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RESEARCH

The expression of TRPV6 and PMCA1 in the mid-secretory endometrium of infertile patients with unexplained infertility and endometriosis

Açıklanamayan infertilite ve endometriozisli infertil hastaların mid-sekretuar endometriyumunda TRPV6 ve PMCA1'in ekspresyonu

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Abstract

Purpose: The goal of this study was to evaluate the expression of transient receptor potential vanilloid 6 (TRPV6) and plasma membrane Ca^{2+} ATPase 1 (PMCA1) in the endometrium of infertile women with unexplained infertility (UI) and endometriosis (E), and to compare the findings to healthy fertile women.

Materials and Methods: Endometrial expression of TRPV6 and PMCA1 proteins was evaluated during the mid-secretory phase using the immunohistochemical method, and mRNA levels were measured by real-time PCR. Calcium concentrations were determined using Inductively coupled plasma-mass spectrometry (ICP-MS).

Results: TRPV6 and PMCA1 protein expression were significantly increased in both luminal and glandular epithelium of UI or E groups compared to the control group. TRPV6 mRNA expression levels were significantly lower in the UI group (0.525 ± 0.087) compared to the control group (0.809 ± 0.073). There was no significant difference in PMCA1 mRNA expression levels between the groups. Calcium concentration was significantly higher in women with UI (median (Q1-Q3): 50.57 [40.67-51.37], compared to the control group (median (Q1-Q3): 24.29 [20.11-25.55]).

Öz

Amaç: Bu çalışmanın amacı, açıklanamayan infertilite (Aİ) ve endometriozisi (E) olan infertil kadınların endometriyumunda Transient Reseptör Potansiyel Vanilloid 6 (TRPV6) ve Plazma Membran Ca^{2+} ATPaz 1 (PMCA1) ekspresyonunu değerlendirmek ve bulguları sağlıklı fertil kadınlarla karşılaştırmaktır.

Gereç ve Yöntem: TRPV6 ve PMCA1 proteinlerinin endometriyal ekspresyonu mid-sekretuar faz sırasında, immünohistokimyasal yöntemle değerlendirilmiş ve mRNA seviyeleri real-time PCR ile ölçülmüştür. Kalsiyum konsantrasyonları İndüktif Eşleşmiş Plazma-Kütle Spektrometrisi (ICP-MS) kullanılarak belirlenmiştir.

Bulgular: TRPV6 ve PMCA1 protein ekspresyonu kontrol grubu ile karşılaştırıldığında, Aİ ve E gruplarının hem luminal hem de glandular epitelinde anlamlı olarak artış saptanmıştır. TRPV6 mRNA ekspresyon seviyeleri Aİ grubunda (0.525 ± 0.087) kontrol grubuna (0.809 ± 0.073) kıyasla anlamlı derecede düşüktür. PMCA1 mRNA ekspresyon düzeylerinde gruplar arasında anlamlı bir fark yoktur. Kalsiyum konsantrasyonu Aİ'li kadınlarda (medyan (Q1-Q3): 50.57 [40.67-51.37], kontrol grubuna (medyan (Q1-Q3): 24.29 [20.11-25.55]) göre anlamlı derecede yüksek bulunmuştur.

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Conclusion: The present findings demonstrate altered expressions of TRPV6 and PMCA1 mRNA and protein, along with differences in calcium concentration among patients, especially those with the UI during the window of implantation. These differences might potentially be considered as one of the underlying causes of infertility. Further studies are needed to elucidate the functional roles of these channels and to develop new strategies for pharmaceutical intervention in the treatment of infertility.
Keywords: Calcium metabolism, endometriosis, TRPV6, PMCA1, unexplained infertility

Sonuç: Mevcut bulgular, implantasyon penceresi sırasında özellikle Aİ'li hastalarda, kalsiyum konsantrasyonlarındaki farklılıkların yanı sıra, TRPV6 ve PMCA1 mRNA ve proteinlerinin değişen ekspresyonlarını göstermektedir. Bu farklılıklar, potansiyel olarak infertilitenin altında yatan nedenlerden biri olarak düşünülebilir. Bu kanalların fonksiyonel rollerini aydınlatmak ve infertilite tedavisinde farmasötik yaklaşımda yeni stratejiler geliştirmek için daha fazla çalışmaya ihtiyaç vardır.
Anahtar kelimeler: Kalsiyum metabolizması, endometriozis, TRPV6, PMCA1, açıklanamayan infertilite

