

Vasoactive intestinal Peptide/Pituitary Adenylate Cyclase activating polypeptide and their receptors in tubal ectopic pregnancy and missed abortion

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide (VIP)/secretin/ glucagon peptide family and functions in reproductive system relative to follicular maturation, ovulation, fertilization, and successful pregnancy.

The aim of this study is to investigate if PACAP, VIP and their receptors (PAC1 and VPAC2) expression in the subepithelium of oviduct are have an important role of ectopic pregnancy (EP) and missed abortion (MA) pathogenesis.

The study was conducted on women with normal oviduct (NonP) (n=7), EP (n=10) and MA (n=10). PACAP, VIP, PAC1 and VPAC2 antibodies were used for immunostaining and their expressions were evaluated by HSCORE technique.

This study showed that PACAP, VIP and their receptors (PAC1 and VPAC2) were expressed in the subepithelium of oviduct on all groups. The NonP group showed a higher PACAP and expression in the subepithelium of NonP than EP and MA group. However, there was no differences PAC1 and VPAC2 expression in the subepithelium of oviduct of NonP, EP and MA group.

In conclusion, the expression of PACAP, VIP, PAC1 and VPAC2 expression in oviduct from women related to missed abortion and ectopic pregnancy. The differential expressions and localizations of these proteins suggest their effective roles in ectopic pregnancy and missed abortion, especially in subepithelium regions.

Keywords: Ectopic pregnancy, missed abortion, PACAP, VIP

Introduction

The oviduct is an essential component of the reproductive system in female that undergoes specific changes in morphology and endocrinology to create an optimized microenvironment for sperm capacitation, fertilization, embryonic development, embryo transport and implantation process (Buhi et al, 2000; Isaac and Sherwood, 2008). Oviductal disorders lead to early pregnancy which is known to be the most common complication of pregnancy where roughly 25% of recognized pregnancies end with missed abortion (MA) and leads to an ectopic pregnancy (EP) (1% to 2%).

Pituitary adenylate cyclase activating polypeptide (PACAP) belongs to the vasoactive intestinal peptide (VIP)/secretin/glucagon peptide family and was discovered from the hypothalamus (Brubel et al, 2010). PACAP exerts its effect through class II G-protein-coupled receptors. The specific PACAP receptor is called PAC1 which binds VIP with much less affinity while VPAC1 and VPAC2 receptors have similar high affinity for VIP and PACAP (Laburthe et al, 2002; Laburthe et al, 2007; Lutz et al, 1999; Muller et al, 2007; Vaudry et al, 2009). PACAP is widely distributed throughout the entire body including the female reproductive organs (Steenstrup et al, 1995; Ko et al, 1999; Reglodi et al, 2012b; Köves et al, 2014; Csanaky et al, 2014).

It is known that the PACAP functions in reproductive system relative to follicular maturation, ovulation, fertilization, and successful pregnancy (Bukowski et al, 2015). As described previously (Reglodi et al, 2012a) there has been a decreased fertility in PACAP knocked out mice. This is in part due to impaired implantation (Isaac and Sheerwood, 2008; Koh et al, 2003) as well as other deficiencies described in mice lacking endogenous PACAP (Reglodi et al, 2012a).

There are two types of PACAP (PACAP1-38 and PACAP1-27) and their receptors have

been found in the human pregnant uterus and placenta (Koh et al, 2005; Scaldeferri et al, 2000). PACAP has been shown to cause a concentration-dependent relaxation on stem villi and intramyometrial arteries suggesting a vasoregulatory role in the uteroplacental unit (Steenstrup et al, 1996). It has been suggested that PACAP plays a role in decidualization and the time-related localization of endometrial-uterine. PACAP has also been implicated in facilitation of endometrial blood flow (Spencer et al, 2001). However, little is known about the expression of PACAP, VIP and their receptors (PAC1 and VPAC2) in the subepithelium of oviduct relative to follicular maturation, ovulation, fertilization, and successful pregnancy. Thus, the aim of this present study was to investigate the role of expression of PACAP, VIP and their receptors (PAC1 and VPAC2) in subepithelium of oviduct on missed abortion (MA) and ectopic pregnancy (EP).

