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Effects of vitamin C and D on the mRNA expression of angiotensin converting enzyme 2 receptor, cathepsin L, and transmembrane serine protease in the mouse lungs

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ABSTRACT

Vitamins (Vit) C and D are widely used as immunogenic supplements among severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infected patients. The SARS-CoV-2 virus enters into the pulmonary endothelial cells through attachment to angiotensin converting enzyme 2 receptor (*Ace2*) and the proteolytic activity of Cathepsin L (*Ctsl*) and transmembrane serine protease 2 (*Tmprss2*) enzymes. This study aimed to determine the influence of Vit C and D on the mRNA expression of *Ace2*, *Tmprss2*, and *Ctsl* genes in the mouse lungs. Vitamins C and D were administered to different groups of mice through intra-peritoneal route in doses equivalent to human for 30 days. Then, the mRNA expression of SARS-CoV-2 entry gene was analyzed using qRT-PCR. It is found that Vit D, but not C, upregulated significantly ($P < 0.05$) the mRNA expression of *Ace2* by more than six folds, while down-regulated the expression of *Ctsl* and *Tmprss2* genes by 2.8 and 2.2 folds, respectively. It can be concluded from this study that Vit D alters the mRNA expression of *Ace2*, *Tmprss2*, and *Ctsl* genes in the mouse lungs. This finding can help us in understanding, at least in part, the molecular influence of Vit D on genes involved in the entry of SARS-CoV-2 into the cells.

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1. Introduction

COVID-19 infection is a global endemic disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1,2]. This virus infects the epithelial cells in the lung through interaction between the viral spike and certain proteins in the host cells [3]. SARS-CoV-2 attaches with the angiotensin converting enzyme type 2 (ACE2) receptor on the host cells [4,5]. Cathepsin isoform L (*Ctsl*) is a protease enzyme which cuts the S-glycoprotein in SARS-CoV-2, which facilitates the attachment of the virus to ACE2 receptor on the host cells. Furthermore, the transmembrane protease serine 2 (TMPRSS2) enhances the entrance of SARS-CoV-2 to the host cells through splitting certain parts in the virus [6]. It was pointed out that alterations in the expression of *Ace2*, *Tmprss2*, and *Ctsl* proteins in the host cells affect the risk and severity of COVID-19 infection [7,8].

Immunogenic agents are widely prescribed for COVID-19 patients [9]. Vitamin (Vit) D affects the immune system and modulates both the innate and adaptive responses. Low plasma level of Vit D was associated with increased susceptibility to COVID-19 infection [10]. Jain et al., 2020, showed that Vit D level was low among critically ill COVID-19 patients [11].

Accordingly, they recommended to prescribe Vit D to high risk and COVID-19 infected patients.

Vitamin C plays a major role in immune defense through enhancing of the innate and adaptive immune system [12]. Chiscano-Camón et al., 2020, found that low level of plasma Vit C was associated with acute respiratory distress syndrome among COVID-19 patients [13]. However, the mechanism of how Vit C protects against acute respiratory distress syndrome is still unknown. Malla et al., 2021, reported that Vit C has a capability for direct inhibition of protease enzymes, which are responsible for SARS-CoV-2 entry [14]. The researchers used *in silico* method and showed that Vit C can inhibit the 3-chymotrypsin protease. Additionally, they reported that Vit C itself can inhibit other protease enzymes expressed in other viruses rather than SARS-CoV-2 [13].

Vitamins C and D are widely used for SARS-CoV-2 infected patients. However, their influences on SARS-CoV-2 entry receptors and enzymes are still not investigated. We hypothesized that Vit C and D decrease the mRNA expression of the SARS-CoV-2 entry genes. The results of the present study can increase the understanding of the influence of Vit C and D on the entrance of SARS-CoV-2 into the host cells.

