Whitney test.

are described in Table 1. All values for DAB intensity passed the D'Agostino & Pearson omnibus normality test, and the unpaired *t* test with Welch correction was used. There was no significant difference between the measured areas in both groups (data not shown). Elafin protein expression was significantly reduced in the mucosa of fallopian tubes with ectopic pregnancy compared with controls (Figs. 2D–G). Its expression was consistently reduced in cases of ectopic pregnancy throughout the Fallopian tube, either at the site of ectopic implantation or in the normal section of the oviduct (Figs. 2F, G). The mean intensity of elafin (mean ± SD) in the mucosa of fallopian tubes was 73.3 ± 19.7 in controls versus 48.9 ± 17.8 in ectopic pregnancy (Fig. 3) (*P* = 0.009).

ANCOVA analysis was conducted to identify whether age between groups had influence on elafin expression. Elafin expression was the dependent variable; fixed factors were the groups, and age was the covariate. The Levene test of equality of error variances was 0.382. After running ANCOVA analysis, age was adjusted, and the *P* value between groups was 0.013, confirming that elafin expression was significantly different, despite the age difference between groups.

DISCUSSION

The most important function attributed to elafin is the protection of tissues against large proteolysis through serine proteinases.^{19,20} The present study was aimed to investigate whether the expression of elafin was increased or reduced in epithelial mucosa of fallopian tubes with and without ectopic pregnancy. An increased expression of elafin would be expected as a physiological response to the invasion of the trophoblastic tissue into the fallopian tube. In contrast, a reduced expression of elafin may suggest that an abnormal innate immune system preexists and predisposes to ectopic implantation. Herein, we verified that levels of elafin were lower in the epithelial mucosa of fallopian tubes with ectopic pregnancy, as opposed to controls. An excess of neutrophil elastase could explain this reduction, as Guyot et al²¹ have reported. Nevertheless, King and colleagues found opposite results from ours. Using 6 cases of fallopian tubes with ectopic pregnancy, these authors showed that elafin mRNA and protein levels in epithelial mucosa with ectopic pregnancy were increased. The possible explanation for this discrepancy could be related to the

expression of elafin in the neutrophils that are presented in the ectopic pregnancy complex (Fig. 2E1). Analysis using the whole tissue would probably yield a false up-regulated expression of elafin, most likely originated from neutrophils, a cell population that is constantly renovated. Indeed, King et al,⁶ in another publication, reported that the high levels of elafin during the menstrual phase were predominantly due to endometrial neutrophil infiltrate. Another finding to support that elafin levels are reduced in epithelial mucosa is provided by an in vitro model of elafin regulation, published by King and colleagues. Using an oviduct cell line infected with *C. trachomatis*, elafin expression was upregulated in the first 24 hours, but after 48 hours, elafin expression was not increased.⁷

We are not able to explain the difference in the immunohistochemical results between our results and those published by King et al,⁷ because detailed data on the immunohistochemical expression of elafin were not presented. It is noteworthy that the reduced expression of elafin in the mucosa of fallopian tubes with ectopic pregnancy was over the whole extension of the fallopian tube (ie, ampulla and isthmus; Figs. 2C, D), even in the absence of the trophoblastic tissue (Fig. 2C). With this finding we postulate that reduced innate mucosal defenses, which prevent tubal infections, may predispose to, rather than be a consequence of, tubal pregnancy, as has been hypothesized by others.¹³ This reduction may be a response to postinflammatory mediators. Our data on reduced expression of elafin in fallopian tubes with hydrosalpinx support this hypothesis.⁹

Positive aspects of our study include sample size calculation and the use of the ImageJ software to quantify DAB intensity. Instead of other semiquantitative methods, such as HSCORE or number of “+,” computer analysis provides a nonsubjective indirect estimation of the amount of antigen present. Moreover, with ImageJ software the area of interest could be selected without the “contamination” of the *muscularis* of the fallopian tube, or the neutrophils presented in the ectopic complex (Fig. 2E1). Furthermore, the use of ImageJ increases objectivity, reproducibility, and calculation of the mean expression of DAB.²² Quantification of the area analyzed did not show a significant difference between groups (data not shown), therefore it does not seem that the difference found was related to the amount of tissue studied. Another aspect is that the difference found