Materials and methods

This prospective study was conducted in Merkezefendi State Hospital, Department of Obstetrics and Gynecology. It was approved by Ethics Committee of Celal Bayar University. All the participants were informed and they gave their consent. Tubal tissue samples were obtained from 10 non-pregnant women during tubal ligation in luteal phase from isthmus of the fallopian tube (NonP group), 10 women with ectopic pregnancy during ampullary salpingectomies (EP group) and 10 women with missed abortion (MA group). All participants had at least one live-born child. As described above, the obtained tubal tissue samples were fixed with suitable fixatives for immunohistochemical procedures.

Immunohistochemistry

Formaline-fixed and paraffin-embedded tuba uterina sections were used for

immunohistochemical staining. Tissue samples were stored at 60°C overnight and then dewaxed with xylene for 30 min. After dehydration process of the sections with ethanol, they were washed with distilled water. Subsequently, the samples were treated with 2% trypsin (ab970, Abcam, Cambridge, UK) at 37°C for 15 min and incubated in 3% H₂O₂ solution for 15 min to inhibit endogenous peroxidase activity. Then, the sections were incubated with Anti-PACAP Primer Antibody (cat no: ab183103), Anti-VIP Primer Antibody (cat no: NBP1-33433), Anti-PAC1 (cat no: LS-A945), Anti-VPAC2 (cat no: ab28624) monoclonal antibody in a 1/100 dilution for 18 h at +4°C. They were then given an additional three ,5-min washes in PBS, followed by the incubation with biotinylated Ig G and administration of streptavidin peroxidase (Histostain Plus kit Zymed 87-9999; Zymed, San Francisco, CA). After washing, the secondary antibodies three times with PBS for 5 min, the sections were stained with DAB substrate system containing diaminobenzidine (DAB, K007, DBS, Pleasanton, CA, USA) to detect the immunoreactivity. Subsequently, the samples were stained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany) for counterstaining. Finally, they were covered with a mounting medium (01730 Surgipath, Cambridge, UK) and observed with a light microscopy (Olympus BX-43, Tokyo, Japan).

Immunostaining for PACAP, VIP, PAC1 and VPAC2 expressions in the tuba uterina samples of each group were evaluated semiquantitatively using H-SCORE analysis. The immunostaining intensities were categorized by the following scores: 0 (no staining), 1 (weak, but detectable, staining), 2 (moderate staining), and 3 (intense staining). A H-SCORE value was derived for each specimen by calculating the sum of the percentage of cells for epithelium, subepithelium, vessel and muscle of tuba

uterina that stained at each intensity category, multiplied by its respective score, using the formula $H\text{-score} = \sum P_i (i+1)$, where i is the intensity of staining with a value of 1, 2 or 3 corresponding to weak, moderate or strong staining, respectively. P_i is the percentage of stained cells for each intensity, varying from 0 to 100%. For each slide, five different fields were evaluated microscopically at 200x magnification. H-SCORE evaluation was performed independently by at least two investigators (K O, E T U, H B) who had no knowledge about the source of the samples as well as to each other's results; the average score of both was then used.

Statistical Analysis

For the statistical analysis, the software SPSS (15.0) was used. The numerical variables are given with mean, standard deviation, median, minimum and maximum values. The Kruskal-Wallis test was used for comparisons of the five groups as the variables do not fit the normal distribution curve and the Mann-Whitney U test was used with Bonferroni correction for post-hoc tests. Statistical comparisons with a p-value below (0.05/10=0.005) are assumed as statistically significant for the immunostaining evaluation and also P-value below (0.05/3=0.016) is assumed as statistically significant for the demographic data.

