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Calcitriol Downregulates ACE1/ACE2, Renin and TMPRSS2 Gene Expression in the Human Placenta

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Abstract: Angiotensin-converting enzyme (ACE)1, ACE2, and renin are components of the renin-angiotensin system (RAS), which regulates blood pressure. ACE2 also serves as a receptor for SARS-CoV-2 and together with the transmembrane serine protease 2 (TMPRSS-2), mediates viral cell-endocytosis. As the placenta expresses all these factors, it acts as a target for SARS-CoV-2 and also as a source of blood pressure modulators. An ACE1/ACE2 ratio imbalance can lead to RAS dysregulation and a bad prognosis in COVID-19 patients. Calcitriol, the vitamin D active metabolite, negatively regulates RAS, reduces inflammation, and enhances antiviral immunity, thereby playing a protective role against COVID-19 severity. Placental calcitriol has been inversely correlated with maternal blood pressure; however, its regulatory role in RAS components and SARS-CoV-2 receptors within the fetomaternal unit has been barely explored. Therefore, we investigated the effects of calcitriol on placental RAS components. Calcitriol downregulated ACE1, ACE2, TMPRSS-2, and renin gene expression in cultured syncytiotrophoblasts and the extravillous trophoblast cell line HTR-8/SVneo. The ACE1/ACE2 ratio was also downregulated by calcitriol. Similar results were obtained in syncytiotrophoblasts treated with calcidiol, the precursor of calcitriol. Altogether, these results support that vitamin D is essential in restricting SARS-CoV-2 placental infection while helping to regulate maternal blood pressure during pregnancy.

Keywords: syncytiotrophoblasts; renin-angiotensin-aldosterone; ACE1; ACE2; TMPRSS2; calcitriol; vitamin D; blood pressure; pregnancy

1. Introduction

A substantial and consistent amount of information has accumulated in recent decades regarding the beneficial effects of vitamin D (VD) during the perinatal period. VD exerts its biological activity mainly at the transcriptional level, through the binding of its active metabolite, calcitriol, to the vitamin D receptor (VDR). The human placenta is a target and a source of calcitriol, as it expresses both the VDR and the vitamin D-activating cytochrome CYP27B1. Aside from its well-known calciotropic properties, the biological effects of VD during pregnancy can be categorized into four main domains. Firstly, VD exhibits anti-inflammatory properties by downregulating systemic and placental inflammatory factors, thereby maintaining a balanced immune response by limiting an exacerbation during inflammatory events [1-3]. Secondly, VD boosts the innate immune response by inducing antimicrobial peptides, aiding in defense against viral and bacterial invasions [3-5]. Thirdly, VD helps regulate the synthesis of placental steroid and protein hormones, contributing to a healthy pregnancy [6-8]. Lastly, VD regulates blood pressure, a crucial factor for pregnant women. In this regard, it is well established that calcitriol downregulates renin gene expression, as demonstrated in renal cells both *in vitro* and *in vivo* [9]. As a result, calcitriol inhibits the renin–angiotensin system (RAS), which is tightly involved in blood pressure modulation and electrolyte and volume