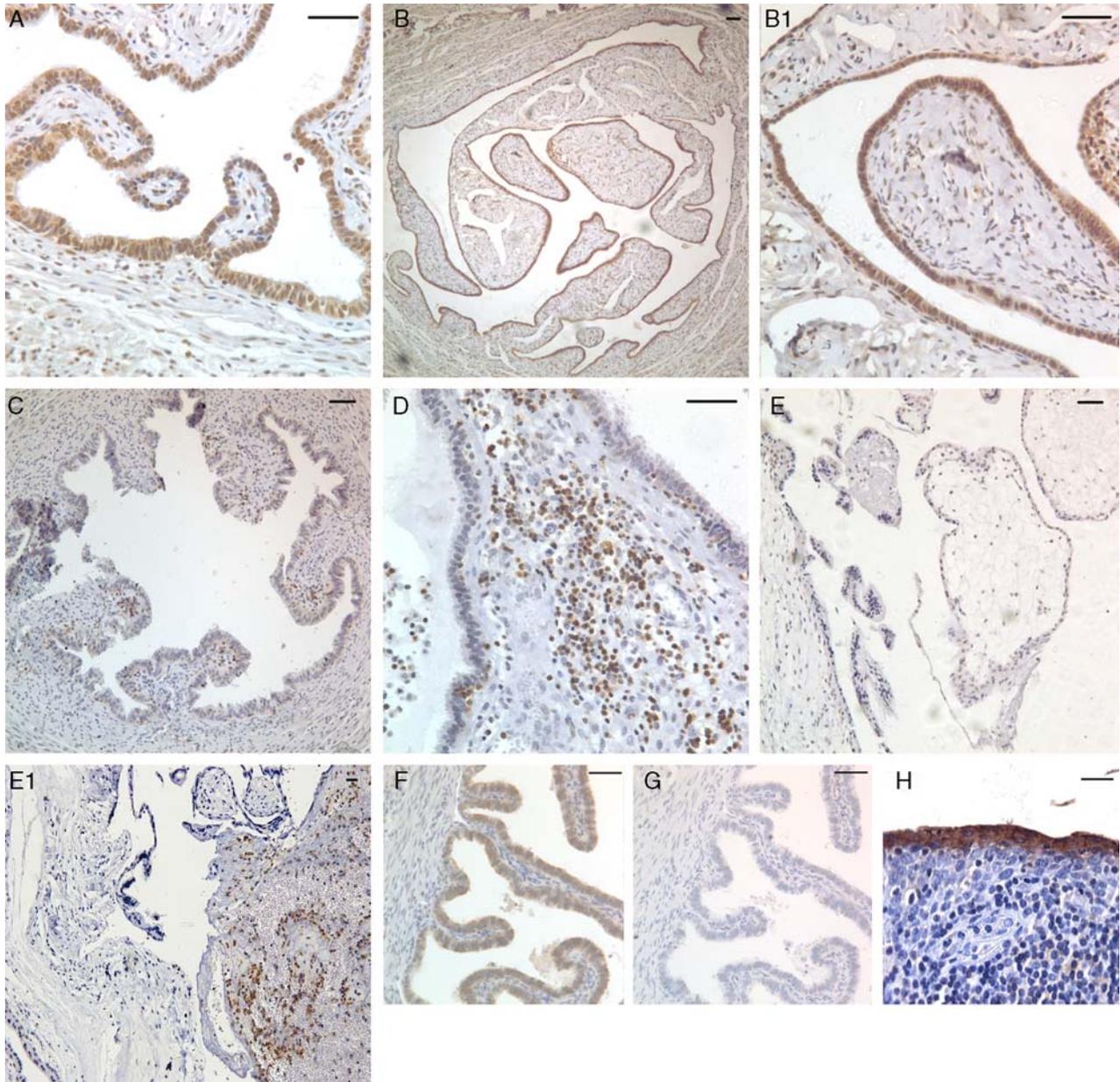


FIGURE 2. Images show representative images of tissue sections in controls and cases of ectopic pregnancy. Note that expression of elafin is constant across different segments of a normal tube (A, ampulla; B, isthmus; B1, isthmus in higher magnification). In tubes with ectopic pregnancy, elafin expression in the mucosa is uniformly reduced, either in the isthmus (C), or in the ampulla (D). Neutrophils are stained inside the villi, serving as positive internal control (D). E, At the implantation site no expression of elafin is seen. E1, Neutrophils are observed in great amount in the ectopic pregnancy material, but not in the mucosa of the fallopian tube. F, Positive control with elafin antibody. G, Negative external control with unspecific antibody. H, Tonsil as second positive external control. Bars represent 50 μm . full color online

in elafin expression was present after age correction with ANCOVA.

There are some limitations that must be addressed in this case-control analysis. The use of laser capture to isolate the epithelial mucosa of the fallopian tube would probably overcome the “contamination” issue caused by the neutrophils. Nonetheless, we have shown that the

protein and mRNA expressions of elafin do not correlated.⁹

In summary, the immunoexpression of elafin is reduced in the tubal epithelium of ectopic pregnancies, compared with nonectopic pregnancy tubes. If the reduced expression of elafin is confirmed by other studies, a new venue is open to investigate the local levels of this

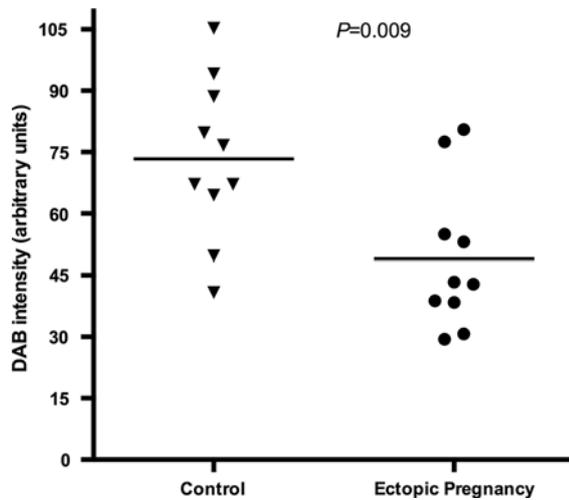


FIGURE 3. Graphical representation of DAB (diaminobenzidine) intensity between groups. Bars represent the mean.

protein as a risk factor for ectopic pregnancy, or to use recombinant elafin to control unwanted proteolysis at inflammatory sites, as shown by others.²³

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