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Original article

ACE inhibitors on ACE1, ACE2, and TMPRSS2 expression and spheroid attachment on human endometrial Ishikawa cells

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ARTICLE INFO

Keywords:

Angiotensin-converting enzymes (ACEs)
ACE inhibitors
TMPRSS2
Endometrium
Spheroid attachment

ABSTRACT

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) enters cells via receptor angiotensin-converting enzyme 2 (ACE2) and co-receptor transmembrane serine protease 2 (TMPRSS2). However, patients with SARS-CoV-2 infection receiving ACE1 inhibitors had higher ACE2 expression and were prone to poorer prognostic outcomes. Until now, information on the expression of ACE1, ACE2, and TMPRSS2 in human endometrial tissues, and the effects of ACE inhibitors on embryo implantation are limited. We found human endometria expressed ACE1, ACE2, and TMPRSS2 transcripts and proteins. Lower ACE1, but higher ACE2 transcripts were found at the secretory than in the proliferative endometria. ACE1 proteins were weakly expressed in endometrial epithelial and stromal cells, whereas ACE2 and TMPRSS2 proteins were highly expressed in luminal and glandular epithelial cells. However, ACE1 and TMPRSS4 were highly expressed in receptive human endometrial epithelial (Ishikawa and RL95–2) cells, but not in non-receptive AN3CA and HEC1-B cells. Treatment of human endometrial epithelial cells with ACE1 (Captopril, Enalaprilat, and Zofenopril) or ACE2 (DX600) inhibitors did not significantly alter the expression of ACE1, ACE2 and TMPRSS2 transcripts and spheroid (blastocyst surrogate) attachment onto Ishikawa cells in vitro. Taken together, our data suggest that higher ACE2 expression was found in mid-secretory endometrium and the use of ACE inhibitors did not alter endometrial receptivity for embryo implantation.

1. Introduction

Angiotensin Converting Enzymes (ACE or ACE1 and ACE2) are the key enzyme involved in the production of Angiotensin II (AngII) from Ang I and the conversion of Ang II to Ang1–7, respectively in many human tissues [1]. The cellular localization and menstrual cycle-based dynamics of renin, angiotensin, and ACE1 in human endometrium have been described previously [2]. Moreover, dysregulation of the renin-angiotensin system in the human endometrium was found to be associated with pregnancy complications like preeclampsia [3]. Interestingly, limited data is available on the expression, interaction, and function of ACE1 and ACE2 in human endometrial receptivity and early

embryo attachment.

Recent studies are focusing on the role of ACEs on the etiology of Covid-19 disease where ACE2 together with another receptor transmembrane serine protease 2 (TMPRSS2) play a major role in the cellular entry of the virus [4]. Therefore, ACE inhibition has become one of the prime drug targets for disease treatment. However, patients with cardiac disease, hypertension, or diabetes, and on treatments (e.g., ACE1 inhibitors) that increase ACE2 expression are at a higher risk for severe SARS-CoV-2 infection [5]. Recent transcriptomic data suggests low level of the ACE1, ACE2, and TMPRSS2 in human endometrial cells at the transcripts level [6]. Nevertheless, information on the expression levels of these proteins in the endometrium are lacking. On the contrary, a

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<https://doi.org/10.1016/j.repbio.2022.100666>

Received 11 April 2022; Received in revised form 25 May 2022; Accepted 29 May 2022

Available online 7 June 2022

1642-431X/© 2022 Society for Biology of Reproduction & the Institute of Animal Reproduction and Food Research of Polish Academy of Sciences in Olsztyn.

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recent transcriptomic analysis indicated that the expression of ACE2 is significantly higher towards the window of implantation in the human endometrium, and TMPRSS2 increases in the human endometrium during the embryo implantation [7]. The human embryo also expresses ACE2 and TMPRSS2 in the trophoblast cells indicating a potential risk of SARS-CoV-2 infection on embryo survival and implantation [8]. Moreover, the expression of ACE2 mRNA and protein increases during human endometrial stromal cell (HESC) decidualization [9], and a recent report suggested that SARS-CoV-2 can cause pregnancy complications such as pre-term birth and miscarriages [10]. A higher expression of ACE2 in the endometrium with advanced maternal age, and during the secretory phase raises concern about the increased susceptibility to Covid-19 during periods of high ACE2 expression [11].

Angiotensin Converting Enzyme Inhibitors (ACEI) have been used as drugs to control many common non-communicable diseases like hypertension, proteinuria, and chronic kidney disease. Sulphydryl-containing drugs (e.g. Captopril, Enalapril, and Zofenopril) are the commonly used ACE inhibitors. However, with the recent coronavirus disease 2019 (COVID-19) caused by SARS-CoV-2, ACEIs got much attention as a means of disease treatment. An early study suggested ACE1 inhibition up-regulates ACE2 in the cardiac system [12]. However, it is unknown whether a similar effect can be found for ACEIs on ACE1, ACE2, and TMPRSS2 expression in human endometrium. Moreover, an imbalance of ACE1 and ACE2 expression has been proposed as one major mechanism of the advancement of Covid-19 in patients. Therefore, the use of ACE inhibitors to restore balance is one of the key therapeutic approaches to preventing disease progression [13]. Although the ACE2 inhibitors are not very much in use clinically, a small molecule DX600 has a very potent inhibitory activity against ACE2.

The current study aimed to examine the transcript and protein expressions of ACE1, ACE2, and TMPRSS2 in human endometrium and endometrial epithelial cell lines and further investigated whether the ACEIs currently in use have any effect on endometrial gene expression and early embryo attachment using a blastocyst surrogate (trophoblast spheroids)-endometrial cells co-culture model.