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homeostasis [10,11]. The RAS mediates its effects by generating angiotensin II (Ang II), a potent vasoconstrictor peptide. Ang II is synthesized after two sequential cleavage reactions, the first one upon angiotensinogen, catalyzed by renin, and the second one upon the resulting product, angiotensin I (Ang I), by the angiotensin-converting enzyme (ACE1). Since renin is the rate-limiting enzyme in Ang II synthesis, factors that modify its transcriptional regulation and secretion are of clinical importance in hypertensive disorders. In this context, and consistently with the inhibition of renin by calcitriol, pre-clinical, clinical and epidemiological studies have shown a negative association between calcitriol and blood pressure, as well as a reduction in blood pressure after patients treatment with calcitriol, its analogs or its precursor cholecalciferol [9,12-17]. Similarly, a negative correlation between calcitriol cord blood levels and maternal blood pressure has been described in a cohort of pregnant women affected by urinary tract infections [18], strongly suggesting that placental calcitriol is involved in lowering maternal blood pressure. Blood pressure during pregnancy must be closely monitored since hypertensive disorders of pregnancy can significantly increase perinatal morbidity and mortality [19]. In this sense, the natural counter-regulatory mechanism of RAS activation is represented by ACE2, which hydrolyzes Ang I and Ang II into Ang-(1-9) and Ang-(1-7), respectively [20]. Particularly, Ang-(1-7) acts as a vasodilator, anti-hypertensive, antioxidant and anti-inflammatory peptide [20]. The human placenta expresses all components of the RAS [21,22], being ACE2 mainly expressed in the syncytiotrophoblast layer [23]. Interestingly, ACE2 also acts as a receptor for SARS-CoV-2, the causal agent of COVID-19 disease, which produced the recent health contingency pandemic [24]. Accordingly, SARS-CoV-2 viral particles have been mainly localized in syncytiotrophoblast cells (STB) at the materno-fetal interface of the placenta [24,25]. In the aftermath, among the most vulnerable groups, the impact of COVID-19 was particularly significant among pregnant women, given that this disease was associated with increased risk of maternal death, cardiovascular disorders, obstetric hemorrhage, hypertension, and preeclampsia [26]. The mechanism of SARS-CoV-2 infection starts with the binding of the coronavirus spike proteins (S) receptor binding domain (RBD) to host cells membrane ACE2, followed by S cleavage by the transmembrane serine protease TMPRSS2. This process allows the viral envelope to fuse with the host cell membrane, after which the viral RNA is released into the cell for translation into structural and accessory viral proteins needed for genome replication. Thus, both ACE2 and TMPRSS2 are fundamental for the SARS-CoV-2 infection, representing potential therapeutic targets [27]. Notably, a significant amount of information has accumulated supporting a protective role of VD against COVID-19 severity and mortality [28,29], which is thought to be mediated through the downregulation of proinflammatory cytokines, the production of antiviral proteins and several other mechanisms [30-32]. However, there are contrasting results in the literature regarding the effects of calcitriol on *ACE* and *TMPRSS2* expression in different tissues and conditions (Supplementary Table S1). At the same time, minimal information is available on the influence of VD in the components of RAS at the placental level. Therefore, in this study, we aimed to explore how calcitriol transcriptionally regulates RAS-related factors in cultured human trophoblast from term and first trimester placentas, to gain a deeper understanding on the potential role of placental VD in regulating COVID-19 susceptibility and maternal blood pressure during pregnancy.

2. Results

2.1. Calcitriol and Calcidiol Downregulate ACE1, ACE2, TMPRSS2 and Renin Gene Expression in Cultured Syncytiotrophoblast from Human Placentas

To investigate the effect of VD upon RAS components in the human placenta, we cultured primary trophoblast from healthy term placentas. These cells undergo spontaneous differentiation within 24-48 hours of culture, fusing into syncytiotrophoblasts (STB), which represent the endocrinologically most active placental cell lineage [33]. Cultured trophoblast cells from term placenta were allowed to differentiate into syncytiotrophoblasts within 24 hours of culture and further incubated for an additional 24 hours in the presence of increasing calcitriol concentrations (0.1 – 100 nM) or 0.1 % ethanol as its vehicle. Afterward, RNA was extracted for RT-qPCR assay. In

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cultured trophoblast cells, calcitriol significantly downregulated, in a concentration-dependent manner, the gene expression of *ACE1*, *ACE2* and renin (*REN*). *TMPRS2* gene expression was also reduced, although not significantly (Figure 1).