INTRODUCTION

Embryo implantation is a complex process involving numerous proteins, genes, steroid hormones, cytokines, growth factors, and adhesion molecules in the endometrium[1]. Several ion channel proteins including transient receptor potential (TRP), voltage-dependent Ca^{2+}, and K^{+} channels are expressed in the endometrial epithelium in this process. Although research on ion channels discovered in the endometrium is limited, the data gathered is critical for the process of endometrial receptivity and embryo implantation. Research suggests that abnormal expression or dysfunction of ion channels may lead to implantation failure[2].

Calcium ions (Ca^{2+}) are important signaling molecules within cells that are essential for fertilization, implantation, and early embryo development[3,4]. Ca^{2+} is found in the cell cytoplasm, intracellular organelles, and extracellular medium. Ca^{2+} influx from the extracellular medium via calcium channels and Ca^{2+} release from internal stores contribute to the calcium mobilization. Intracellular free Ca^{2+} binds to calcium-binding proteins, such as calbindin, calmodulin, and parvalbumin, all of which have buffering capacities. These proteins take part in the transport process of cytosolic Ca^{2+} from the apical to the basolateral cell membrane[5]. However, the regulation of calcium homeostasis in the endometrium is not completely understood yet.

The superfamily of TRP channels is a family of non-voltage-dependent Ca^{2+}-permeable cation channels. Currently, 28 different TRP channel genes are identified in mammals. Transient receptor potential vanilloid 6 (TRPV6) is one of the highly Ca^{2+} selective channels in the TRP superfamily. TRPV6 mediates Ca^{2+} uptake into the cell and is localized in the plasma membrane in many organs[6]. The highest TRPV6 mRNA level was detected in the endometrium of pigs during implantation[7]. TRPV6 knockout mice showed reduced fertility, disordered Ca^{2+} homeostasis, increased urinary Ca^{2+} excretion, defective intestinal Ca^{2+} absorption, inability to gain weight, and lower maternofoetal Ca^{2+} transport[8].

Plasma membrane Ca^{2+} ATPase 1 (PMCA1; ATPase Ca^{2+} transporting plasma membrane also called ATP2B) is an ATP-dependent transporter that pumps calcium from the cell to the extracellular environment. It cooperates with other transport systems and with Ca^{2+}-binding proteins. PMCAs help to keep the cells' cytoplasmic Ca^{2+} levels stable. In humans and other mammals, the PMCA family contains four distinct isoforms that are encoded by particular genes (PMCA1-4). The PMCA1 pump is widely expressed in all tissues[9]. PMCA1 is expressed in the endometrium of rats[10], porcine[11], and humans[12]. Yang et al.[12] reported that PMCA1 and TRPV6 were expressed in human endometrium which indicates that both proteins are essential for implantation, calcium homeostasis, and endometrial cell proliferation. PMCA1 knockout mice have been reported to be embryonically lethal[13].

Although the roles of TRPV6 and PMCA1 in the endometrium have been studied, there is no evidence regarding their roles in the endometrium of infertility women. Therefore, our study aims to contribute valuable information concerning the potential role of these proteins in promoting fertility. This study hypothesizes that differences in calcium concentration and altered expression of TRPV6 and PMCA1 channels, which are involved in calcium regulation during implantation, may be one of the causes of infertility. Thus, this study investigated whether TRPV6 and PMCA1 mRNA and protein expression levels and calcium concentration altered in the mid-secretory phase endometrium of infertile patients (unexplained infertility (UI) and endometriosis (E)) and control group.

MATERIALS AND METHODS

Patients and tissue preparation

Mersin University Clinical Research Ethics Committee (Approval no: 2015/35, dated: 29/01/2015) granted permission for the study. Initially, the study was initiated by acquiring informed consent from the couples involved in the research. Respondents to the informed consent form were provided with the assurance that the original data would be kept confidential and would not be shared. The age, body weight, height, and additional details of patients were collected through the hospital information system. Biopsy materials were collected from patients by the experienced obstetrician using an endometrial suction catheter at the Assisted Reproduction Techniques (ART) Clinic at the University of Health Sciences. All the women, under the age of 38 with a minimum of 2 years of infertility, reported a regular menstrual cycle (26-32 days), a current desire for conception, and no hormonal treatments for at least 3 months before surgery. Infertility patients with no abnormal surgical findings and no abnormal laboratory findings were included in the group of women with UI[14].