Results

We observed in the H&E staining tuba uterina slides of control group, exhibits relatively thin longitudinal folds that project into the lumen throughout its length. The epithelium lining of tuba uterina is simple columnar with two population cells- ciliated and nonciliated cells. The lamina propria has connective tissue cells and fibers. The muscularis layer is composed of a thick inner layer of circularly arranged fibers and an outer layer of longitudinal fibers. The serosa

is thin layer of connective tissue covered by mesothelium (Data are not shown.).

In the examination of the epithelium with PACAP antibody, the immunoreactivity of MA group was higher than NonP and EP groups. When examined, the subepithelial regions, PACAP immunoreactivity was observed higher NonP group than MA and EP groups, and a statistically significant difference was determined. There were no differences the PACAP immunoreactivity of the muscle layer among the groups. The immunoreactivity on PACAP on the vessels of tuba was weak in the EP group than NonP and MA groups (Figure-1). Statistical results of the groups' H-Scores of immunohistochemical staining for PACAP was shown in Table-1.

When examining the VIP immuno-reactivity, there were no differences on the epithelium of the all groups. However, in the subepithelium VIP immunoreactivity was strong both NonP and EP groups than MA group. In the muscle layer, there was no any differences among the groups. The VIP immunoreactivity on vessel of the NO and MA groups were strong than the EP group

(Figure-2). Statistical results of the groups' H-Scores of immunohistochemical staining for VIP was shown in Table-2.

The PAC1 immunoreactivity on the epithelium of EP and MA groups were increased compared the NonP. In the subepithelium and vessel, PAC1 immunoreactivity was similar on the all groups. However, In the EP group the PAC1 immunoreactivity was weak than NonP and MA groups (Figure-3). Statistical results of the groups' H-Scores of immunohistochemical staining for PAC1 was shown in Table-3.

The VPAC2 immunoreactivity was similar almost all group the tuba uterine tissue samples but only on the vessel the VPAC2 immunoreactivity was different. In the EP group, the VPAC2 immunoreactivity was weak than the other groups (NonP, MA) (Figure-4). Statistical results of the groups' H-Scores of immunohistochemical staining for VPAC2 was shown in Table-4.