2. Materials and methods

2.1. Patient samples

The study recruited women who attended the Assisted Reproduction Unit of the Department of Obstetrics and Gynaecology, Queen Mary Hospital, Hong Kong for in vitro fertilization (IVF) treatment. Endometrial biopsies were collected from women with regular menstrual cycles, whose husbands had male infertility, and had not taken hormonal treatments for the past 3 months. Samples were collected during the proliferative (n = 12) and secretory (n = 16) phases of the cycle. The study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB) (UW17-458). Informed written consent was obtained from all participants in the study.

2.2. Immunohistochemical staining

Human endometrial biopsies were fixed in 4 % paraformaldehyde and embedded in paraffin. Section (5 µm thick) were mounted on polylysine coated slides and de-waxed followed by antigen retrieval as reported previously [14]. Antibodies against ACE (1:50, PA5-83080, Invitrogen), ACE2 (1:100, MA5-31394, Invitrogen), TMPRSS2 (1:100, ab92323, Abcam). A positive signal was detected by 3,3'-diaminobenzidine (DakoCytomation) staining and nuclei were counter-stained with hematoxylin. A human testis sample was used as a positive control for ACE2 staining. The stained sections were observed under a light microscope (Axioskop, Zeiss, Germany) with bright-field optics.

2.3. Human endometrial epithelial and trophoblastic cell lines

Human endometrial epithelial Ishikawa (ECACC, 99040201), RL95-2 (ATCC, CRL1671), AN3CA (ATCC, HTB-111), and HEC1-B (ATCC, HTB-113), and trophoblastic choriocarcinoma BeWo (ATCC, CCL-98) cells were used in this study. Ishikawa, AN3CA, and HEC1-B cells were maintained in minimum essential medium (MEM, M0268, Sigma) supplemented with 1 % penicillin/streptomycin (Gibco, USA), 1 % L-glutamine (Gibco, USA), and 10 % fetal bovine serum (FBS, Thermo Fisher). BeWo and RL95-2 cells were maintained in Dulbecco's Modified Eagle Medium nutrient mixture/F12 (DMEM/F12, D8900, Sigma) supplemented with 1 % penicillin/streptomycin, 1 % L-glutamine, and 10 % FBS. All cells were sub-cultured every 2-3 days and maintained at 37 °C at 5 % CO₂.

2.4. ACE inhibitors and treatments

ACE inhibitors were obtained from a library of small molecules (LOPAC 1280, Sigma). The ACE inhibitors (Captopril, Enalaprilat, and Zofenopril) and ACE2 inhibitor (DX600, Merck) were used to treat the human receptive endometrial epithelial Ishikawa cells at doses of 0.3, 1, 3, 10, and 30 µM for 24 h. Dihydroouabain (3 µM, DHO) was used as a positive control for the co-culture assay.

2.5. RNA extraction and qPCR analysis of ACE1, ACE2, and TMPRSS2 expressions

Total RNAs were extracted from human endometrial tissue samples and ACEIs-treated cell lines using the MirVANA PARIS RNA isolation kit (Thermo Fisher) and followed the manufacturer's instructions. Total RNA (100 ng) was reversed transcribed to cDNA using TaqMan 2X Universal PCR Master Mix (Life Technologies). Real-time PCR was carried out using TaqMan probes specific for human ACE, ACE2, and TMPRSS2 (Hs00174179_m1, Hs01085333_m1, and Hs01122322_m1, Applied Biosystems) using a real-time PCR platform (QuantStudio 5, Applied Biosystems). Eukaryotic 18 S (4318839, Applied Biosystems) was used as the internal control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of mRNA [15].

2.6. Protein extraction and Western blotting

Total cellular protein was extracted by dissolving cells in RIPA buffer. Protein concentration was measured using the Pierce BCA protein assay kit (23225, Thermo Fisher). Samples with normalized total protein concentrations were denatured at 95 °C for 5 min and separated by SDS-PAGE, and then transferred to a PVDF membrane and blocked in 5 % skimmed milk in PBST for 1 h. Rabbit anti-ACE (1:1000, PA5-83080, Thermo Fisher), rabbit anti-ACE2 (1:1000, ab108252, Abcam), rabbit anti-TMPRSS2 (1:1000, ab92323, Abcam), mouse anti-β-actin (1:5000, Sigma) or mouse anti-α-tubulin (1:5000, Santa Cruz) in blocking buffer were added and incubated overnight at 4 °C. After washing with PBST, the membranes were incubated with anti-rabbit or anti-mouse secondary antibody conjugated with horse-radish peroxidase (1:5000, GE Healthcare) for 1 hr and washed. Membranes were developed using a chemiluminescence kit (Advansta, Griffin Biotech). The normalization of protein loading was confirmed with β-actin or α-tubulin expression.

2.7. Effects of ACE1 and ACE2 inhibitors on spheroid co-culture

The attachment of BeWo spheroids (blastocyst surrogates) onto human endometrial epithelial Ishikawa cells were quantified by spheroid-endometrial cells co-culture assay as previously described [16-18] with modifications. Briefly, BeWo cells were trypsinized into single cells and seeded at a density of 1.2×10^5 cells per well in an AggreWell 400 (STEMCELL Technologies). After centrifugation and 24 h

aggregation, BeWo spheroids formed with sizes similar to that of human embryos. Spheroids with a diameter of around 100 μm were transferred onto confluent monolayers of Ishikawa cells with or without ACE inhibitor pre-treatment. The co-culture was incubated for 30 min without the inhibitor at 37 °C in 5 % CO_2 in a humidified environment. Unattached spheroids were removed by shaking the plate at 8 xg for 10 min and washing with 1 mL PBS twice. Attached spheroids were counted under a light microscope and the rate of attachment was expressed as a percentage of the total number of spheroids used.

2.8. Statistical analysis

All results were expressed as means \pm SEM. Statistical analysis was performed using GraphPad Prism 6.0 software. The spheroid attachment rate was compared between treatment groups using one-way ANOVA or Kruskal-Wallis test. A probability value < 0.05 ($p < 0.05$) was considered statistically significant. Each in vitro experiment was independently repeated at least three times.

