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RESEARCH ARTICLE

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Estradiol reduces *ACE2* and *TMPRSS2* mRNA levels in A549 human lung epithelial cells

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Abstract

Epidemiologic studies suggest slightly higher risk of severe Covid-19 symptoms and fatalities following SARS-CoV-2 infection in men compared with women from similar age groups. This bias was suggested to reflect differences in the male and female immune system regulation, driven by different sex hormone levels in men and women, in particular, higher plasma estradiol in women. SARS-CoV-2 infects respiratory tract epithelial cells by binding to their cell membrane ACE2, followed by priming for cell entry by the host cell membrane serine protease TMPRSS2. The cell protease FURIN facilitates cell exit of mature SARS-CoV-2 virions. Our study examined the effects of in vitro treatment of A549 human lung epithelial cells with 17-β-estradiol on mRNA expression of genes coding for these proteins. Treatment of A549 human lung epithelial cells with 17-β-estradiol reduced the cellular mRNA levels of *ACE2* and *TMPRSS2* mRNA, while not affecting *FURIN* expression. Our findings suggest that 17-β-estradiol may reduce SARS-CoV-2 infection of lung epithelial cells, which may in part explain the reduced incidence of severe Covid-19 and fatalities among women compared with men of similar age. Studies into the molecular pathways by which 17-β-estradiol reduces *ACE2* and *TMPRSS2* mRNA expression in lung epithelial cells are needed for assessing its potential protective value against severe Covid-19.

KEYWORDS
A549 lung epithelial cells, *ACE2*, Covid-19, estrogen replacement therapy, *TMPRSS2*

1 | INTRODUCTION

Older age is a key risk factor for developing severe Covid-19 following SARS-CoV-2 infection, while most infected individuals under age 60 are either asymptomatic or present mild symptoms (Del Rio & Malani, 2020). Besides older age, comorbidities such as chronic respiratory illness, type-2 diabetes, or immune deficiency increase risk for severe Covid-19 following SARS-CoV-2 infection (Richardson et al., 2020; Wang et al., 2020). Male Covid-19 patients typically experience more severe symptoms and face higher fatality risk compared with female patients of similar age groups (Bwire, 2020; Griffith et al., 2020), albeit this sex bias was not always evident in studies analyzing epidemiological data sets. For example, Leong et al. analyzed Covid-19 epidemiologic records (January 2020 to January 2021) from nine countries with a population over one million people. Their analysis found higher proportions of Covid-19 fatalities among males than females in seven of these countries (India, Italy, Peru, Portugal, South Korea, United Kingdom, and the United States), while records from Canada and Netherlands did not show sex bias in Covid-19 fatalities (Leong et al., 2021). The possible protective roles of

estradiol against severe inflammatory diseases, including Covid-19, were reviewed by Khan (2020).

The key cellular proteins essential for SARS-CoV-2 infection are angiotensin-converting enzyme 2, encoded by *ACE2*, which serves as the SARS-CoV-2 Spike protein binding site, and transmembrane serine protease 2, encoded by *TMPRSS2*, which serves as the priming protease for SARS-CoV-2 cell entry (Hoffmann et al., 2020). Another important player in Covid-19 pathology is the cell membrane protease paired basic amino acid cleaving enzyme, encoded by *FURIN*, which facilitates cell exit of mature SARS-CoV-2 virions (Johnson et al., 2021; Peacock et al., 2021). Several transcriptomic studies with Covid-19 patient tissues, including lungs, reported dysregulated immune and coagulation pathway genes (Delorey et al., 2021), hyperinflammation, and fibrosis (Wang et al., 2021) as implicated in disease severity. Based on bioinformatics search for repressors of *ACE2* expression, estradiol was proposed among tentative Covid-19 treatments (Glinsky, 2020). Additionally, bioinformatics analysis of transcriptomic data from golden hamsters infected with SARS-CoV-2 identified estradiol among potential Covid-19 therapeutics (Zou et al., 2021). Our study therefore examined the effects of in vitro 17-β-estradiol treatment of cultured A549 human lung epithelial cells on the transcription of *ACE2*, *TMPRSS2*, and *FURIN*, the key genes implicated in SARS-CoV-2 infection.