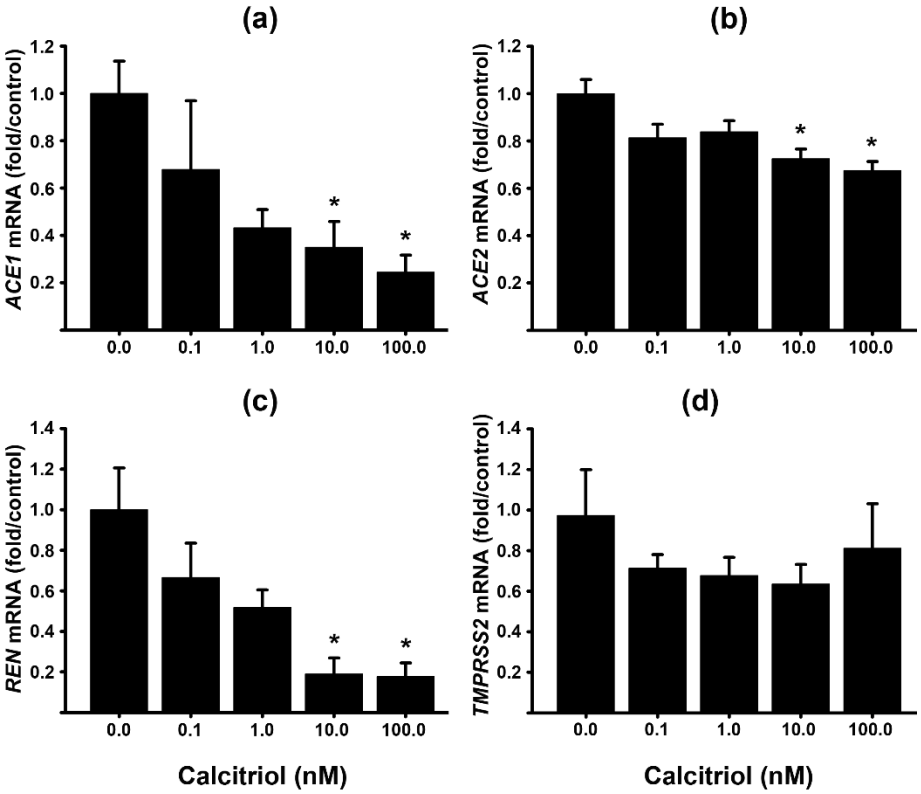

Figure 1. The gene expression of *ACE1, ACE2, TMPRSS2* and *REN* is downregulated by calcitriol in a concentration-dependent manner in human placental syncytiotrophoblasts. Cultured trophoblast cells from term human placentas were allowed to differentiate into syncytiotrophoblasts within 24 hours of culture. They were further incubated for another 24 hours in the presence of increasing calcitriol concentrations or its vehicle (0.1% ethanol). Afterward, RNA was extracted and gene expression was analyzed by RT-qPCR using specific primers for the human genes (a) *ACE1*, (b) *ACE2*, (c) *REN* and (d) *TMPRSS2*. Results were normalized against the gene expression of GAPDH, and vehicle values were set to 1 in all cases for normalization. At least 4 different cell cultures from different placentas were used. Bars represent the mean ± SEM. * $P < 0.05$).

While calcitriol is the active hormonal VD form, its precursor 25-hydroxyvitamin D_3 (calcidiol) must be biotransformed into calcitriol by CYP27B1 to activate the VDR and exert its biological effects. Since the placenta expresses both CYP27B1 and the VDR [7,34,35], using calcidiol provides additional information on the viability of cultured placentas cells by reflecting intracrine calcitriol production, as previously described [35]. Therefore, as a control, we cultivated trophoblasts with a single calcidiol concentration (1 µM), which was chosen as considered previously [36]. As shown in Figure 2, and similarly as observed with calcitriol, *ACE1, ACE2* and *REN* gene expression was inhibited by calcidiol, whereas *TMPRSS2* was not modified by calcidiol (Figure 2).

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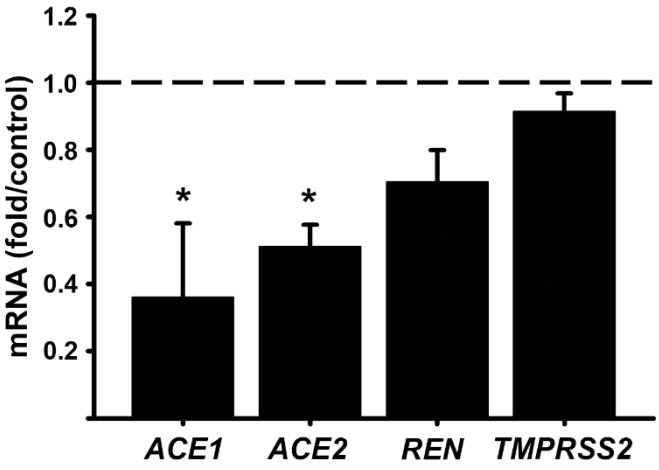

Figure 2. Calcidiol, the precursor of calcitriol, reduces RAS components gene expression in cultured human syncytiotrophoblasts. Cultured trophoblasts were allowed to differentiate into syncytiotrophoblasts for 24 hours, and then were incubated in the presence of 1 calcidiol (µM) or its vehicle (0.1% ethanol) for additional 24 hours. The day after, RNA was extracted and gene expression was analyzed by RT-qPCR using specific primers for the RAS components. Results were normalized against the gene expression of GAPDH, and vehicle values were set to 1 (the dotted line representing control values). Three different cell cultures were used. Bars represent the mean ± SEM. * $P < 0.05$ *vs.* control.