Endometrial samples obtained from 27 infertile patients (15 UI and 12 E) and 14 healthy fertile women with proven fertility were included in the study. Clinical descriptions of the patients are given in Table 1. The Noyes criteria were employed for the histological dating of secretory phase endometrial biopsies to evaluate whether the endometrium displays a histological response appropriate to the duration of progesterone exposure[15]. Endometrial histology was prospectively examined using Noyes' criteria[15] by an independent histologist. No significant differences were observed in serum progesterone and 17β estradiol values among infertile and control groups. The serum progesterone levels suggested that, in these infertile groups, ovulation had occurred.

Endometrial biopsies were performed on 18-24 days. The collected tissue was washed in phosphate buffer saline (PBS) and cut into three sections: the first section was fixed in 4% paraformaldehyde solution buffered with 0.1 M sodium phosphate (pH 7.4) and embedded in paraffin for histopathological examination and immunohistochemistry. The second section was used to isolate mRNA to study TRPV6 and PMCA1 gene expression using quantitative real-time PCR. To conduct an additional examination, the third section was immediately collected into Eppendorf tubes and kept at -20 ^{o}C.

Table 1. Clinical characteristics of the groups

Parameter	Control Group (n=14) Mean ± SD	UI Group (n=15) Mean ± SD	E Group (n=12) Mean ± SD
Age	30.1 ± 4.4	30.9 ± 3.5	29.3 ± 3.4
BMI	24.2 ± 3.6	24.5 ± 2.4	23.7 ± 2.6
Serum oestradiol values (pg/ml)	102.8 ± 38.4	103.6 ± 30.8	109.4 ± 33.7
Serum progesterone values (ng/ml)	19.6 ± 5.1	22.8 ± 7.4	20.6 ± 5.1

Data were presented as Mean ± Standard Deviation (SD); UI group: patients with unexplained infertility; E group: patients with endometriosis; BMI: Body Mass Index.

Immunohistochemistry

The endometrial sections were deparaffinized and boiled in sodium citrate buffer (0.01 M, pH 6.0) for antigen retrieval. The slides were then subjected to washing in phosphate-buffered saline (PBS), treated with 3% H_2O_2 for 10 min, and subsequently, an ultra-V block (ab93697, Abcam, Waltham, MA, USA) was applied. The sections were incubated with primary antibodies; TRPV6 (1:100, orb158655, Biorbyt, Cambridge, UK) and PMCA (1:50, ab3528, Abcam, Waltham, MA, USA) overnight at 4 ^{o}C. On washing a PBS rinse, the sections were incubated with secondary antibody for 30 min and washed with PBS, then incubated with streptavidin-conjugated peroxidase for 10 min. They were treated with diaminobenzidine (DAB; ab64238, Abcam, Waltham, MA, USA) after being rinsed in PBS, and Mayer's hematoxylin was used to counterstain for 2 minutes. For analysis, prepared sections were converted to the 16-bit color format. Luminal epithelium (LE), the glandular epithelium (GE), and stromal cells were evaluated for five different areas at 400X magnification in all sections using an image analysis software Image J (National Institutes of Health, Bethesda, MD) and photographed under an

Olympus BX50 microscope (Olympus, Tokyo, Japan). Calibration was set based on 256 levels of the grey scale. The data were calculated using the pixel intensity values for the aforementioned antibodies.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the endometrial tissue samples using "Ribozol RNA Extraction Reagent (Ambresco, Solon, OH, USA). Complementary DNA was synthesized (cDNA) following the protocol of a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) in a thermal cycler (Techne Flexigene, Cambridge, UK). Gene expression levels were measured by ABI PRISM 7500 Real-Time PCR System (Applied Biosystems, USA). Sequence data were obtained from the GeneBank Sequence database (Table 2). Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) was used to design both the PCR primers and the TaqMan probes (Metabion International AG, Martinsried/Munich/Deutschland) (Table 2). The ACTB was employed as an endogenous control gene (housekeeping genes) and the reference RNA sample was Taqman Control Human RNA (Applied Biosystems in the USA). The $2^{-\Delta\Delta CT}$ method was used to evaluate the differences in gene expression.