3. Results

3.1. Expression of ACE1, ACE2, and TMPRSS2 transcripts and proteins in human endometrial samples

The gene expression analysis was performed using human endometrial aspirates obtained at both proliferative and secretory phases. The qPCR results showed significantly ($p < 0.05$) lower expressions of ACE1 in the secretory phase (Fig. 1A). However, a higher expression of ACE2 (Fig. 1B) ($p < 0.05$) was observed in the secretory phase than in the proliferative phase endometrium. Although a lower expression of TMPRSS2 was found in the secretory endometrium, the difference was not statistically significant ($p < 0.05$, Fig. 1C). Immunohistochemical staining of ACE1, ACE2, and TMPRSS2 proteins was performed in human endometrial biopsies taken at the mid-secretory phase (LH+7 days) corresponding to the window of implantation (WOI). The expression of ACE1 protein was mainly localized to the glandular epithelium (GE) and luminal epithelium (LE, Fig. 1D). The expression of ACE2 protein was high in both luminal and glandular epithelial cells than in the stromal cells, and the expression of TMPRSS2 protein was high in both the luminal and glandular epithelial cells compared to the stromal cells (Fig. 1D). The human testis sample was used as a positive control for ACE1, ACE2 and TMPRSS2 protein staining, and the endometrial sample omitted with primary antibody was used as a negative control for comparison.

3.2. Expression of ACE1, ACE2, and TMPRSS2 transcripts and proteins in the human endometrial epithelial cell lines

Quantitative PCR and Western blotting were used to examine ACE1, ACE2, and TMPRSS2 transcripts and proteins expression in the four human endometrial epithelial cell lines: Ishikawa, RL95–2, AN3CA, and HEC1-B. The receptive endometrial epithelial Ishikawa and RL95–2 cells expressed higher levels of ACE1 and TMPRSS2 transcripts and proteins than the non-receptive endometrial cell lines AN3CA and HEC1-B cells (Fig. 2A & B). The expression of ACE2 transcript varied among the four cell lines, however, the non-receptive AN3CA and HEC1-B cells seem to express higher levels of ACE2 protein than the receptive Ishikawa and RL95–2 cells (Fig. 2A & B).

3.3. Effects of ACE inhibitors on the expression of ACE1, ACE2, and TMPRSS2 in Ishikawa cells and spheroid attachment in vitro

Quantitative PCR was used to study how ACE inhibitors (Captopril, Enalaprilat, and Zofenopril) and ACE2 inhibitor (DX600) modulate ACE1, ACE2, or TMPRSS2 expressions in human endometrial epithelial cell lines. Ishikawa cells were treated with different concentrations

(0.3–30 μM) of the ACE or ACE2 inhibitors for 24 hrs. Total RNA was extracted, cDNA was synthesized and then subjected to quantitative PCR analysis using gene-specific TaqMan probes. Treatments of ACE or ACE2 inhibitors did not significantly affect the expression of ACE1, ACE2, or TMPRSS2 transcripts in Ishikawa cells (Fig. 2C). No change in ACE1, ACE2, and TMPRSS2 expression when the Ishikawa cells were not treated with chemicals or with 1 % DMSO (solvent control). We next investigated whether the ACE1 and ACE2 inhibitors could alter the attachment of blastocysts-surrogate trophoblastic spheroids onto treated Ishikawa cells which mimics the initial embryo attachment to the receptive endometrial epithelium. Ishikawa monolayer and Jeg-3 spheroids were prepared for coculture study (Fig. 3A). ACE inhibitors were used to treat Ishikawa cells for 24 h before co-culture. It was found that the ACE (Captopril, Enalaprilat, and Zofenopril) and ACE2 inhibitors (DX600) did not affect the spheroid attachment onto Ishikawa cells when compared to the controls (untreated or 0.1 % DMSO, Fig. 3B). The small molecule, dihydroouabain (3 μM , DHO), was used as the positive control to suppress spheroid attachment.

4. Discussion

The present study examined the expression of ACE1, ACE2, and TMPRSS2 transcripts and proteins in human endometrial epithelial cell lines and tissues to evaluate the possible impact of ACE inhibitors on the endometrial receptivity and embryo implantation. We found the expression of ACE1 mRNA decreased while the ACE2 mRNA increased in the secretory endometrium when compared to proliferative endometrium. ACE2 and TMPRSS2 protein were strongly expressed in the luminal and glandular epithelium of human endometrium; while ACE1 protein was weakly expressed in these compartments. Human endometrial epithelial cell lines express ACE1, ACE2, and TMPRSS2 transcripts, and higher expression of ACE1 and TMPRSS2 protein, while lower expression of ACE2 protein was found in receptive endometrial epithelial Ishikawa and RL95–2 cells. ACE1 (Captopril, Enalaprilat, and Zofenopril) and ACE2 (DX600) inhibitors did not affect the expression of ACE1, ACE2, and TMPRSS2 transcripts expression or spheroid attachment on Ishikawa cells, suggesting that patients receiving ACE inhibitors treatment may not affect endometrial receptivity and embryo implantation.

Many non-communicable diseases like hypertension, cardiac events, kidney diseases, and diabetes have etiological associations with ACEs, thus ACEs have been a popular target in treating such complications. Recently the attention on ACE was renewed with the SARS-CoV-2 infection leading to serious respiratory diseases including pneumonia in patients of all ages [19]. The new SARS-CoV-2 uses the same receptor ACE2 and co-receptor TMPRSS2 for entering epithelial cells [4]. We found ACE1, ACE2, and TMPRSS2 transcripts and proteins in the human endometrial cell lines and tissues, suggesting that the virus can infect the female reproductive tissue including the endometrium.