2.2. Calcitriol Inhibits RAS Components in the Placental Cell Line HTR-8/SVneo

In this study, we aimed to compare the potential differential effects of calcitriol in the placenta depending on the pregnancy trimester and cellular type. HTR-8/SVneo (HTR-8) is a human trophoblast cell line derived from a first-trimester placenta, with characteristic features of extravillous invasive trophoblast (EVT). Therefore, we used this cell line to compare EVTs and STB placental cells. As seen in Figure 3, and similarly to placental STB, calcitriol significantly downregulated *ACE1, ACE2,* and *REN* gene expression (Figure 3). Contrary to STBs, calcitriol robustly inhibited *TMPRSS2* mRNA at all concentrations tested (0.1 – 10 nM). *ACE1* was also robustly inhibited starting from the lowest dose tested (0.1 nM), while *ACE2* was significantly inhibited from 1.0 nM. Therefore, EVTs were more sensitive to calcitriol inhibitory effects upon the RAS axis than STB.

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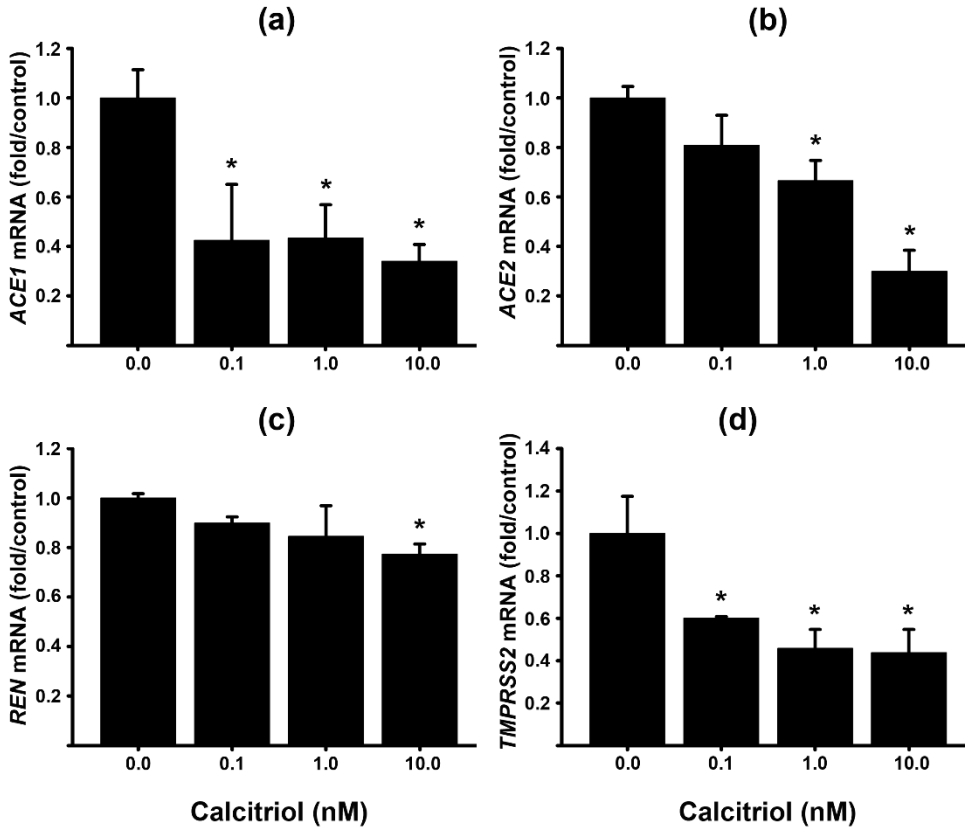

Figure 3. Calcitriol inhibits the gene expression of RAS components in the placental EVT cell line HTR8. Cultured HTR8 cells were incubated with increasing calcitriol concentrations or its vehicle (0.1% ethanol) for 24 hours. The day after, RNA was extracted and gene expression was analyzed by RT-qPCR using specific primers for the human genes (a) ACE1, (b) ACE2, (c) REN, and (d) TMPRSS2. Results were normalized against GAPDH, used as a housekeeping gene, and vehicle values were set to 1 for normalization. N = 3. Bars represent the mean ± SEM. * $P < 0.05$.