Table 2. Sequences of primers and probes

	Primer/probe sequence*	NCBI Reference Sequence Number**
TRPV6 forward	5'-TGGTTCCTGCGGGTGGA-3'	
TRPV6 reverse	5'-CCTGTGCGTAGCGTTGGAT-3'	NM_018646.5
TRPV6 probe	5'-FAM-ACAGGCAAGATCTCAACCGGCA-BHQ-1-3'	
PMCA1 forward	5'-TTACGGGCGCCTGCAT-3'	
PMCA1 reverse	5'-TGAGGTTTACCCACAGCATCTG-3'	NM_001001323.1
PMCA1 probe	5'-FAM-A(pdC)T(pdC)AAGA(pdC)T(pdC)AC(pdC)G(pdC)TTA-BHQ-1-3'	
ACTB forward	5'-GGCACCCAGCACAATGAAG-3"	
ACTB reverse	5'-GCCGATCCACACGGAGTACT-3'	NM_001101.3
ACTB probe	5'-Yakima Yellow-TCAAGATCATTGCTCCTCCTGAGCGC-BHQ-1-3'	

*: http://www.ncbi.nlm.nih.gov/RefSeq/, **: pdC: Substitution of C-5 propynyl-dC (pdC) for dC is an effective strategy to enhance base pairing. Using these base substitutions, duplex stability and melting temperatures are raised by C-5 propynyl-dC 2.8° per substitution.

Inductively coupled plasma-mass spectroscopy (ICP-MS) analysis

Each tissue sample was transferred to a polypropylene tube and prepared for microwave digestion[16]. The sample was digested in a solution containing 1 mL of nitric acid and 100 µL of hydrogen peroxide and was then digested in a microwave oven at 500 W/110 °C on the defrost setting for 30 seconds each time. Then, the fluid was transferred into a second polypropylene tube, and 1% nitric acid was added until a 10 mL volume was reached (Agilent 7500ce; Agilent Technologies).

Statistical analysis

To assess sample size, power calculations were carried out considering a desired study power of 80% and an α error of 0.05, two-tailed. The minimum sample size was determined as 14 women for the control group, 15 women for the UI group, and 12 women for the E group, respectively. Data analyses were conducted using SPSS for Windows, version 22 (IBM SPSS for Windows version 22, IBM Corporation, Armonk, New York, United States). The variables were tested for normal distribution using the Shapiro-Wilks test. The one-way analysis of variance (ANOVA) was used to compare mRNA expression levels between the groups, followed by post-hoc tests including the Least Significant Differences (LSD test) and Dunnett's test. The General Linear Model Repeated-ANOVA test (Wilk's lambda & Huynh-Feldt) was used to analyze repeated quantitative measurements in comparison of the immunoreactivity intensity of the calcium channels between groups, and LSD-Bonferroni was conducted for pairwise comparisons. The Kruskal-Wallis H test was used to evaluate the Ca^{2+} concentrations between the groups, and the Mann-Whitney U test with the Bonferroni correction was applied to pairwise comparisons of the groups to determine the cause of the difference. The data was presented as mean ± standard error of the mean (SEM), mean ± standard deviation (SD), and median

(Q1-Q3). Statistical significance was established as $p < 0.05$.

RESULTS

As shown in Figure 1 and Figure 2, there were moderate immunostaining cells in luminal epithelium (LE), glandular epithelium (GE), and stromal cells of the control group, when the sections with TRPV6 (Figure 1b) and PMCA1 (Figure 2b) immunoreactivity were examined. The intensity of TRPV6 and PMCA1 immunoreactivity increased significantly in LE and GE of the UI (Figure 1c, 2c) and E (Figure 1d, 2d) groups, compared to the control group ($p < 0.05$; Figure 1e, 2e). No significant differences were observed in TRPV6 and PMCA expression in stromal cells among infertile women and the control group ($p > 0.05$; Figure 1e, 2e). Furthermore, there was no difference in these parameters when comparing the UI group with the E group ($p > 0.05$; Figure 1e, 2e).