2 | METHODS

2.1 | A549 human lung epithelial adenocarcinoma cells

A549 cells, obtained at early passage from the American Type Culture Collection (ATCC), were a generous gift from Dr Yael Ziv (Tel Aviv University). Cells were grown in T-75 tissue culture plates under optimal conditions (37°C and 5% CO_2) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/L penicillin and 100 μg/l streptomycin. DMEM, FBS and antibiotics were purchased from Biological Industries (Israel). 17-β-estradiol was purchased from Sigma-Aldrich (cat. E8875; Israel) and kept as 0.5 M stock solution in ethanol. Dilutions into PBS were stored at −20°C. A549 cells were plated within 1 week of reaching confluence. About 2×10^5 A549 cells per well were plated (10^5 cells/ml) in 12-well plates (Corning, ME, United States). The indicated final concentrations of 17-β-estradiol were added on the third day from plating, when the A549 cells were confluent. The applied range of 17-β-estradiol concentrations (37 nm to 144 nM) and the exposure period of 24 h were chosen based on studies on its in vitro effects in cultured cells (Jarzynka et al., 2006; Lemes et al., 2021). Our preliminary experiments indicated that lower 17-β-estradiol concentrations or longer in vitro exposure periods had less consistent effects on the expression of the selected genes. Experiments were run in 6-plicates for each 17-β-estradiol concentration. Cells were harvested following the treatment period by removing the media, gentle blotting of plates on tissue paper, and their immediate freezing at −80°C until RNA extraction.

2.2 | RNA extraction

RNAs were extracted from A549 cells by lysing the frozen cells using Tri-reagent (T9424, Sigma-Aldrich, Israel), followed by RNA separation using chloroform and precipitation with isopropanol, and next washing with 80% cold ethanol, as described (Oved et al., 2013). RNA was quantified using a NanoDrop spectrophotometer (ND-1000), with 260/280 nm >1.8 and 260/230 nm >2.0.

2.3 | Real-time PCR experiments

Real-time quantitative PCR (qPCR) reactions were performed using RNAs extracted from A549 cells as described (Voinsky et al., 2019) with cDNA samples prepared from 1 μg RNA samples using qScript cDNA Synthesis Kit (Quanta Bio, MA, United States). Reverse transcription was performed using a thermal cycler over three steps (22°C for 5 min, followed by 42°C for 30 min and 85°C for 5 min). Real-time PCR reactions were done with 10 μl mixtures containing 10 ng of cDNA, PerfeCTa SYBR® Green FastMix Kit (Quanta Bio) and Integrated DNA Technologies, Inc. (Leuven, Belgium) primers (listed below). *GAPDH* (beta-glucuronidase) was used as reference gene (Li et al., 2014). The primer sequences applied for the real-time PCR reactions were:

ACE2 forward: CAAGAGCAAACGGTTGAACAC
ACE2 reverse: CCAGAGCCTCTCATTGTAGTCT
TMPRSS2 forward: GTCCCCACTGTCTACGAGGT
TMPRSS2 reverse: CAGACGACGGGGTTGGAAG
FURIN forward: GCAAAGCGACGGACTAAACG
FURIN reverse: TGCCATCGTCCAGAATGGAGA
GAPDH forward: GGAGCGAGATCC CTCCAAAAT
GAPDH reverse: GGCTGTTGTCATACTTCTCATGG

2.4 | Statistics analysis

Real-time PCR data analysis was conducted as described (Voinsky et al., 2019) using GraphPad Prism v.6 (San Diego, CA, United States). Normality of data distribution was evaluated using the Shapiro–Wilk test; variables between groups were analyzed by one-way analysis of variance; outliers were detected and removed by using Grubbs test. p values <.05 were considered significant. The Dunnett's test was used for post hoc analysis (significance was set at $p < .05$).