2.3. Syncytiotrophoblast Cells Exibit Greater Expression of RAS Components than HTR8 EVT Cells, While ACE1/ACE2 Ratio is Higher in the Latter

It has been reported that ACE2 protein is predominantly found in the STB layer of chorionic villi, as compared to EVT or other placental cell types [23,37]. Therefore, we were interested in knowing if this difference was also valid at the mRNA level in our cultured STB and EVT cells. In addition, we compared the basal gene expression of the other RAS components. In line with a previously reported immunohistochemical study [37], we found that ACE2 gene expression was several folds more expressed in STB than in EVT HTR8 cells. This result was comparable to the other RAS components, except for ACE1, which was found within a similar range (Table 1). However, the ACE1/ACE2 ratio was higher in HTR8 cells (7.4×10^{-2}) than that calculated for STB cells (9.4×10^{-5}).

Table 1. Basal gene expression of RAS genes in syncytiotrophoblast cells (STB) and EVT (HTR8).

Gene	STB	HTR8	*P*
ACE1	$2.7 \times 10^{-6} \pm 7.2 \times 10^{-7}$	$1.7 \times 10^{-6} \pm 3.4 \times 10^{-7}$	0.58
ACE2	$2.9 \times 10^{-2} \pm 4.6 \times 10^{-3}$	$2.3 \times 10^{-5} \pm 3.8 \times 10^{-6}$	< 0.001
TMPRSS2	$1.8 \times 10^{-2} \pm 8.8 \times 10^{-3}$	$1.8 \times 10^{-4} \pm 6.5 \times 10^{-5}$	< 0.001
REN	$3.2 \times 10^{-2} \pm 5.3 \times 10^{-3}$	$8.7 \times 10^{-5} \pm 3.3 \times 10^{-5}$	< 0.001

The gene expression is presented relative to that of GAPDH. Results are shown as the mean ± SEM from a minimum of 3 experiments, each with triplicate replicates. Statistical significance was determined using the Rank Sum Test, comparing the gene expression of each gene in STB and HTR8.

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2.4. Calcitriol Reduces ACE1/ACE2 Ratio

An increased expression of ACE1 relative to ACE2 has been associated with inflammation and various pathologies' morbidity. Indeed, an imbalance in the ACE1/ACE2 ratio may result in increased blood pressure, inflammation, and susceptibility to COVID-19 infection [38,39]. Therefore, we analyzed the *ACE1/ACE2* ratio in function to the exposure of cells to different calcitriol concentrations. Figure 4 shows the calcitriol-dependent reduction of *ACE1/ACE2* ratio in STB (Figure 4a) and HTR8 EVT cells (Figure 4b). As illustrated, the *ACE1/ACE2* ratio in STB cultures was downregulated progressively with increasing calcitriol concentrations. Interestingly, although this ratio was also inhibited by calcitriol in HTR8 cells, there was a divergence in the trend's pattern, as the highest reduction on ACE1/ACE2 ratio was achieved with the lowest calcitriol concentration.

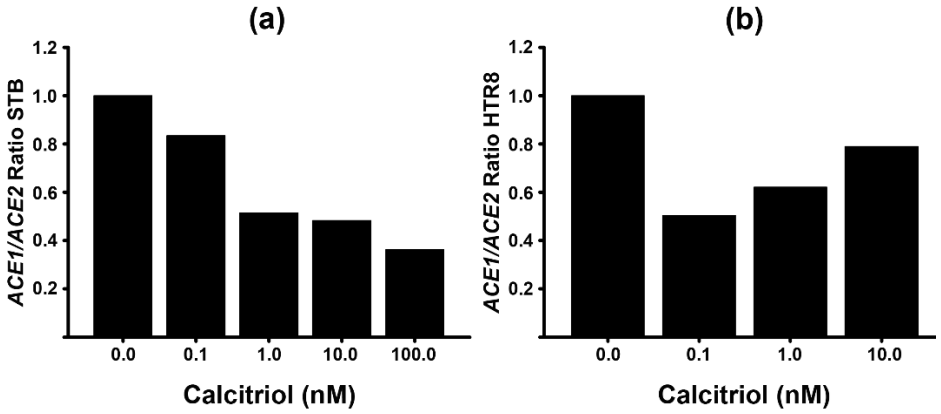

Figure 4. ACE1/ACE2 ratio in human placental cells. The ACE1/ACE2 ratio, based on the gene expression of both enzymes, was calculated in syncytiotrophoblasts (STB) (a) or in HTR8 cells (b) treated with different calcitriol concentrations.