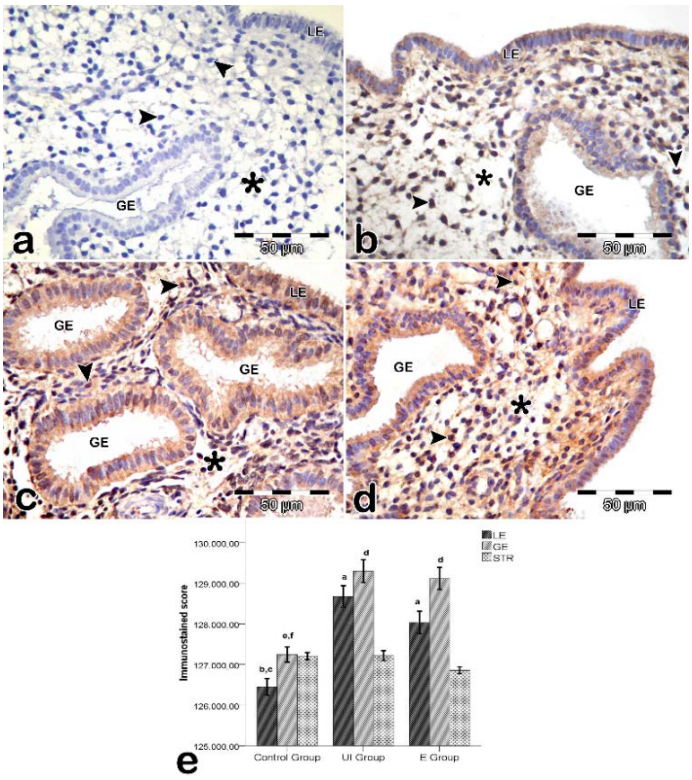

Figure 1. Light microscopic images of TRPV6 immunoreactivity in the endometrium (400x). (a) negative control, without anti-TRPV6 treatment, (b) Control group shows moderate immunostaining cells, (c) UI and (d) E groups show intense TRPV6 expression in LE and GE. (e) Comparison of TRPV6 expression between groups.

General Linear Model Repeated-ANOVA test (Wilk's lambda & Huynh-Feldt) - For pairwise comparisons: LSD-Bonferroni. [a]The difference between this group and the control group for LE values was statistically significant. (respectively, UI group - Control group p=0.001; E group - Control group p=0.005). [b]The difference between this group and the UI group for LE values was statistically significant (p=0.001). [c]The difference between this group and the E group for LE values was statistically significant (p=0.005). [d]The difference between this group and the control group for GE values was statistically significant (respectively UI group - Control group p=0.009; E group - Control group p=0.003). [e]The difference between this group and the UI group for GE values was statistically significant (p=0.009). [f]The difference between this group and the E group for GE values was statistically significant (p=0.003). LE, luminal epithelium; GE, glandular epithelium; STR, stromal cells; stromal cells (arrowheads) and stroma (asterisk).