3 | RESULTS

Treatment of confluent A549 cells with the studied 17-β-estradiol concentrations for 24 h did not result in noticeable changes in cell morphology or viability, as assessed by microscopy (not shown). Findings from our real-time PCR measurements of *ACE2*, *TMPRSS2*, and *FURIN* are presented in Figure 1. Treatment of A549 cells with 37, 74,

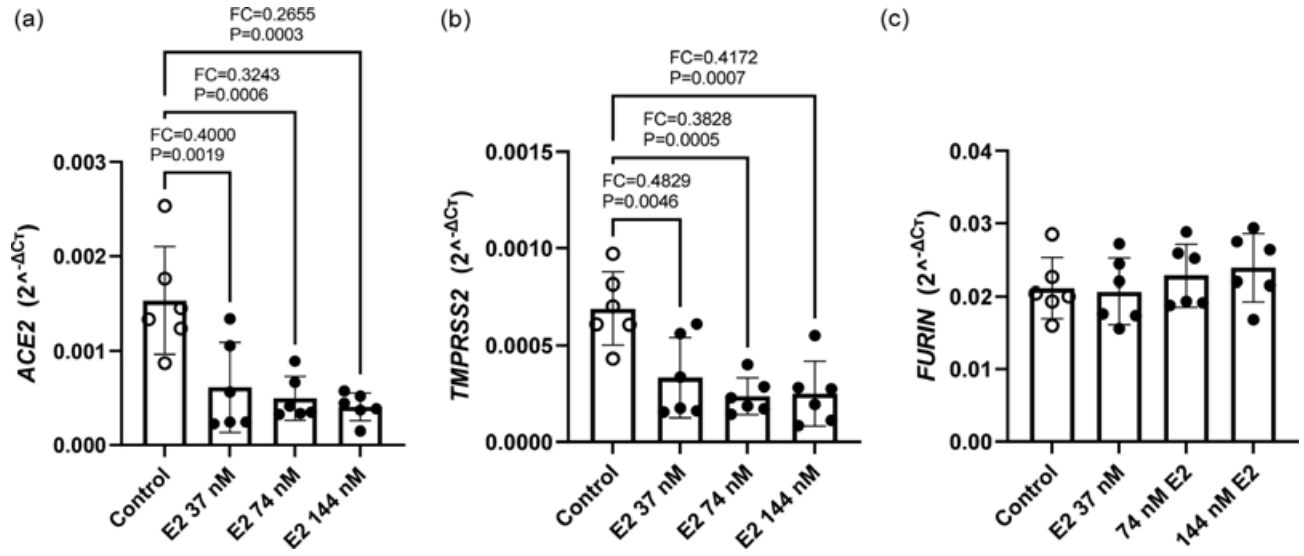

FIGURE 1 Treatment with 17-β-estradiol increases *ACE2* and *TMPRSS2* mRNA expression in A549 human lung epithelial cells. A549 lung epithelial cells were grown on 12-well plates. Confluent cells were exposed to the indicated concentrations of 17- β-estradiol (E2) for 24 h followed by removal of medium, extraction of RNA, and real-time PCR experiments. The cellular mRNA expression levels were compared to those of control cells grown in parallel for (a) *ACE2*, (b) *TMPRSS2*, (c) *FURIN*. *GAPDH* was used as the control gene. The fold-change (FC) and *p* values for statistically significant differences between the indicated concentrations of 17-β-estradiol (E2) treated and control cells are shown in the relevant panels. The Shapiro–Wilk normality test indicated that all the data had normal distribution. See Section 2 for further details. Results are from a representative experiment, which was replicated at least twice with closely similar findings for each of the measured mRNAs

or 144 nM 17-β-estradiol for 24 h resulted in over twofold reduced mRNA expression of both *ACE2* and *TMPRSS2* compared with untreated A549 cells. No changes were observed in the expression of *FURIN* mRNA in the same experiments. The findings presented in Figure 1 were replicated by at least two independent experiments for each of the above genes.