3. Discussion

It is a well-known fact that SARS-CoV-2 uses ACE2 as a receptor in host cells and that TMPRSS2 primes S protein to facilitate viral RNA entry into the cells [27], highlighting the importance of these proteins in cell infection and COVID-19 therapeutic opportunities. The human placenta expresses *ACE2* and *TMPRSS2*, whose levels vary depending on cell type and pregnancy trimester [40]. While conflicting information on the transmission of SARS-CoV-2 from mother to child exists, it is now well recognized that this virus can vertically infect the fetus by bypassing placental barriers, albeit at a low rate [41,42]. Importantly, STB is highly susceptible to SARS-CoV-2, representing the primary target of infection among placental cells, most probably due to the high levels of *ACE2* and *TMPRSS2*; thus, supporting SARS-CoV-2 efficient entry and replication [41,43,44]. Accordingly, ACE2 knockdown in cultured placental cells abolished viral infection, drastically decreasing the SARS-CoV-2 genome presence in supernatants and cell lysates [43]. Similarly, the SARS-CoV-2 infection of STB cells was prevented by using anti-ACE2 antibodies [44]. This background strongly supports ACE2 and TMPRSS2-targeting as a strategy to prevent placental infection and vertical transmission of the virus. In the present study, we show that calcitriol and its precursor calcidiol significantly downregulated *ACE2* gene expression both in primary STB and HTR8 cell cultures, firmly suggesting that intracrine and exogenous calcitriol can diminish the risk of viral entry into trophoblast cells, placental SARS-CoV-2 infection, and consequently, fetal infection. Our results are supported by an *in silico* analysis identifying the VDR as a putative repressor of *ACE2* gene expression [45]. Moreover, calcitriol also diminished *TMPRSS2* gene expression in first trimester HTR8 cells. Notably, in *in vitro* and experimental animal studies, *TMPRSS2* KO contributed to reduced viral lung replication, low proinflammatory reaction, and mild lung pathology [46], while the functional inhibition of this protease blocked SARS-CoV-2 entry [27], demonstrating a positive defensive outcome through the suppression of TMPRSS2. Interestingly, TMPRSS2 is an androgen-stimulated gene, containing multiple androgen response elements upstream of its gene transcription start site [47,48], partially

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explaining the male bias in susceptibility to severe COVID-19 disease and mortality [49,50]. Accordingly, TMPRSS2 expression has been found significantly higher in placentas from male fetus compared to those from female [51]. Thus, its downregulation by calcitriol might have special positive implications for male offspring vulnerability, deserving further studies.

If our results are replicated in other cell types, they could contribute to explain the acknowledged preventive role of VD sufficiency upon COVID-19 infection and severity [28,29]. However, we did not anticipate the outcomes on *TMPRSS2* and *ACE2* gene expression, given the contrasting information reported in different tissues and conditions, with ACE2 being either upregulated or downregulated by VD derivatives (Supplementary Table S1). Nevertheless, our findings complement other studies postulating additional mechanisms by which active VD metabolites can impede viral entry. For instance, computational and functional analyses have demonstrated the potential for VD derivatives to physically bind ACE2 and TMPRSS2, thereby affecting their ability to recognize and prime the SARS-Cov2 S protein [52,53]. Similarly, molecular simulations have shown the feasibility of the bonding between VD derivatives and the spike protein, stabilizing it in the locked conformation, thus inhibiting its interaction with the host receptor [53,54]. Moreover, VD hydroxymetabolites have shown the potential to inhibit SARS-CoV-2 infection by restricting its replication cycle, through targeting SARS-CoV-2 replication enzymes [55]. Altogether, this information identifies VD active compounds as potential natural therapeutic agents for preventing placental SARS-CoV-2 infection.