2. Materials and methods

2.1. Chemicals

Vitamins C and D were bought from Sigma-Aldrich (St. Louis, USA). The Trizol solution was purchased from Thermo Fisher Scientific (Massachusetts, USA). The cDNA synthesis kit was obtained from Applied Biosystems (Massachusetts, USA). The SYBER GREEN master mixture for real-time PCR was bought from Takara (Tokyo, Japan). The oligo DNA primers were purchased from Integrated DNA technologies (Coralville, Iowa, USA).

2.2. Experimental animals

Twenty-eight male Balb/c mice (*Mus musculus*), with an average weight between 24 and 28 g, were obtained from the animal house of Al-Zaytoonah University (Amman, Jordan). The mice were handled according to the guide of Canadian Council on Animal Care [15] and the ethical committee at Al-Zaytoonah University approved the protocol of this study. The animals were housed at $23 \pm 1^\circ\text{C}$, and a 12 hours light/12 hour dark cycle. All mice were fed with standard animal pellets *ad libitum*.

2.3. Experimental protocol

The mice were grouped according to the Vit administration, as follows:

Negative control group: the mice were administered the drug vehicle 10% DMSO through the intraperitoneal route once daily.

Vitamin C group: the mice were administered 179 mg/kg vit C, dissolved in 10% DMSO, through a single intraperitoneal dose once daily.

Vitamin D group: the mice were administered 357 units/kg vit D, dissolved in 10% DMSO, through a single intraperitoneal dose once daily.

Each group contained seven mice. The Vit C and D were administered to the animals for 1 month. The used doses of Vits, in this study, were equivalent to the daily dose of humans, depending on the surface area of the mouse's body [16].

2.4. RNA extraction and cDNA synthesis

The mice were sacrificed at the last day of drug administration. Then, around 100 mg of lung was

isolated from each mouse. The RNA was isolated from the mouse lungs using Trizol solution, as described by the instructions of the factory. After that, the isolated mRNA was converted to cDNA as following: 1 μg of total RNA was reconstituted in a reaction mixture containing 100 pmol oligo deoxythymine, 2.5 mM deoxy-nucleic triphosphate mixture, 0.1 M dithiothreitol, 1X reverse transcriptase buffer, and 10 units of reverse transcriptase isolated from Moloney Murine Leukemia Virus. This reaction mixture was incubated at 37°C for 50 minutes.

2.5. Gene expression analysis

The mRNA expression of *Ace2*, *Ctsl*, *Tmprss 2* genes was analyzed in this research using quantitative real-time polymerase chain reaction (qRT-PCR). The primer sequence, amplicon size, and the annealing temperature for each amplified gene are shown in Table 1. Briefly, 10 ng of the synthesized cDNA was added to a reaction mixture containing 1X of SYPER GREEN master mix and 10 pmoles of sense and antisense primers. The PCR reaction consisted of 40 cycles. Each cycle was as the followings: denaturation step at 95°C for 12 sec, annealing step at $53\text{--}58^\circ\text{C}$ for 30 sec, and lastly the extension step at 72°C for 25 sec. β -Actin gene was used as a housekeeping gene. The relative mRNA expression of the targeted genes was calculated using $\Delta\Delta\text{CT}$ method [17].

Histological analysis

The lungs of the mice were isolated and washed with 0.9% normal saline, then fixed in 10% formalin. After that, dehydration of the lung samples was done through passing the samples through a graded series of alcohol followed by xylene. Then, the samples were embedded in paraffin wax. Lastly, hematoxylin and eosin were used to stain the sample sections.

2.6. Statistical analysis

The relative mRNA expression was expressed as a fold change in comparison with the negative control group. One-way ANOVA with Tukey's post-test was used as a statistical tool for comparison between the mRNA expression of the tested genes of the control and Vit C and D treated groups. *P* value was considered significant when it was less than 0.05.

Table 1. The primer sequence, amplicon size, and annealing temperature of *Ace2*, *Ctsl*, and *Tmprss* genes.