4 | DISCUSSION

Over 2 years into the Covid-19 pandemic, old age and severe comorbidities remain the major risk factors for Covid-19 intensive care hospitalizations and fatalities. By comparison, male sex poses a lesser Covid-19 fatality risk. For example, Zhu et al. reported an odds risk of 1.34 (95% CI 1.29–1.39) for Covid-19 fatality in male compared with female patients, based mostly on U.S. Covid-19 records by January 2021 (Zhu et al., 2021). The above report is in close agreement with data presented on The Sex, Gender, and COVID-19 Project website (https://globalhealth5050.org/the-sex-gender-and-covid-19-project/). Data collected by this project from 135 countries as of late December 2021 shows 1.30-fold more male than female Covid-19 fatalities globally (based on data only from open sources with Covid-19 patient demographics by age and sex). Notably, health records from the 2003 SARS pandemic indicated that men had much higher fatality rates than women (OR = 1.66; $p < .0001$; Karlberg et al., 2004). The 2003 SARS pandemic was caused by a coronavirus which infected lung epithelial cells via their ACE2 receptor, similarly to SARS-CoV-2; thus, this observation suggests common pathways which afford women some protection from both SARS-CoV-1 and SARS-CoV-2. Aside from of its modulatory effects on *ACE2* and *TMPRRSS2* expression, as observed in our current study with A549 lung epithelial cells, estradiol likely affords partial protection from coronavirus-mediated diseases by enhancement of immune functions (Dhindsa et al., 2021; Khan, 2020; Ramírez-de-Arellano et al., 2021). Notably, "Long Covid," the persisting neurological symptoms affecting some Covid-19 patients for many months following their recovery, is most common among premenopausal women, who have higher estradiol levels than either men or postmenopausal women; for such patients, higher endogenous estradiol levels were suggested as the underlying cause (Bai et al., 2021; Sigfrid et al., 2021). Thus, while estradiol may protect younger women from severe Covid-19, it also predisposes them to "Long Covid" following recovery from SARS-CoV-2 infection.

Our real-time PCR findings indicate that exposure of A549 lung epithelial cells to 37, 74, or 144 nM 17-β-estradiol for 24 h resulted in reduced mRNA expression levels of both *ACE2* and *TMPRSS2*, while not affecting their *FURIN* expression (Figure 1). Lower expression levels of *ACE2* and *TMPRSS2* in lung epithelial cells following 17-β-estradiol treatment may suggest partial protection from spread of SARS-CoV-2 to further tissues following lung infection, and thus lower incidence of severe Covid-19 symptoms and fatalities. SARS-CoV-2 in vitro infection of cultured VERO E6 African green monkey kidney cells increased their *ACE2* and *TMPRSS2* expression, an effect which was attenuated by 17-β-estradiol co-incubation (1 or 100 nM), while also reducing viral release to the medium (Lemes et al., 2021). Our current study confirms and extends these earlier observations by showing that 17-β-estradiol reduces the mRNA levels of both

ACE2 and *TMPRSS2* in human lung epithelial cells regardless of SARS-CoV-2 infection, thus suggesting an a priori protective effect against SARS-CoV-2 infection in the most relevant target cells for Covid-19 pathology. By contrast, *FURIN* expression per se is not essential for infection; rather, it promotes viral spread to further cells and tissues (Johnson et al., 2021; Peacock et al., 2021).

4.1 | Clinical relevance

Active clinical trials listed on ClinicalTrials.gov as of late December 2021 include three studies for estradiol medication in hospitalized Covid-19 patients (NCT04865029; NCT04853069; NCT04539626). The first of these studies aims to treat hospitalized male and female Covid-19 patients with both estradiol and progesterone in addition to standard of care (Lovre et al., 2021). As of late December 2021, none of these trials published their findings. Another study measured endogenous estradiol and reported lack of correlation between plasma estradiol and Covid-19 symptom severity in SARS-CoV-2 infected women (Schroeder et al., 2021). However, the pre-infection endogenous estradiol status in these patients was not available; thus, the interpretation of these findings is uncertain, as estradiol production might be affected by viral infection and the resulting inflammation.