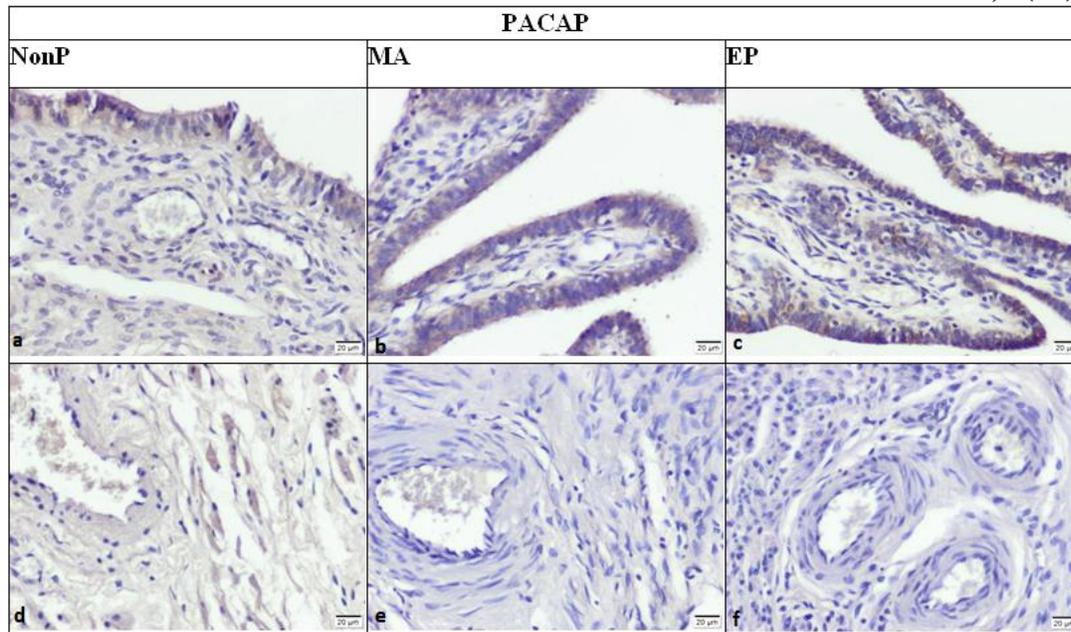


Figure-1. The expression of PACAP in epithelium, subepithelium, muscle and vessel regions of NonP, MA and EP groups. Immunoreactivity to PACAP was mainly found in epithelium of all groups (a-c). The epithelium of MA group showed higher PACAP expression compared with NonP group (b) and the subepithelium of MA and EP group showed lower PACAP expression compared with NonP group (d-f). Moreover, the EP group showed decreased PACAP expression in vessel region (f). Magnification: x200.

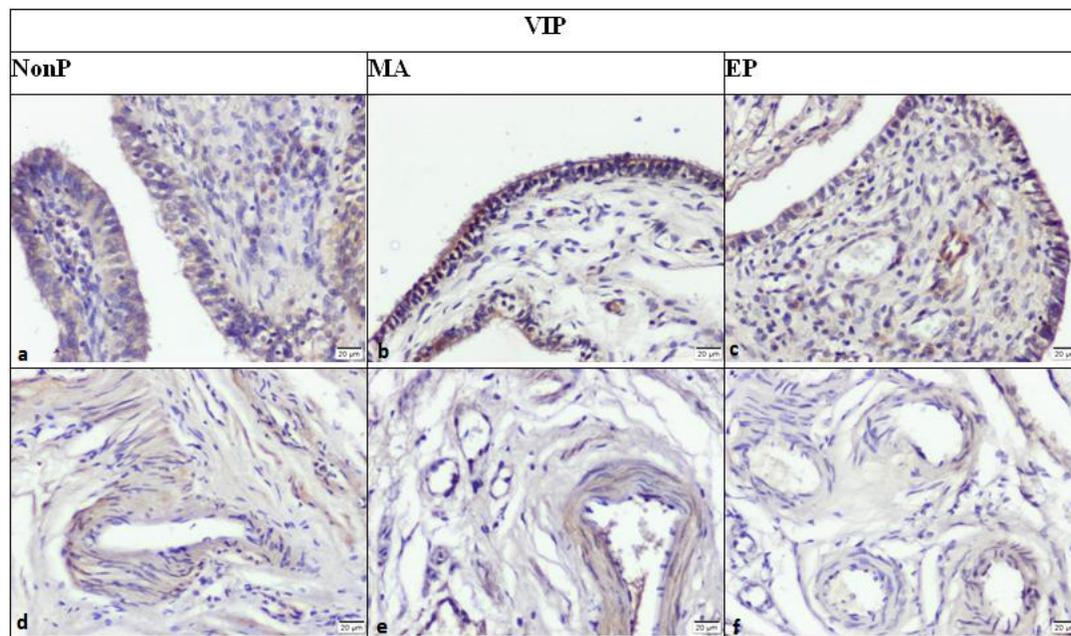


Figure-2. The expression of VIP in epithelium, subepithelium, muscle and vessel regions of NonP, MA and EP groups. Immunoreactivity to VIP was mainly found in epithelium of all groups but there is no difference between groups, statistically (a-c). The subepithelium of MA group showed lower VIP expression compared to other groups (a-c). EP group showed decreased VIP expression in vessel region (f). Magnification: x200