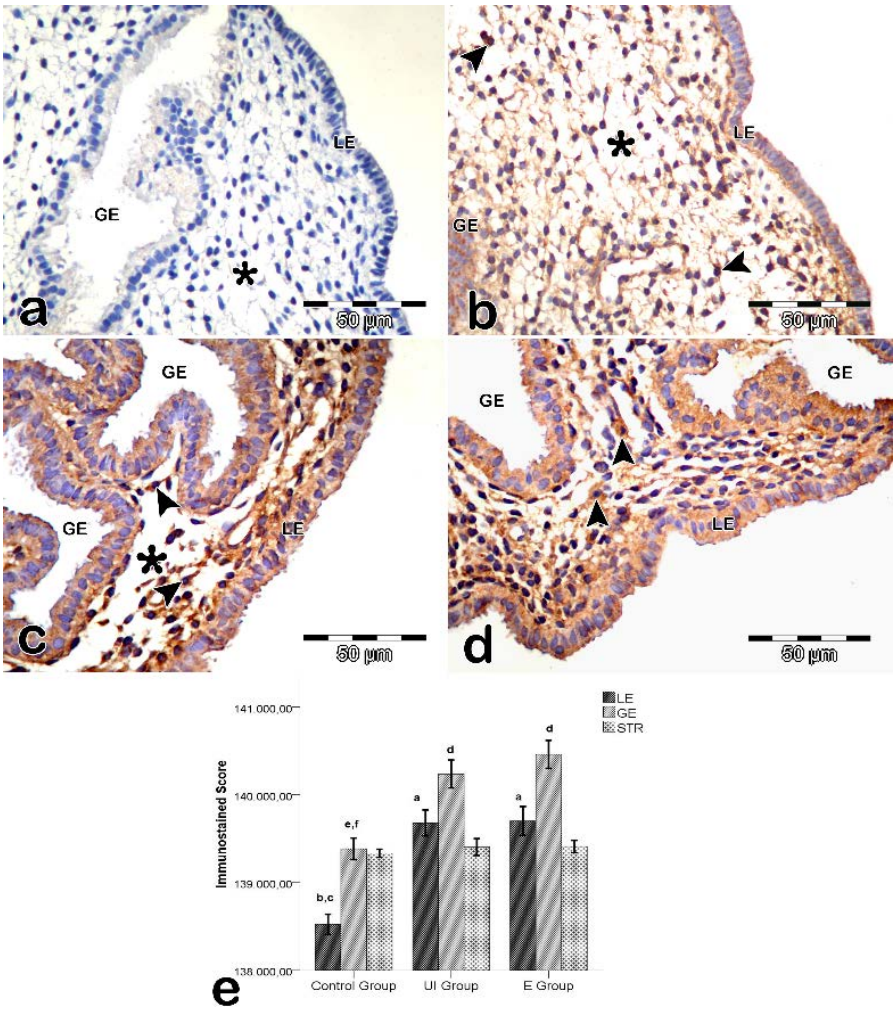

Figure 2. Light microscopic images of PMCA1 immunoreactivity in the endometrium (400x). (a) negative control, without anti-PMCA1 treatment, (b) Control group shows moderate immunostaining cells, (c) UI and (d) E groups show intense PMCA1 expression in LE and GE. (e) Comparison of PMCA1 expression between groups.

General Linear Model Repeated-ANOVA test (Wilk's lambda & Huynh-Feldt) - For pairwise comparisons: LSD-Bonferroni. [a]The difference between this group and the control group for LE values was statistically significant (respectively, UI group - Control group p=0.003; E group - Control group p=0.001). [b]The difference between this group and the UI group for LE values was statistically significant (p=0.003). [c]The difference between this group and the E group for LE values was statistically significant (p=0.001). [d]The difference between this group and the control group for GE values was statistically significant (respectively, UI group - Control group p=0.028; E group - Control group p=0.002). [e]The difference between this group and the UI group for GE values was statistically significant (p=0.028). [f]The difference between this group and the E group for GE values was statistically significant (p=0.002). LE, luminal epithelium; GE, glandular epithelium; STR, stromal cells; stromal cells (arrowheads) and stroma (asterisk).

TRPV6 mRNA expression levels were significantly lower in the UI group compared to the control group (p=0.027, Figure 3a). Comparing the infertile groups to the control group revealed that although PMCA1 mRNA expression levels increased, this difference was non-significant ($p > 0.05$, Figure 3b). There were no differences in TRPV6 and PMCA1 mRNA expression levels between infertile groups ($p > 0.05$, Figure 3).

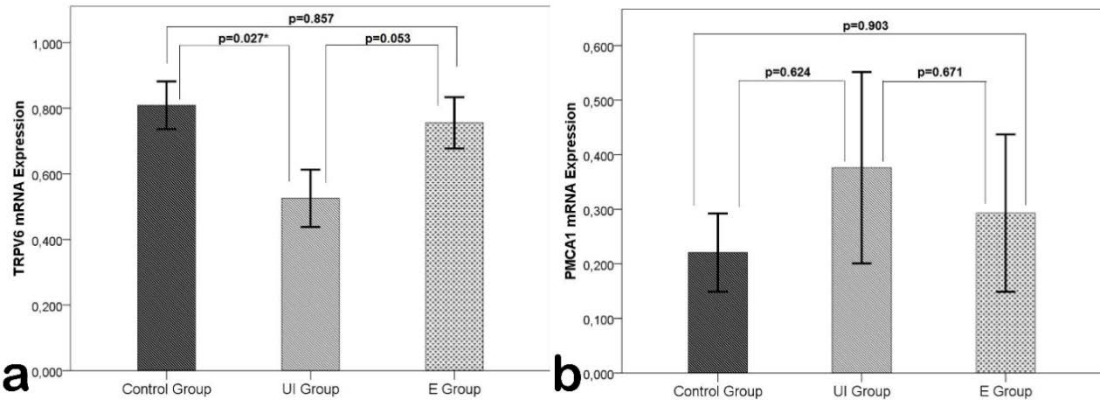

Figure 3. Results of qRT-PCR for TRPV6 and PMCA1 mRNA expression in the endometrium of control and infertile groups.