On the other hand, it is well established that VD limits RAS activity by inhibiting renin expression [9-11]. Renin is released by the kidneys into the bloodstream when blood pressure drops. Then, renin catalytically cleaves circulating angiotensinogen to form Ang I, which ACE1 converts into Ang II, a potent vasoconstricting peptide hormone that efficiently raises blood pressure. Notably, experimental studies in mice have shown that renin from placental origin is released into maternal circulation and can cause hypertension [56]. In addition, it has been reported that the risk of developing hypertensive disorders of pregnancy in patients who tested positive for COVID-19 is over 70% higher than in those who did not [57], which is in line with the described association between severe COVID-19 and hypertension [58]. Therefore, an equilibrated placental RAS regulation, emphasizing renin expression, is crucial for an adequate pregnancy outcome, especially under the threat of COVID-19 infection. In this regard, we found in this study that REN gene expression was significantly downregulated by calcitriol in STBs and EVT cells. Both types of placental cells play distinct and important roles in the placenta, STBs primarily contributing to hormonogenesis, nutrient exchange, and immune regulation, while EVTs are involved in establishing the placental-uterine interface and maternal spiral arteries remodeling. The fact that calcitriol downregulated placental *REN* gene expression might be associated with its capacity to lower maternal blood pressure, which is in line with previous findings from our laboratory showing a negative correlation between cord blood calcitriol and maternal systolic and diastolic blood pressure in a cohort of patients with urinary tract infections [18]. While hypertension is associated with maternal mortality and perinatal morbidity, the causative mechanisms are not yet fully clarified. However, several conditions have been associated with this disorder, including placental ischemia, abnormal EVT invasion of spiral arteries, endothelial dysfunction, and increased production of placental vasoconstrictor factors [59]. Additional risk factors are placental and systemic inflammation, characterized by exacerbated inflammatory cytokines production [60]. Notably, the cytokine storm is a characteristic feature of COVID-19 infection, while placental inflammation and malperfusion have been reported in SARS-CoV-2-infected placentas [61]. Thus, the ability of calcitriol to restrain inflammation by downregulating placental inflammatory cytokines production [2] and to inhibit REN gene expression in trophoblast cells, as shown herein, may help to regulate blood pressure both under normal and COVID-19 infection conditions. Our results concur with Chen Chun-yan et al., who described that the treatment with calcitriol or calcidiol significantly reduced REN gene expression in cultured decidual epithelial cells [62].

An imbalance in RAS and its ACE1/ACE2 components has been proposed as a driver of COVID-19 pathobiology [38,63]. Indeed, SARS-CoV-2 infection causes inhibition of ACE2 in infected cells,

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increasing Ang II signaling [64]. In our study, the ratio *ACE1*/*ACE2* was significantly reduced by calcitriol in placental cells, showing different trends depending on the hormone concentration. This result could reflect a positive outcome *in vivo*, given that a high ACE1/ACE2 ratio has been recognized as a critical predictor for COVID-19 severity [63]. Although calcitriol reduced this ratio in both cell types, the observed concentration-dependent differences might be consequential to the basal expression levels of both enzymes in each cell type, being ACE2 increased in STBs as compared to HTR8 cells, while ACE1 was within a similar expression rate. Also, the robust calcitriol-dependent inhibitory effect upon ACE1 in STBs compared to HTR8 might play a role, given that EVT cells required higher calcitriol concentrations for ACE1 downregulation compared to STBs. Based on the baseline expression levels of RAS components, it appears that STBs play a more preponderant role in the RAS placental system than EVT cells.

Previously, as an extrapolation of VD inhibiting the RAS, it had been postulated that VD restricts SARS-CoV-2 viral entry through the downregulation of ACE2 expression [32,65]. However, contradictory and limited information exists on ACE2 transcriptional regulation by calcitriol (Supplementary Table S1). At the same time, there is no information on the effects of VD derivatives on *ACE2* and other RAS components' expression at the feto-maternal unit, aside from one study regarding *REN* in decidua [62]. Thus, to our knowledge, this is the first report showing the transcriptional regulation of placental RAS components by VD natural derivatives in two different cell types.

While ACE2 helps to regulate blood pressure by shifting the balance towards vasodilators and anti-hypertensive factors such as Ang-(1-7), it also facilitates SARS-Cov-2 entry by binding to the viral S protein. These facts highlight the role of ACE2 as a double-edged sword in the context of COVID-19. In this scenario, our results showing *ACE2* and *TMPRSS2* downregulation by calcitriol suggest the reduction of the entry gate of SARS-CoV-2 to the placenta. At the same time, the inhibition of *ACE1* and *REN* may account for blood pressure attenuation. The latter is supported by the reduced *ACE1*/*ACE2* ratio in calcitriol-treated cells observed in this study. This outcome could translate into therapeutic opportunities for the prevention and mitigating effects of COVID-19.

Among the limitations of our study, the lack of RAS components protein analysis, Ang(1-7) quantification, and *in vivo* experiments, remain areas that warrant further exploration. In addition, since the RAS placental system is involved in placentation by participating in spiral artery remodeling, trophoblast invasion and placental angiogenesis [22], further studies are needed to understand the impact of VD as a modulator factor of placental RAS.