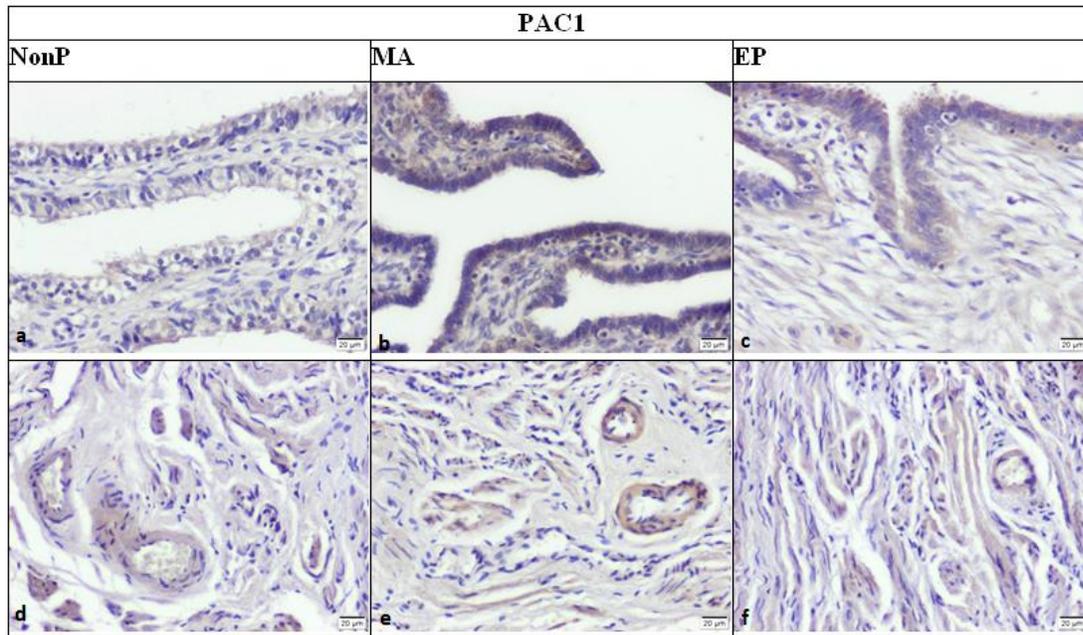


Figure-3. The expression of PAC1 in epithelium, subepithelium, muscle and vessel regions of NonP, MA and EP groups (a-f). Immunoreactivity was found in all regions of all groups but the subepithelium of MA and EP groups showed higher PAC1 expression (a-c). In the other regions, there were no difference between groups, statistically. Magnification: x200