Although a higher ACE1 transcript expression was found in the receptive endometrial cell lines, the expression level of ACE1 decreased in the secretory endometrium when compared to the proliferative endometrium. However, the glandular endometrial epithelium expressed a higher level of ACE1 protein when compared to the luminal epithelium. Moreover, it has been previously shown that ACE1 has the highest expression in the endometrium in the late-secretory phase which was postulated to be involved in the menses [2]. The difference in ACE1 expression in receptive endometrial epithelial cell lines than the receptive luminal epithelium may be cancerous origin of the cell lines or the cells may be originated from the glandular endometrial epithelial cells.

In this study, we found that ACE1 was mainly expressed in the glandular epithelium, as well as endothelial cells of the human endometrium. It is well known that ACE1 functions to convert Ang I into Ang II, whereas ACE2 hydrolyzes Ang I to Ang-(1–9), and also cleaves Ang II to Ang-(1–7) [20]. Ang-(1–7) facilitates vasodilation and counteracts

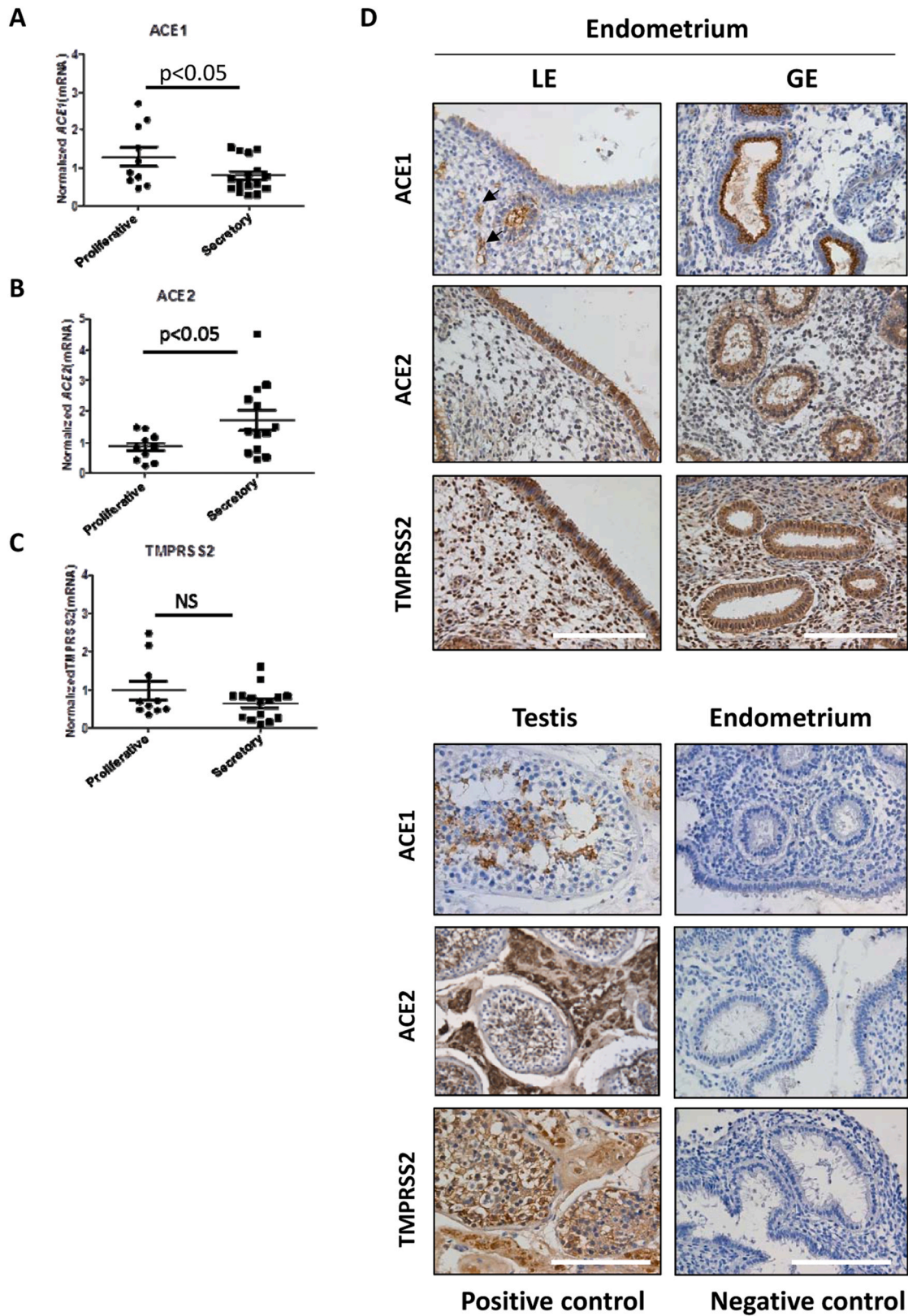


Fig. 1. Expression of ACE1, ACE2, and TMPRSS2 transcripts and proteins in Human endometrial tissues. Human endometrial samples were taken at proliferative and secretory phases of the cycle were used to analyze the expression of (A) ACE1, (B) ACE2, and (C) TMPRSS2 transcripts. (D) Immunohistochemical staining of ACE1, ACE2, and TMPRSS2 proteins on human endometrial samples were taken at LH+ 7 days of the cycle. Brown staining for ACE1 protein was found in the glandular epithelium (GE) and endothelial cells (arrows). ACE2 was expressed mainly in the luminal (LE) and glandular epithelium, mildly in stromal cells and weakly in endothelial cells of endometrial tissues. TMPRSS2 was expressed mainly in the luminal and glandular epithelium, as well as in stromal cells. Human testis samples were used as positive control for ACE1, ACE2 and TMPRSS2 protein, and an endometrial sample omitted with primary antibody was used as a negative control. * denotes significant differences ($P < 0.05$) between groups ($n = 4-7$ per group). Scale bar = 100 μ m.