One Way Anova; α:0.05; Post-Hoc: LSD test, Dunnett's test; *The difference between the control and UI groups was statistically significant (p=0.027).

A significantly higher level of calcium in the endometrial tissues of the UI group was observed compared to the control and E groups ($p < 0.05$, Table 3). Additionally, the difference between the E and UI groups was statistically significant ($p < 0.001$, Table 3).

Table 3. Ca^{2+} concentrations in endometrial tissues

	Control Group (n=14)	UI Group (n=15)	E Group (n=12)	P value
Ca (ppm) Median (Q1-Q3)	24.29 [20.11-25.55]	50.57*,†[40.67-51.37]	25.2 [22.4-26.6]	0.001*

Kruskal Wallis H test; *Difference between UI and control group was statistically significant (p=0.007). †Difference between E and UI groups was statistically significant ($p < 0.001$); (Q1:25% - Q3:75%).

DISCUSSION

Cell adhesion between the endometrium and conceptus during the implantation period requires Ca^{2+} [17,18]. Calcium also acts as an activator of cell adhesion molecules including integrins, cadherins, and selectins for ligand binding[19]. An influx of Ca^{2+} into the epithelial cells through TRPV6 may affect cell adhesion between LE and blastocyst[20]. Secretion of Ca^{2+} into the uterine lumen and absorption by the endometrium is active at the time of implantation. Endometrial glands and LE secrete histotroph which includes transport proteins, enzymes, ions, glucose, growth factors, and other molecules that have a role in the implantation and conceptus development[21]. Ca^{2+} may be involved in the exocytotic secretion of various histotrophic molecules because Ca^{2+} triggers exocytosis[22]. Defective development and inadequate function of GE may be an underlying cause of pregnancy failure in humans[23]. Previous research has found that TRPV6 protein expression increased in the preeclamptic placenta[24], the placenta of pregnant rats under hypoxic stress[25], the colon tissue of patients with ulcerative colitis[26], and cystic fibrosis human bronchial epithelial cells[27]. These reports were consistent with the data which shows increased TRPV6 expression in both epithelial cells of infertile women compared to the control group in the present study, suggesting increased TRPV6 expression/activity may lead to impaired Ca^{2+} homeostasis by stimulating Ca^{2+} entry to the epithelial cells and these results demonstrate that the absence of feedback mechanism contributes to dysregulation of TRPV6 channel[27].

A sustained high Ca^{2+} concentration in the cytoplasm is toxic to the cell. Therefore, the cell develops mechanisms to eliminate increased cytosolic Ca^{2+} [5,28]. Several calcium-regulatory molecules are reported to

mediate the extrusion of calcium ions from the cell including PMCA1, the sodium/calcium exchanger, and the potassium-dependent sodium/calcium exchanger[29]. Several studies have reported overexpression of PMCA1 in breast cancer lines[30], insulin-secreting ß-cells (BRIN-BD11)[31], and preeclamptic placenta[24]. Notably, the lack of ATP production impairs the activity of the PMCA pumps for Ca^{2+} extrusion which contributes to sustained Ca^{2+} overload in pancreatic acinar and ductal cells in acute pancreatitis[33]. Increased PMCA1 expression was observed in response to raised intracellular calcium upon exposure to oxidative stress in human cataractous lenses, as previously shown[32]. In line with these findings, it was observed that infertile women in both groups had significantly higher PMCA1 expression in both LE and GE than the control group. The increased expression of this pump in endometrial cells may be part of the response interfering with compensatory mechanisms that are inactive in restoring Ca^{2+} [30]. There was no difference in PMCA1 protein expression in the epithelial cells of both infertile groups.