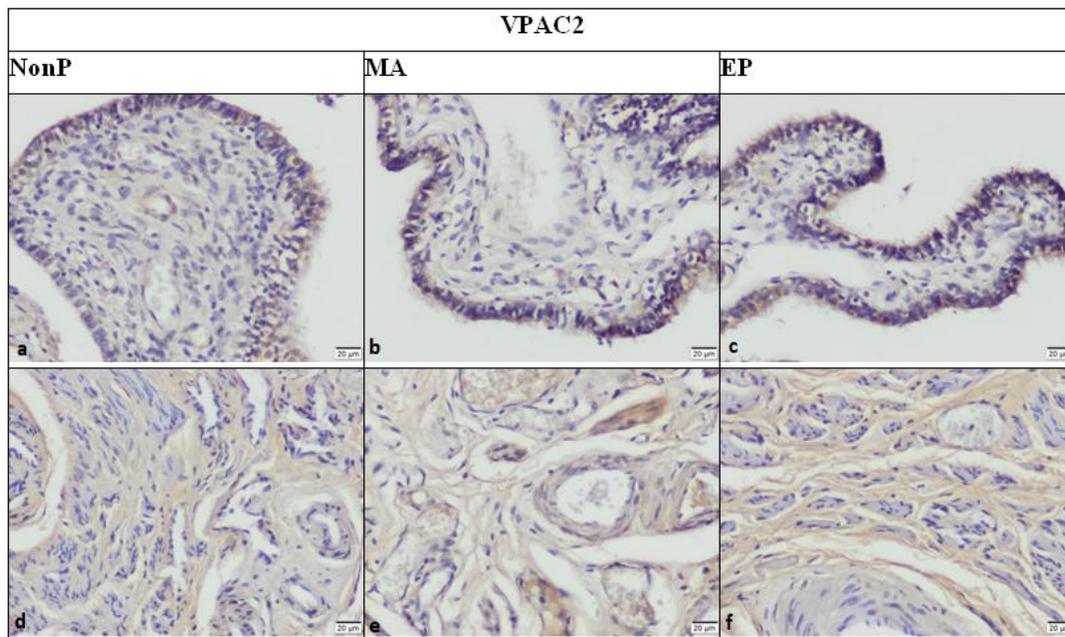


Figure-4. The expression of VPAC2 in epithelium, subepithelium, muscle and vessel regions of NonP, MA and EP groups (a-f). All regions of all groups showed VPAC2 expression (a-f). The VPAC2 expression decreased in vessel region of EP group, statistically (d-f). Magnification: x200

Table-1: The groups' H-Scores of immunohistochemical staining for PACAP antibody for each region.

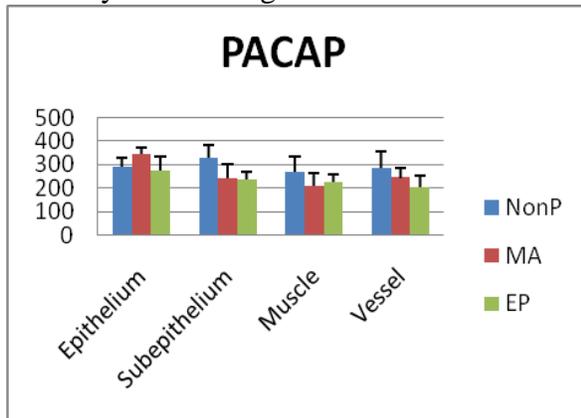


Table-4: The groups' H-Scores of immunohistochemical staining for VPAC2 antibody for each region.

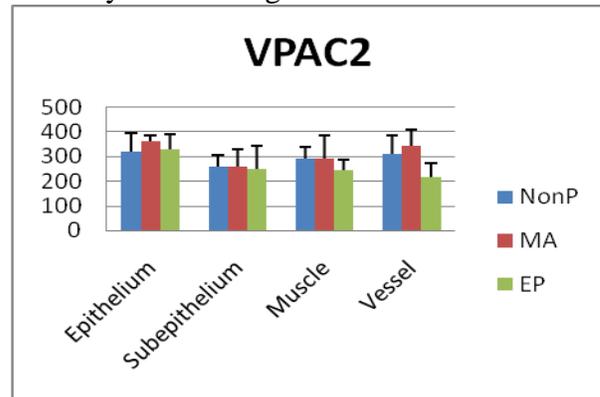


Table-2: The groups' H-Scores of immunohistochemical staining for VIP antibody for each region.

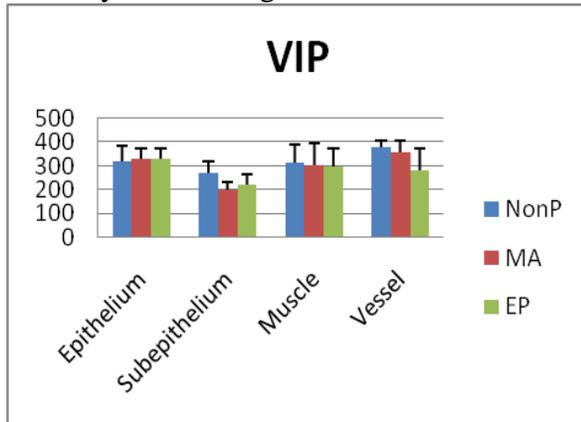
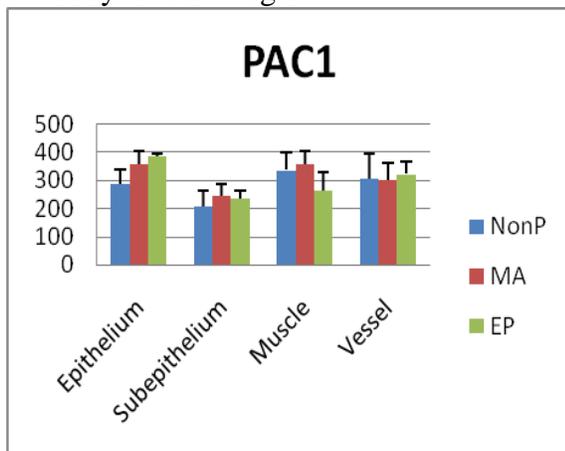