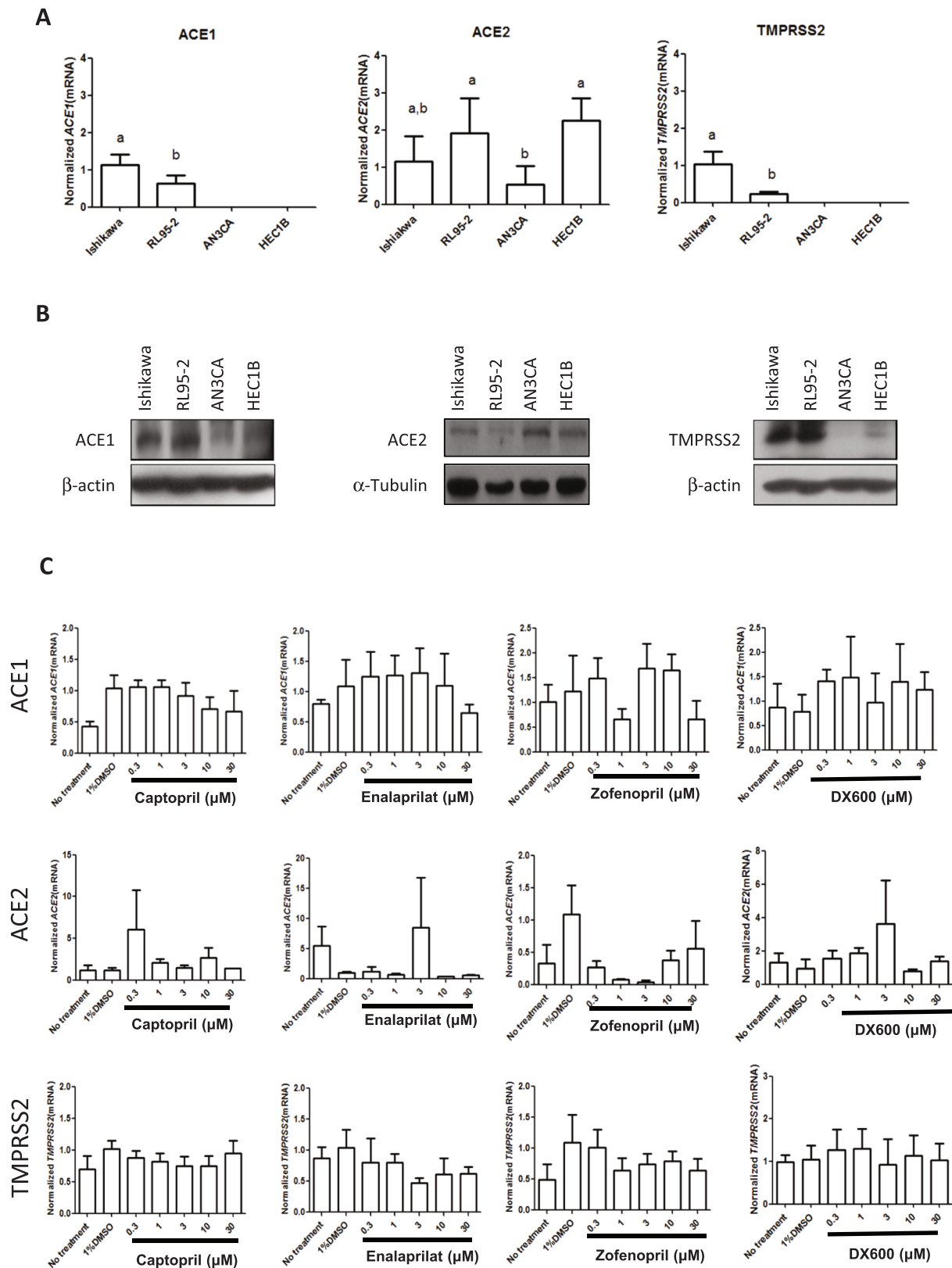


Fig. 2. Effect of ACE inhibitors on the expression of ACE1, ACE2, and TMPRSS2 in human endometrial cell lines. The expression of ACE1, ACE2, and TMPRSS2 transcripts in four human endometrial cell lines including two receptive (Ishikawa and RL95-2) and two non-receptive (AN3CA and HEC1-B) cell lines were measured by quantitative PCR. (B) The expression of ACE1, ACE2, and TMPRSS2 proteins were analyzed by Western blotting and normalized to β-actin or α-tubulin expression. (C) Expression of ACE1, ACE2, and TMPRSS2 transcripts in Ishikawa cells treated with different concentrations of ACE1 (Captopril, Enalaprilat, and Zofenopril) and ACE2 (DX600) inhibitors (0.3 – 30 μM) for 24 h. Untreated Ishikawa cells or cells treated with 0.1 % DMSO solvent were used as controls. a-b denotes a significant difference between groups (P < 0.05).