Gene name	Forward	Reverse	Size	Annealing temp
<i>Ace2</i>	ATTCACCCAACACTTGAGCC	TGTCCATCGAGTCATAAGGGT	213	55
<i>Ctsl</i>	AGGAAAATGGAGGTCTGGACT	GCAACAGAAAATAGGCCCCAC	205	58
<i>Tmprss 2</i>	CGTTCCCGTATACTCCAGGT	CGTTCCCGTATACTCCAGGT	221	58
β -Actin	CCCCTGAGGAGCACCGTGTG	ATGGCTGGGGTGTGAAGGT	106	53

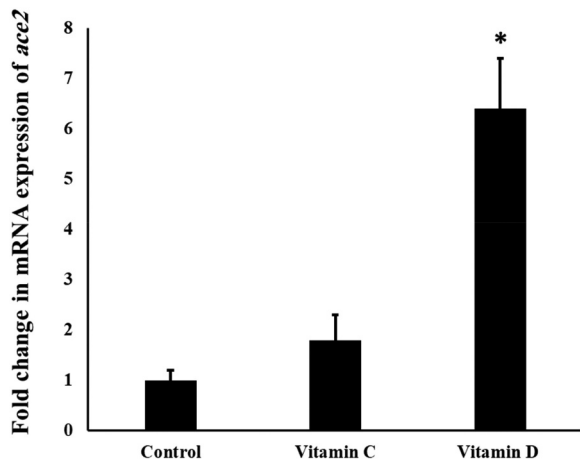


Figure 1. The influence of Vit C and D on the mRNA expression of *Ace2* gene in the mouse lungs.

3. Results

3.1. mRNA levels of *Ace2* gene after administration of Vit C and D

Figure 1 shows the effect of Vit C and D on the mRNA levels in the mouse lungs. It is found that Vit D upregulated significantly (P value <0.05) the mRNA expression of *Ace2* in the lungs by six folds. The level of *Ace2* mRNA was increased slightly (1.7 folds) after Vit C administration, however it failed to reach the statistical difference (P value >0.05).

* indicates statistical significance (P value <0.05) using one-way ANOVA test.

3.2. mRNA levels of *Tmprss* gene after administration of Vit C and D

It was found in this study that Vit D downregulated significantly (P value <0.05) the mRNA expression of *Tmprss2* gene in the mouse lungs by 2.8 folds, while Vit C did not show any significant influence on the expression of *Tmprss2* gene, as represented by Figure 2.

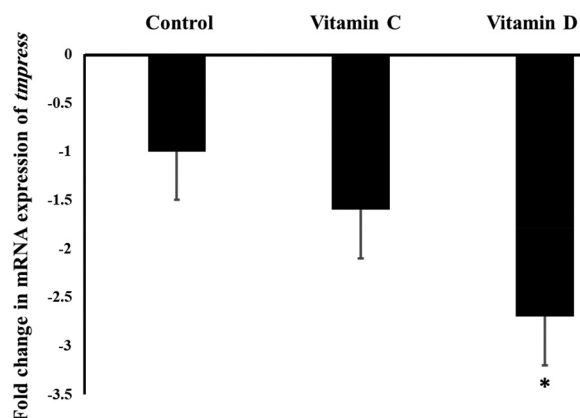


Figure 2. The influence of Vit C and D on the mRNA expression of *Tmprss2* gene in the mouse lungs.

* indicates statistical significance (P value <0.05) using one-way ANOVA test.

4. mRNA levels of *Ctsl* gene after administration of Vit C and D

As shown in Figure 3, the expression of *Ctsl* gene in the mouse lungs was downregulated significantly (P value <0.05) after Vit D treatment by 2.2 folds, while Vit C did not show any significant (P value >0.05) influence on the expression of *Ctsl* gene.

* indicates statistical significance (P value <0.05) using one-way ANOVA test.

Histological examination

Figure 4 represents the histological analysis of the lung sections after administration of Vit C and D to the mice for 1 month. The results showed that administration of Vit C and D did not induce pathohistological alterations in the mouse lungs.