Table-3: The groups' H-Scores of immunohistochemical staining for PAC1 antibody for each region.



Discussion

PACAP is a pleiotropic substance having a broad spectrum of biological functions; the peptide can act as a hormone, neurohormone, autocrine/paracrine substance, neurotransmitter, neuromodulator, neurotrophic factor and immuno-modulator. In this study, we showed that PACAP, VIP and their receptors (PAC1 and VPAC2) were expressed in the subepithelial layers of the oviducts in the NonP, MA and EP groups whereas PACAP and VIP expression were specifically found in subepithelial of NonP group. However, a significant loss of PACAP and VIP expressions were detected in the subepithelium of MA and EP groups.

PACAP is one of the most important regulatory peptides in the reproductive system, and PACAP and the PACAP-specific receptor, PAC1, are detected within granulosa, thecal and corpora luteal cells (Moretti et al, 2012) and also present in human follicular (Brubel et al, 2010). In this study, we observed the different levels of the PACAP expressions in the oviduct subepithelium of NonP, MA and EP groups and we thought that PACAP could be considered as having the embryo implantation, uterine decidualization and also MA and EP pathogenesis.

PACAP, as well as their receptors, PAC1, have been found in the human pregnant

uterus and placenta (Koh et al, 2005; Scaldeferri et al, 2000) and PACAP mRNA levels in the gravid uterus and placenta during peak embryonic development on gestation days 9-15 (Spencer et al, 2001). Isaac and Sherwood (2008) reported that implantation was the main defect in mice lacking PACAP and indicate the decrease in implantation following a single mating event with a male, as only 13% had implanted embryos at 6.5 days following mating. It was claimed that the effect may have related with decrease of progesterone levels, because PACAP may stimulate the corpus luteum and progesterone secretion. The PACAP $-/-$ mouse were normal ovulated and fertilized however this reduced fertility due to decreased progesterone receptor A and prolactin receptor. However, RU 486 (anti-progesterone) reduced the uterine PACAP mRNA expression on day 9 of pseudopregnancy and gestation (Spencer et al, 2001).

In humans, the decidualization is an essential component of implantation and subsequent embryonic development. Spencer et al (2001) are reported that PACAP is expressed in the rat endometrium during decidualization and claimed that PACAP may enhance vasodilator dynamics and facilitate access to the increased nutritional demands that are required for endometrial transformation during decidualization, as well as for placental formation and embryonic development during pregnancy. The decidualization of the uterine stromal cells was accompanied by increased secretions of progesterone, decidual prolactin, prostaglandin E2 and cAMP (Telgmann and Gellersen, 1998).