Successful embryo implantation requires effective communication between the embryo, endometrial epithelial, and stromal cells. However, the current knowledge about the distribution and function of TRP and PMCA channels in stromal cells is incomplete. Further studies are needed to investigate their functional roles in the endometrium. Reports showed that the expression of TRPV6 could be detected in human endometrial biopsies by qRT–PCR whereas it could not be detected in pure stromal cell cultures[34]. TRPV6 mRNA expression was previously observed only in a culture of murine endometrial epithelial cells[35]. These studies demonstrated that limited pharmacological agents for TRPV6 restricted the functional analysis. Its regulation is controlled by ovarian hormones. The differentiation of stromal cells into decidual cells could alter TRP channel expression. It has been shown that PMCA1 mRNA in LE, GE, and stromal cells of the porcine endometrium in pregnancy was expressed[11]. No significant differences were observed in TPRV6 and PMCA1 protein expression in stromal cells among the groups in our study. The role of these channels in signaling between stromal and endometrial epithelial cells can be further studied.

The altered expression pattern of mRNA and protein expression was reported in different tissues[36-38]. Bax mRNA expression was decreased in metastatic lesions whereas protein expression was higher in primary breast cancer tumors and brain metastases[36]. Although CD20 mRNA levels were normal, there was a decrease in CD20 protein expression in chronic lymphocytic leukemia cells of the patients[37]. There was no significant correlation between the mRNA and protein levels of matrix metalloproteinases 2 and 9 and the tissue inhibitor of metalloproteinases 1 in human prostate cancer versus control[38]. In the current study, TRPV6 mRNA expression levels were lower in the UI group compared to the control group. In contrast to mRNA evaluation, immunohistochemical data revealed higher expression levels of TRPV6 in the UI group. However, our results showed an increased PMCA1 expression on the protein level but not on the mRNA level. Posttranslational or posttranscriptional regulation mechanisms might be involved in this case[39]. When comparing the UI and E groups, no significant difference was observed in TRPV6 and PMCA1 mRNA expression levels.

Little data exists about the accumulation of elements in the female reproductive system, particularly in the uterus. Some metals, such as lead, mercury, zinc, and cadmium can trigger endometrial changes including increased uterine volume, endometrial hyperplasia, and the promotion of spontaneous abortions[40]. A previous study has reported that higher Ca concentration in malign ovary tissues than the noncancerous ovary tissues, which was measured using atomic absorption spectrometry[41]. A similar result was obtained for Ca concentration in uterine cancer tissue[42]. Our study is, to our knowledge, the first to identify the difference in Ca^{2+} levels in endometrial biopsies of the infertile groups and control groups. Among the infertile groups, particularly, the UI group had a significantly higher Ca concentration than the control group which is consistent with the literature. The difference between UI and E groups was also significant. Given the ability of TRPV6 and PMCA1 to regulate Ca^{2+}, we believe that their increased expressions could mediate the imbalance in the calcium homeostasis possibly related to the disease by causing Ca^{2+} accumulation.

The limitations of this study are that we were unable to test the channels associated with calcium using molecular approaches such as Western blot, as we demonstrated through immunohistochemical methods.

In conclusion, our study demonstrates that there are altered expressions of TRPV6 and PMCA1 protein,

mRNAs, as well as differences in calcium concentration, in the endometrium of infertile women, especially in the UI group. Additional studies are required to elucidate the functional roles of TRPV6 and PMCA1 channels for Ca^{2+} homeostasis in the infertility pathophysiology and to specifically develop further knowledge of these proteins and new strategies for pharmaceutical intervention in the treatment of infertility. Therefore, the contribution of mechanisms controlling TRPV6 and PMCA1 channel activity in infertility to the maintenance of calcium metabolism, which is necessary for endometrial survival, should be studied.

Author Contributions: Concept/Design: TOM, BCY; Data acquisition: TOM, NY; Data analysis and interpretation: TOM, BCY, MEE, GOT, IBK, AD; Drafting manuscript: TOM, BCY; Critical revision of manuscript: TOM, NY, BCY, SNY; Final approval and accountability: TOM, NY, BCY, SNY, MEE, IBK, GOT, AD; Technical or material support: TOM, BCY, NY; Supervision: TOM, BCY; Securing funding (if available): n/a.
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