In summary, our results support the beneficial effect of VD sufficiency during pregnancy as a protective factor against hypertensive disorders and, possibly, COVID-19 vertical transmission.

4. Materials and Methods

4.1. Reagents

The RNA extraction solution Trizol was purchased from Life Technologies, Carlbad, USA. The LightCycler TaqMan Master reaction was bought from Roche (Roche Applied Science, IN, USA). Reverse transcription (RT) system (Maxima™ Reverse Transcriptase) was from Thermo (Thermo-Scientific, St. Louis, MO, USA). Calcidiol and calcitriol were from Sigma-Aldrich (St. Louis, MO).

4.2. Cell Cultures

The placentas from uncomplicated pregnancies were processed and cultured as previously described [33,36]. Briefly, villous tissue was enzymatically treated and trophoblasts were separated on Percoll gradients. After estimating cell viability (0.4% trypan-blue), the cells were seeded in supplemented medium (DMEM-HG, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal bovine serum) and maintained under standard culture conditions. Trophoblasts were incubated for 24 hours and then exposed to calcidiol (1 μM), calcitriol at different concentrations, or their vehicle (ethanol 0.1%), for one more day. The HTR8 cell line was from the American Type Culture Collection (ATCC, HTR-8/SVneo CRL-3271) and was maintained with supplemented medium. This cell line was

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derived from a first-trimester placenta, specifically from the cell population that grew out from chorionic villi explants. As described by ATCC, these cells express proteins typical of extravillous invasive trophoblast cells.

The study methodologies conformed to the standards set by the Declaration of Helsinki. The placentas used in this study were obtained from protocols approved by the applicable Medical Ethical Committee from the Instituto Mexicano del Seguro Social (R-2013-785-033) and the Ethical and Research Committees from Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (REF 2221).

4.3. Reverse Transcription and Real-Time PCR Amplifications (qPCR)

The total RNA was extracted from cultured trophoblasts following instructions from the TRIzol reagent manufacturer (Invitrogen, Carlsbad, CA). RNA concentration was estimated spectrophotometrically at 260/280 nm, and 2 μg of RNA were used to synthesize cDNA using the Maxima First Strand cDNA Synthesis Kit (ThermoFisher Scientific). The cDNA was then submitted to standard qPCR conditions using the LightCycler 480 Instrument (Roche Diagnostics, Mannheim, Germany). The expression of the housekeeping gene *GAPDH* was used for normalization purposes. The oligonucleotides were synthesized by Integrated DNA Technologies (IDT), and their sequences and number of hydrolysis probes are described in Table 2.

Table 2. Primers sequence and probes for RT-qPCR.

Gene	Upper primer	Lower primer	Probe number
ACE1	ctgctcatctgctgggagac	ttgtctgggaaaggcaccac	33
ACE2	ttctgtcacccgattttcaa	tcccaacaatcgtgagtgc	4
TMPRSS2	acctgatcacaccagccatg	tcaccctggcaagaatcgac	4
GAPDH	agccacatcgctgagacac	gcccaatacgaccaaatcc	60
REN	taccttttggtctcccgacag	ttgagggcattctcttgagg	77

Hydrolysis probes are from the Universal Probe Library (Roche).

4.5. Statistical Analysis

The statistical differences were calculated by one-way ANOVA, using a specialized software package (SigmaStat, Jandel Scientific). For the comparisons between the relative gene expression of RAS genes in STB and EVT, the statistical significance was calculated by the Rank Sum Test. Differences were considered statistically significant at $P < 0.05$.

5. Conclusions

Vitamin D metabolites inhibited the gene expression of RAS components and *ACE1*/*ACE2* ratio in placental cells. These results have important implications for COVID-19 susceptibility and maternal blood pressure regulation, suggesting that VD supplementation may not only decrease the risk of SARS-CoV-2 infection, but also reduce the likelihood of COVID-19 worsening during pregnancy.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Vitamin D effect on RAS components in different tissues and/or conditions.

Author Contributions: Validation, methodology, investigation, formal analysis, writing-review and editing, R.V.-C.; validation, methodology, investigation, formal analysis, writing-review and editing, J.G.-Q. and A.O.-O.; methodology, formal analysis and visualization, writing-review and editing, E.A.; writing-review and editing, F.L.; conceptualization, validation, methodology, investigation, formal analysis, visualization, writing-original draft preparation, supervision, project administration and funding acquisition, L.D. All authors have read and agreed to the published version of the manuscript.

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