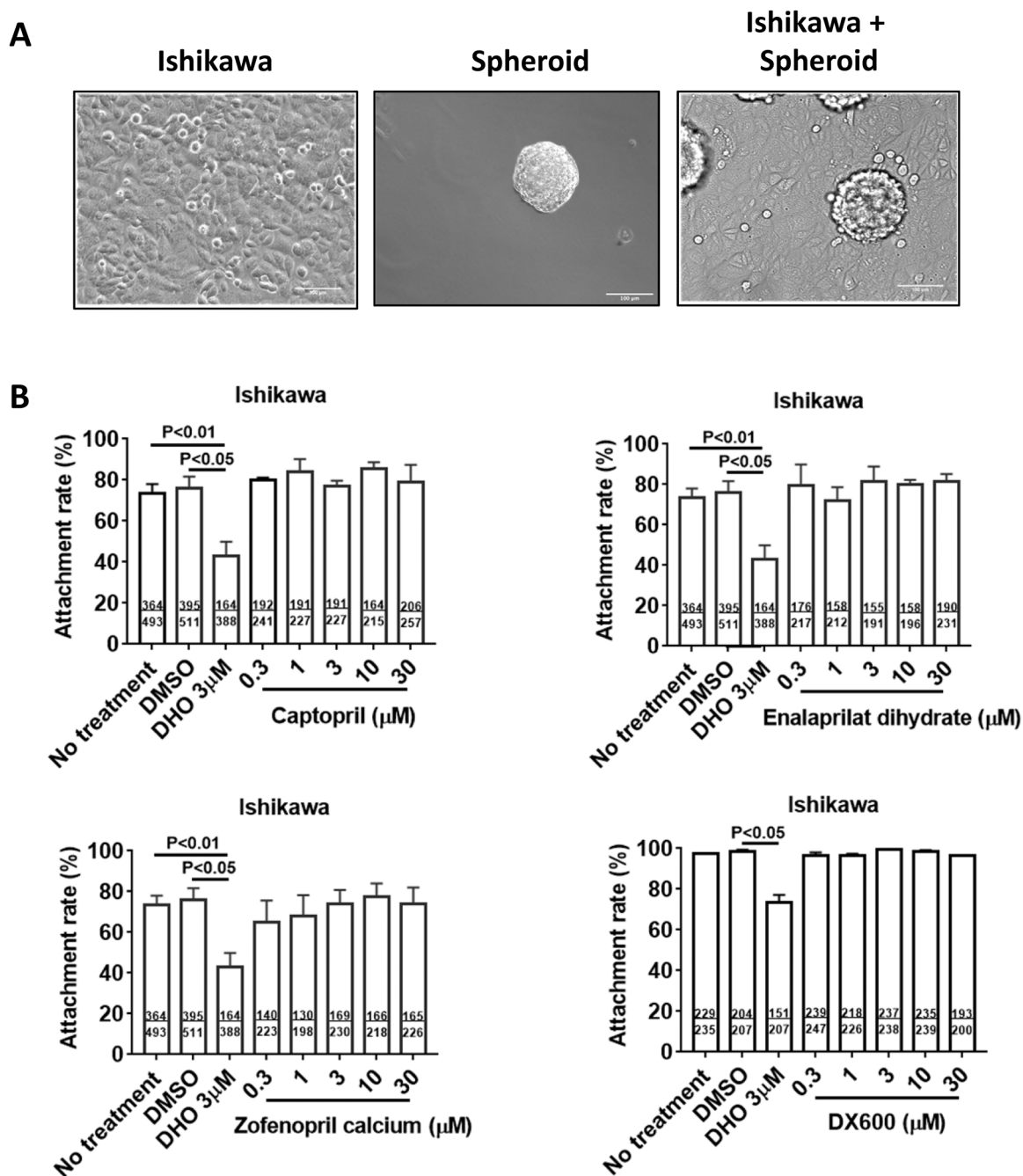


Fig. 3. Effect of ACE1 inhibitors (Captopril, Enalaprilat, and Zofenopril) and ACE2 inhibitors (DX600) on the attachment of Jeg-3 spheroid on Ishikawa cells. (A) Photomicrograph of Ishikawa cell, spheroids, and spheroid attached to Ishikawa monolayer cells. (B) The attachment rate of spheroids on Ishikawa cells treated with different concentrations of ACE1 (Captopril, Enalaprilat, Zofenopril) and ACE2 (DX600) inhibitors. Dihydroouabain (DHO) was used as a positive control for spheroid attachment. No treatment or 0.1 % DMSO was used as a negative control. Scale bar = 100 μ m.

metabolic syndrome, whereas Ang II contributes to vasoconstriction, cell proliferation, and hydro-salinity balance [21]. Interestingly, Ang II also increases uterine contraction in rats [22]. In line with this, angiotensin II type 1 (AT1) receptor (AT1-R) and angiotensin II type 2 (AT2) receptor (AT2-R) were expressed in all endometrial compartments, whereas their altered expressions were found in women with recurrent miscarriage [23]. Even though our data supports more endocrine system-related regulation of the ACEs, it is also important to note that the uterus has been identified as an organ with an active renin-angiotensin system (RAS) which may be under more paracrine control and can independently modulate the tissue functions [24]. However, the exact physiological role of ACEs in the human

endometrium is yet to be disclosed with more empirical evidence.

The increased ACE2 expression in the secretory endometrium could be mediated by steroid hormones. It is known that estrogen stimulates ACE2 expression in differentiated airway epithelial cells [25]. In line with the ACE2 expression pattern in the present study, a previous study reported that the secretory phase endometrium expressed a higher level of Ang-(1–7) suggesting a higher ACE2 expression in the endometrium during the secretory phase of the cycle [26]. Nevertheless, recent data supported the balance of ACE and ACE2 in the human body which controls the expression of both molecules through the Ang II and Ang 1–7 concentrations in the body, and this phenomenon has been linked to the Covid-19 infection pathway as well [27]. Our present in vivo and in

vitro data also suggested reverse expression levels between ACE1 and ACE2 proteins in endometrial epithelial cells. However, further confirmation on the down-regulation of ACE1 and up-regulation of ACE2 in the secretory endometrium through steroid hormones and other molecules await further investigations.

All three ACEIs used in this study are known to be very potent inhibitors. However, it is important to note that none of them had any significant effect on the expression of their receptors in the Ishikawa cells at different concentrations. An earlier study suggested that ACE1 inhibitor reduces Ang II formation and Ang-(1–7) metabolism due to increased Ang II metabolism by ACE2 [12]. A recent report also suggested that patients receiving ACE1 inhibitors for the treatment of chronic disease were at risk of SARS-CoV-2 infection, partly due to the elevated expression of ACE2 receptor [5].