5. Discussion

The entry of SARS-CoV-2 virus in pulmonary cells requires the host *Ace2*, *Tmprss*, and *Ctsl* [18]. It is hypothesized that alterations in the functionality and number of *Ace2*, *Tmprss*, and *Ctsl* in the lung affect the severity and the spread of SARS-CoV-2 infection [19]. Vitamins C and D are widely administrated to SARS-CoV-2 infected patients, and as prophylaxis against COVID-19 infection [20]. Some clinical studies found that these Vits have favorable effects among SARS-CoV-2 infected patients [21]. Therefore, this study investigated the influence of Vit C and D on the mRNA expression of SARS-CoV-2 entry genes. It is found in this study that Vit D altered the mRNA expression of SARS-CoV-2 entry genes, while Vit C did not show a significant influence. Vitamin D upregulated ($P < 0.05$) the mRNA expression of *Ace2*, while downregulated significantly ($P < 0.05$)

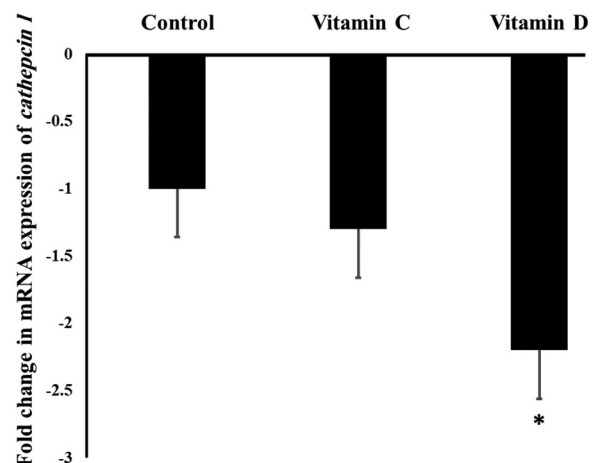


Figure 3. The influence of Vit C and D on the mRNA expression of *Ctsl* gene in the mouse lungs.

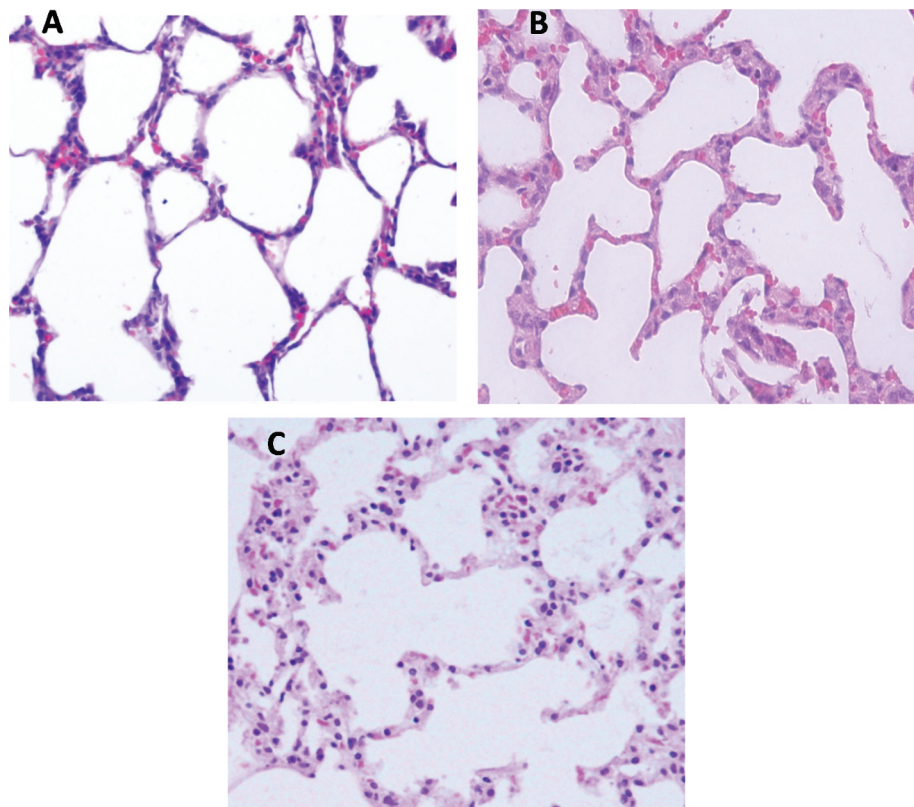


Figure 4. Light micrograph in the lung sections of mice received (A) the vehicle 10% DMSO, (B) Vit C dissolved in 10% DMSO, and (C) Vit D dissolved in 10% DMSO.