An indirect but fast method for repurposing existing therapeutics for Covid-19 is to analyze large electronic health record data sets and search for correlations between chronic medications and disease outcome in SARS-CoV-2 infected individuals (Gurwitz, 2020; Satterfield et al., 2021). Indeed, a retrospective study comparing clinical records of women aged over 50 years who were prescribed estrogen replacement therapy (ERT) prior to SARS-CoV-2 infection had a fatality risk of 0.33 (95% CI 0.18, 0.62) compared with age-matched women not receiving ERT (Seeland et al., 2020). The same study reported a similar Covid-19 fatality risk for younger (premenopausal) women, irrespective of ERT; this probably reflects their higher endogenous estradiol (Seeland et al., 2020). Additionally, a small randomized trial ($n = 40$) in hospitalized Covid-19 postmenopausal females treated with oral estradiol supplementation (2 mg per day for 7 days) found faster recovery, while its small size ($n = 40$) did not allow to identify a reduction in Covid-19 fatalities (Seth et al., 2021). Larger cohorts are definitely required to assess the potential of prescribing estradiol to Covid-19 patients.

4.2 | Study limitations

Lung epithelial cells constitute the primary location of lung infection by SARS-CoV-2. Thus, A549 human lung epithelial carcinoma cells serve as appropriate tool for studying the in vitro effects of estradiol on the expression levels of key genes implicated in SARS-CoV-2 lung infection, However, our study has several limitations, as A549 is a human cancer cell line. Due to their unstable genomes, cancer cell lines accumulate somatic mutations during continued in vitro culture, which may lead to irreproducible findings. We therefore used early passage of A549 cells (see Section 2), and thawed new stock following nine in vitro passages. Another limitation is that A549 cells were established from a male lung carcinoma patient, and findings might have less relevance for female Covid-19 patients. Studies using both female and male animal models are required for assessing the in vivo effects of estradiol treatment both prior to and following SARS-CoV-2 infection.

Our study examined the mRNA levels of selected genes following treatment of cultured A549 cells with 37, 74, or 144 nM 17-β-estradiol for 24 h. Serum estradiol concentrations of premenopausal women vary in the range of 0.1–2.9 nM (highest during ovulation); while they are typically <0.07 and <0.14 nM for postmenopausal women and men, respectively (Stanczyk & Clarke, 2014). Thus, the estradiol concentrations applied in our study are much higher than serum estradiol levels in either women or men. Albeit, some of the added estradiol was likely metabolized during the 24 h exposure period, requiring higher than physiological hormone levels. Indeed, the range of 17-β-estradiol concentrations applied in our present study was similar to that of published studies on its in vitro effects on cultured cells (Jarzynka et al., 2006; Lemes et al., 2021).

We focused our study on only three genes (*ACE2*, *TMPRSS2*, and *FURIN*), while several immune, inflammation, coagulation, and fibrosis pathway genes were implicated in Covid-19 (COVID-19 Host Genetics Initiative, 2021; Delorey et al., 2021; Pairo-Castineira et al., 2021; Wang et al., 2021). Being based on a candidate gene approach and in vitro studies in human lung carcinoma epithelial cells, our findings should be considered preliminary, and require validation and further exploration applying a genome-wide approach (ideally, RNA-seq) with animal models for SARS-CoV-2 infection.

5 | CONCLUSIONS

Our findings suggest that estradiol may decrease SARS-CoV-2 infection in human lung epithelial cells by reducing their expression of *ACE2* and *TMPRSS2*, genes coding for two cell membrane proteins required for SARS-CoV-2 cell binding and entry, respectively. Our findings highlight the tentative potential of estradiol against severe Covid-19, as evaluated by ongoing clinical trials. Clarifying the sex bias and the possible role of estradiol in Covid-19 morbidity and fatality requires animal model studies. Establishing the role of estradiol and additional hormones in Covid-19 pathology may lead to the development of novel Covid-19 therapeutics.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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