PACAP immunoreactivity in the oviduct subepithelium is low in the EP group than the NonP group. The decreased expression of PACAP in the subepithelium of oviduct in the EP group may be related with EP pathogenesis. In this condition, tubal transport delays the passage of the embryo

along the fallopian tube which results in early implantation (Shaw et al, 2010). The role of PACAP in oviduct is not known completely but we speculate that PACAP in the subepithelium may affect the epithelial ciliary movement. However, Mnkkönen et al (2008) reported that PACAP reduced ciliary beat frequency in the rat ependymal cilia on culture condition. Moreover, it is known that galectin-3 is a centrosome-associated protein which leads to abnormal morphology of primary cilia in renal epithelial cells once it is knocked out (Koch et al, 2010). Nio-Kobayashi et al (2014) reported that the percentage of epithelial cells expressing galectin-3 in cilia in the fallopian tube tended to be reduced ($p = 0.0685$) with an accompanying loss of a normal ciliary structure while the nuclear galectin-3 increased ($p < 0.05$) in ectopic pregnancies.

We observed the presence of PACAP immunoreactivity in the oviduct subepithelium of MA group but low than the NonP group. The statistically significant decrease of the PACAP expression in MA may related with MA pathogenesis. However, presence the PACAP expression in the subepithelium may prevent to abortion PACAP is known to enhance survival many cell types such as retinal cells (Atlasz et al, 2014) and trophoblast cells (Horváth et al, 2014). PACAP is also enhance the invasive ability of trophoblast and enhance the angiogenesis in placenta. (Horváth et al, 2014; Ozcakil et al, 2015).

In this study, VIP and VPAC 2 immunoreactivity were seen in subepithelium of NonP, MA and EP groups indicating the idea that VIP has a very important role in tubal functions. Fraccaroli et al (2016) reported that VIP induces decidualization associated with the expression of decidualization markers. VIP also displays an immunomodulatory role in this process since VIP decidualized cells were able to control the immune microenvironment by conditioning DC to a tolerogenic profile

similar to the positive control of differentiation. VIP emerges as a key regulator factor with immunomodulatory effects on maternal leukocytes, for example inducing Tregs through a mechanism involving TGF- β 1 the maternal–fetal interface recruits immunoregulatory Treg cells “helping to form an immune tolerant microenvironment (Gallino et al, 2016).

Previous studies performed on the rabbit oviduct indicated that there was a discrete presence of VIP was found in the ciliated cells and in the muscle layer, in both ampulla and isthmus (Menghi et al, 1990). There is no evidence about the role of VIP in the oviduct in patients who has ectopic pregnancy and miscarriage however our results showed that VIP statistically reduced in the subepithelium of EP groups. The oocytes after fertilization were stayed in the oviduct for 2 to 3 days and then rapidly transferred through the isthmus into the uterine cavity. A sphincter-like mechanism which is found in the isthmus part of the oviduct is controlled by VIP-containing nerve fibers (Helm et al, 1981). It was known that VIP in vitro reduces tubal motor activity and produces pronounced relaxation of the isthmus sphincter. However, the decrease of VIP immunoreactivity in the subepithelium of EP group may lead to ischemic contraction and thus blastocyst can't reach the uterus.

MA may occur due to chromosomal anomalies, hormonal problems, uterine abnormalities, infections and autoimmune disorders but the etiology of MA is not fully understood. In addition to this, Cao et al (2014) reported that CD4+CD25+ Treg cells play a pivotal role in MA pathogenesis. According to our results, VIP immunoreactivity was diminished in the missed abortion group than the NonP group. A difference in the frequency of CD4+CD25+ Treg cells in the peripheral blood of MA patients and normal early pregnancy and non-pregnant subjects was demonstrated; normal pregnant patients

demonstrated an expansion of CD4+CD25+ cells at the periphery compared with non-pregnant subjects. Furthermore, significantly lower frequencies of Treg were found in MA patients. Analyses have suggested that decreasing levels of Treg cells indicate that they have a role in maternal alloantigen tolerance during pregnancy.

In conclusion, here in we describe the expression of PACAP, VIP, PAC1 and VPAC2 expression in fallopian tubes from women related to healthy pregnancy, missed abortion and ectopic pregnancy. The differential expressions and localizations of these proteins suggest their effective roles in ectopic pregnancy and missed abortion, especially in subepithelium regions.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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