the mRNA expression of *Tmprss2* and *Ctsl* genes in the treated mice. These results suggest that Vit D has a molecular influence on SARS-CoV-2 entry genes that can prevent or reduce the transmission of SARS-CoV-2 virus within the epithelial cells in the lung. Further investigations are needed to determine the role of Vit D in the prevention of the entry of SARS-CoV-2 into the epithelial cells.

It was found that Vit D levels were negatively correlated with the severity of COVID-19 infection symptoms [22]. Accordingly, it was suggested that Vit D plays a major role in reducing the severity of the disease symptoms, since Vit D has a boosting immunogenic effect [23]. The present study added another possible mechanism regarding the favorable effect of Vit D against COVID-19 infection that Vit D downregulated the mRNA expression of *Ctsl* and *Tmprss2* while upregulated *Ace2* that may affect the entry of SARS-CoV-2 into the epithelial cells.

Tmprss2 is considered as a target for the treatment of viral infections, including COVID-19 [24]. It was found that mucolytic ambroxol decreased influenza infection through inhibition of *Tmprss2* protein [25]. Furthermore, Vit D has an anti-influenza effect [26]. In the current study, it was found that Vit D reduced the mRNA levels of *Tmprss2*, in addition to *Ctsl*. Therefore, it can be speculated that Vit D reduces the number of functional *Tmprss2* and *Ctsl* and hence reduces the transmission of viral diseases.

The role of *Ace2* in the severity of COVID-19 infection is still not fully understood. It was found that *Ace2* expression level is negatively associated with the severity of COVID-19 infection [27]. It was reported that upregulation of *Ace2* receptor decreases the activation of the renin-angiotensin-aldosterone system and hence reduces the possible harmful effects on the hearts and kidneys [28]. The results of this study showed that *Ace2* was sharply upregulated by more than 6 folds in the mouse lungs after 30 days administration of Vit D. Interestingly, administration of Vit D can protect against cardiovascular diseases [29,30].

It was reported previously that Vit C has a potentiality to alter the expression of SARS-CoV-2 entry genes [31]. However, the present study did not find any significant influence of Vit C on the mRNA expression of SARS-CoV-2 entry genes in the mouse lungs. The difference between Vit C and D regarding their influence on SARS-CoV-2 entry genes might be due to the different physio-chemical properties of both Vits, where Vit D is more lipophilic than Vit C. Different water solubility can affect the distribution of Vits to specific organs, such as the lung. It was found that more lipophilic drugs can be widely distributed within the body [32]. In addition, more lipophilic drugs can enter the cells and activate transcriptional factors, while more water-soluble compounds have lower capacity to enter the cells [33].

There are some limitations in this study. First: the protein expressions of the tested genes were not analyzed. Second: this study evaluated only a single time point of 1-month administration of Vits. Further studies are needed to find out the influence of chronic administration of these Vits on the expression of *Ace2*, *Tmprss2*, and *Ctstl*.

6. Conclusion

The present study investigated the influence of the most commonly used Vits among humans during COVID-19 pandemic on the mRNA expression of SARS2-CoV-2 entry genes and found that Vit D induced the expression of *Ace2* and reduced the expression of *Tmprss2* and *Ctstl* genes in the mouse lungs. These findings may increase our understanding of the molecular regulation of SARS2-CoV-2 entry genes by Vits.

Ethics approval and consent to participate

The ethical committee at Al-Zaytoonah University approved the protocol of this study.

Human and animal rights

The mice were handled according to the guide of Canadian Council on Animal Care.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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