Given the current knowledge on ACE's action together with TMPRSS2 as receptors for SARS-CoV-2 virus entry, the human endometrium was proposed to be one of the putative infection sites for viral infection and subsequently leading to pregnancy loss. The TMPRSS2 is a serine-associated protease and it was known to be involved in the prostate gland functions in an androgen-dependent manner [28,29]. The serine proteases are known to play critical roles in many physiological processes, like protein catabolism, cell growth and migration, tissue rearrangement, and inflammation [30]. Interestingly, many of those mentioned processes are vital in human endometrial epithelial functions including embryo implantation. The result from this study suggested that TMPRSS2 expression is independent of the menstrual cycle stage. However, the strong staining intensity in both glandular and luminal epithelium suggests a functional role in embryo implantation. Higher TMPRSS2 protein and transcript expression were found in receptive endometrial Ishikawa and RL95–2 cells, arguing the role of TMPRSS2 expression and endometrial receptivity, and embryo implantation.

The spheroid-endometrial cells co-culture model was used in the present study. This model has been commonly used to evaluate the effect of different factors on early embryo attachment and has proven to be a well-accepted model for functional study [14,17,18,31]. We found the three ACE1 inhibitors; Enalaprilat, Captopril, and Zofenofril, and ACE2 inhibitor DX 600 did not affect the attachment of spheroids (embryo surrogate) onto the endometrial Ishikawa cells. Although the existing understanding of ACEIs in pregnancy is not clear, results from some studies supported the adverse effect on the fetus, but others are not [32–34]. However, given the role of angiotensins in uterine smooth muscles contraction and relation plus endothelial cells/blood vessels functions, it may be worth investigating the roles of the angiotensin system related to the human endometrial receptivity.

5. Conclusion

In conclusion, the human endometrium expresses ACE1, ACE2, and TMPRSS2 transcripts and proteins. Similar findings have been reported in recent studies [35–37]. The commonly used human receptive endometrial Ishikawa and RL95–2 cells both express ACE1, ACE2, and TMPRSS2 transcripts and proteins making them suitable for in vitro studies on endometrial receptivity and embryo implantation. Importantly, the ACEIs used in the current study did not alter the expression of ACE1, ACE2, and TMPRSS2 expressions, and the spheroid attachment onto Ishikawa cells, suggesting that the use of ACEIs for disease treatment may not affect the endometrial receptivity and subsequent embryo implantation. Yet, the effects of ACE inhibitors on subsequent pregnancy events including trophoblast invasion, stromal cell decidualization, and placentation remain to be investigated.

Funding

This study was supported in part by a Ferring COVID-19 investigational grant in Reproductive Medicine and Maternal Health (RMMH) and a GRF grant (17120720) from the Hong Kong Research Grant

Council to KFL.

Author's contributions

S.R.F., S.P.K., and K.F.L. conceived the concept of this study, designed and experimental approach. S.R.F., X.C., K.W.C., P.C.W., S.Q., and L.J. performed the experiments. E.H.Y.N. provided the clinical samples, and clinical insight into the study design, and edited the manuscript. S.P.K., E.H.Y.N., W.S.B.Y., and K.F.L. provided critical intellectual support, assisted with data analysis, and edited the manuscript. S.R.F., and K.F.L. wrote the manuscript. All authors contributed to the final editing and reviewed the manuscript.

Declaration of Competing Interest

None of the authors have relevant financial or non-financial interests to disclose.

References

- [1] Fountain JH, Lappin SL. Physiology, Renin Angiotensin System. StatPearls [Internet]. Treasure Island ((FL)). StatPearls Publishing; 2021.
- [2] Li XF, Ahmed A. Compartmentalization and cyclic variation of immunoreactivity of renin and angiotensin converting enzyme in human endometrium throughout the menstrual cycle. *Hum Reprod* 1997;12(12):2804–9.
- [3] Anton L, Merrill DC, Neves LAA, Diz DI, Corthorn J, Valdes G, et al. The uterine placental bed renin-angiotensin system in normal and preeclamptic pregnancy. *Endocrinology* 2009;150(9):4316–25.
- [4] Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell* 2020;181(2):271–80.
- [5] Fang L, Karakiulakis G, Roth M. Are patients with hypertension and diabetes mellitus at increased risk for COVID-19 infection? *Lancet Respir Med* 2020;8(4):e21.
- [6] Vilella F, Wang W, Moreno I, Roson B, Quake SR, Simon C. Single-cell RNA sequencing of SARS-CoV-2 cell entry factors in the preconceptual human endometrium. *Hum Reprod* 2021;36(10):2709–19. Sep 18.
- [7] Henarejos-Castillo I, Sebastian-Leon P, Devesa-Peiro A, Pellicer A, Diaz-Gimeno P. SARS-CoV-2 infection risk assessment in the endometrium: viral infection-related gene expression across the menstrual cycle. *Fertil Steril* 2020;114(2):223–32 (Aug).
- [8] Weatherbee BAT, Glover DM, Zernicka-Goetz M. Expression of SARS-CoV-2 receptor ACE2 and the protease TMPRSS2 suggests susceptibility of the human embryo in the first trimester. *Open Biol* 2020;10(8):200162.
- [9] Chadchan SB, Popli P, Maurya VK, Kommagani R. The SARS-CoV-2 receptor, angiotensin-converting enzyme 2, is required for human endometrial stromal cell decidualization. *Biol Reprod* 2021;104(2):336–43. Feb 11.
- [10] Baud D, Greub G, Favre G, Gengler C, Jaton K, Dubruc E, et al. Second-trimester miscarriage in a pregnant woman with SARS-CoV-2 infection. *JAMA* 2020;323(21):2198–200. Jun 2.
- [11] Lee WY, Mok A, Chung JPW. Potential effects of COVID-19 on reproductive systems and fertility; assisted reproductive technology guidelines and considerations: a review. *Hong Kong Med J* 2021;27(2):118–26 (Apr).
- [12] Ferrario CM, Jessup J, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, et al. Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation* 2005;111(20):2605–10. May 24.
- [13] Sriram K, Insel PA. A hypothesis for pathobiology and treatment of COVID-19: The centrality of ACE1/ACE2 imbalance. *Br J Pharmacol* 2020;177(21):4825–44.
- [14] Kottawatta KS, So KH, Kodithuwakku SP, Ng EH, Yeung WS, Lee KF. MicroRNA-212 regulates the expression of Olfactomedin 1 and C-terminal binding protein 1 in human endometrial epithelial cells to enhance spheroid attachment in vitro. *Biol Reprod* 2015;93(5):109.
- [15] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 2001;25(4):402–8.
- [16] Hohn HP, Linke M, Denker HW. Adhesion of trophoblast to uterine epithelium as related to the state of trophoblast differentiation: in vitro studies using cell lines. *Mol Reprod Dev* 2000;57(2):135–45.
- [17] Kodithuwakku SP, Ng PY, Liu Y, Ng EH, Yeung W, Ho PC, et al. Hormonal regulation of endometrial olfactomedin expression and its suppressive effect on spheroid attachment onto endometrial epithelial cells. *Hum Reprod* 2011;26(1):167–75.
- [18] Fernando SR, Kottawatta KSA, Jiang L, Chen X, Cheng KW, Wong BP, et al. Differential expression of protein disulfide isomerase (PDI) in regulating endometrial receptivity in humans. *Reprod Biol* 2021;21(2):100498.
- [19] Li Q, Guan X, Wu P, Wang X, Zhou L, Tong Y, et al. Early transmission dynamics in Wuhan, China, of novel coronavirus-infected pneumonia. *New Engl J Med* 2020;382(13):1199–207.
- [20] Chappell MC, Ferrario CMACE. and ACE2: their role to balance the expression of angiotensin II and angiotensin-(1-7). *Kidney Int* 2006;70(1):8–10 (Jul).

- [21] Vickers C, Hales P, Kaushik V, Dick L, Gavin J, Tang J, et al. Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J Biol Chem* 2002;277(17):14838–43.
- [22] Lalanne C, Mironneau C, Mironneau J, Savineau JP. Contractions of rat uterine smooth muscle induced by acetylcholine and angiotensin II in Ca²⁺-free medium. *Br J Pharm* 1984;81(2):317–26.
- [23] Qi R, Li TC, Chen X. The role of the renin-angiotensin system in regulating endometrial neovascularization during the peri-implantation period: literature review and preliminary data. *Ther Adv Endocrinol Metabol* 2020;11:2042018820920560.
- [24] Casalechi M, Dela Cruz C, Lima LC, Maciel LP, Pereira VM, Reis FM. Angiotensin peptides in the non-gravid uterus: Paracrine actions beyond circulation. *Peptides* 2018;101:145–9.
- [25] Stelzig KE, Canepa-Escaro F, Schiliro M, Berdnikovs S, Prakash YS, Chiarella SE. Estrogen regulates the expression of SARS-CoV-2 receptor ACE2 in differentiated airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2020;318(6):L1280–1.
- [26] Reis FM, Reis AM. Angiotensin-converting enzyme 2 (ACE2), angiotensin-(1-7) and Mas receptor in gonadal and reproductive functions. *Clin Sci* 2020;134(22):2929–41.
- [27] Zheng H, Cao JJ. Angiotensin-converting enzyme gene polymorphism and severe lung injury in patients with coronavirus disease 2019. *Am J Pathol* 2020;190(10):2013–7 (Oct).
- [28] Vaarala MH, Porvari K, Kyllönen A, Lukkarinen O, Vihko P. The TMPRSS2 gene encoding transmembrane serine protease is overexpressed in a majority of prostate cancer patients: detection of mutated TMPRSS2 form in a case of aggressive disease. *Int J Cancer* 2001;94(5):705–10. Dec 1.
- [29] Chen Y, Lee M, Lucht A, Chou F, Huang W, Havighurst TC, et al. Lin C. TMPRSS2, a serine protease expressed in the prostate on the apical surface of luminal epithelial cells and released into semen in prostasomes, is misregulated in prostate cancer cells. *Am J Pathol* 2010;176(6):2986–96.
- [30] Bond JS. Plasma membrane proteases: introductory remarks. *Biomed Biochim Acta* 1991;50(4–6):775–80.
- [31] Heneweer C, Kruse LH, Kindhauser F, Schmidt M, Jakobs KH, Denker HW, et al. Adhesiveness of human uterine epithelial RL95-2 cells to trophoblast: rho protein regulation. *Mol Hum Reprod* 2002;8(11):1014–22.
- [32] Steffensen FH, Nielsen GL, Sorensen HT, Olesen C, Olsen J. Pregnancy outcome with ACE-inhibitor use in early pregnancy. *Lancet* 1998;351(9102):596.
- [33] Ratnapalan S, Koren G. ACE inhibitors during pregnancy: are they safe? *Can Fam Physician* 2002;48:1047–9.
- [34] Moretti ME, Caprara D, Drehtuta I, Yeung E, Cheung S, Federico L, et al. The fetal safety of angiotensin converting enzyme inhibitors and angiotensin II receptor blockers. *Obstet Gynecol Int* 2012;2012:658310.
- [35] D'Ippolito S, Turchiano F, Vitagliano A, Scutiero G, Lanzone A, Scambia G, et al. Is there a role for SARS-CoV-2/COVID-19 on the female reproductive system? *Front Physiol* 2022;13:845156.
- [36] Liu A, Raja Xavier J, Singh Y, Brucker SY, Salker MS. Molecular and physiological aspects of SARS-CoV-2 infection in women and pregnancy. *Front Glob Women's Heal* 2022;3:756362.
- [37] Haouzi D, Entezami F, Tuailon E, Gala A, Ferrières-Hoa A, Brouillet S, et al. SARS-CoV-2 and Implantation Window: Gene Expression Mapping of Human Endometrium and Preimplantation Embryo. *Life*, 11. Switzerland: Basel; 2021. p. 1378.