Therapeutic application of estrogen for COVID-19: Attenuation of SARS-CoV-2 spike protein and IL-6 stimulated, ACE2-dependent NOX2 activation, ROS production and MCP-1 upregulation in endothelial cells

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ABSTRACT

The outbreak of COVID-19 has remained uncontained with urgent need for robust therapeutics. We have previously reported sex difference of COVID-19 for the first time indicating male predisposition. Males are more susceptible than females, and more often to develop into severe cases with higher mortality. This predisposition is potentially linked to higher prevalence of cigarette smoking. Nonetheless, we found for the first time that cigarette smoking extract (CSE) had no effect on angiotensin converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) expression in endothelial cells. The otherwise observed worse outcomes in smokers is likely linked to baseline respiratory diseases associated with chronic smoking. Instead, we hypothesized that estrogen mediated protection might underlie lower morbidity, severity and mortality of COVID-19 in females. Of note, endothelial inflammation and barrier dysfunction are major mediators of disease progression, and development of acute respiratory distress syndrome (ARDS) and multi-organ failure in patients with COVID-19. Therefore, we investigated potential protective effects of estrogen on endothelial cells against oxidative stress induced by interleukin-6 (IL-6) and SARS-CoV-2 spike protein (S protein). Indeed, 17β-estradiol completely reversed S protein-induced selective activation of NADPH oxidase isoform 2 (NOX2) and reactive oxygen species (ROS) production that are ACE2-dependent, as well as ACE2 upregulation and induction of pro-inflammatory gene monocyte chemoattractant protein-1 (MCP-1) in endothelial cells to effectively attenuate endothelial dysfunction. Effects of IL-6 on activating NOX2-dependent ROS production and upregulation of MCP-1 were also completely attenuated by 17β-estradiol. Of note, co-treatment with CSE had no additional effects on S protein stimulated endothelial oxidative stress, confirming that current smoking status is likely unrelated to more severe disease in chronic smokers. These data indicate that estrogen can serve as a novel therapy for patients with COVID-19 via inhibition of initial viral responses and attenuation of cytokine storm induced endothelial dysfunction, to substantially alleviate morbidity, severity and mortality of the disease, especially in men and post-menopause women. Short-term administration of estrogen can therefore be readily applied to the clinical management of COVID-19 as a robust therapeutic option.

1. Introduction

The outbreak of the coronavirus disease 2019 (COVID-19) has turned into a global pandemic of more than 197 million cases and 4.2 million deaths (as of Aug 3rd, 2021) [1]. The uncontained outbreak remains challenging to manage with new strains/mutants of the virus emerging constantly. Currently, four variants of alpha (B.1.1.7) [2–4], beta (B.1.351) [4,5], gamma (P1, B.1.1.28.1) [5] and delta (B.1.617) [6], first identified in United Kingdom, South Africa, Brazil, and India respectively, have been classified as variants of concern (VOC) due to their high transmissibility and potentially more challenging features for disease management [1,7]. Intensive research efforts have been devoted to understanding molecular mechanisms of viral infection and disease pathogenesis since the outbreak to result in many breakthroughs including very rapid identification of the viral strains and their RNA sequences, identification of viral receptor ACE2 and its partner of

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transmembrane protease serine 2 (TMPRSS2, primes S protein for host cell entry of SARS-CoV-2) for entry into host cells [8], development of multidimensional diagnostic methodologies and applications, and remarkable progress of vaccine development. Nonetheless, the molecular mechanisms underlying detailed pathological processes of COVID-19 have remained far from being fully understood.

We have previously reported sex difference of COVID-19 for the first time indicating male predisposition [9]. Epidemiological studies have shown that males are more susceptible to COVID-19 than females [9-12]. In a report of 1,099 COVID-19 cases from 552 hospitals in 30 provinces in China, 58.1% of the patients were men [11]. In another study, 62% (119 out of 191) of patients examined between December 29th, 2019 and January 31st, 2020 in China were men [12]. In addition, more males (60.6%, 238 out of 393) than females were admitted to hospital due to COVID-19 in New York City in March 2020 [10]. Of note, male gender was found to be independently associated with increased in-hospital mortality, oxygenation requirements, and outcome of intu

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post-menopause women at both early stage of viral infection and later stage of cytokine storm mediated systematic injuries.

2. Materials and methods

2.1. Cell treatment and siRNA transfection

Primary bovine aortic endothelial cells (BAECs, Genlantis, passages 4 to 6) were cultured in M199 supplemented with 10% fetal bovine serum (FBS), 1% vitamin, and 1% L-glutamine as previously described [36–39]. Primary human aortic endothelial cells (HAECs, Lonza, passages 4 to 6) were cultured in EGM-2 media supplemented with 10% fetal bovine serum and EGM-2 SingleQuots (†C-C4176, Lonza) as previously described [40]. Confluent BAECs were starved overnight in M199 containing 5% FBS, and treated with different concentrations of cigarette smoke extract (CSE, 0, 2.5%, 5%, 10%), recombinant human interleukin-6 (IL-6, 100 ng/mL, #206-IL-010, R&D), or recombinant SARS-CoV-2 Spike protein (S protein, 500 ng/mL, #10549-CV-100, R&D) for 24 h before harvested for analyses. In the experiment when estrogen was applied, cells were pre-treated with IL-6 (100 ng/mL) or S Protein (500 ng/mL) for 30 min prior to exposure to various doses of 17β-estradiol (10 nmol/L to 10 μmol/L, #E2758, MilliporeSigma) for 24 h.

In additional experiments, BAECs at 80% confluence were transfected with siRNA targeting bovine NOX2 (#sc-270683, Santa Cruz Biotechnology) (200 pmol) or scrambled control siRNA (#sc-37007, Santa Cruz Biotechnology) according to the manual of Lipofectamine RNAiMAX reagent (#13778150, ThermoFisher). Forty-eight hours post-transfection, cells were starved overnight and then treated with IL-6 (100 ng/mL) or S protein (500 ng/mL) for 24 h. To examine specificity of ACE2 antibody, two μg of pcDNA3.1-hACE2 (#145033, Addgene) or 200 pmol of siRNA targeting ACE2 (customized #CTM-785249, Dharmacon) was used for overexpression of human ACE2 or knockdown of ACE2. For experiments of ACE2 neutralization, 1 μg/mL of ACE2 antibody (#ab15348, Abcam) or control Rabbit IgG (#N101, Calbiochem) was used to pre-treat endothelial cells for 30 min prior to S protein stimulation.

2.2. Cigarette smoking extract (CSE) preparation

Fresh CSE was prepared by bubbling the smoke generated from one 3R4F reference cigarette (University of Kentucky) (350 mL smoke) into 10 mL M199 according to previous publications [41,42]. The resulted CSE was considered as 100% stock (350 mL smoke/10 mL medium = 35 mL smoke/mL medium) and diluted as required for experimentation after filtering through a 0.22 μm filter. The concentration of the cigarette smoke in 10% dilution of the CSE stock (3.5 mL smoke/mL medium) is approximately equivalent to the concentration of cigarette smoke in one’s circulation for human subjects who consume more than 2 packs of cigarettes per day (2 packs * 20 cigarettes/pack * 350 mL smoke/cigarette/5–7 L blood = 2.8–2.8 mL smoke/mL blood) [43,44].

2.3. Western blotting and RT-PCR

After indicated treatment protocols, BAECs and HAECS were harvested in lysis buffer (20 mM/L Tris-HCl pH 7.4, 150 mM/L NaCl, 1 mM/L EDTA, 1 mM/L EGTA, 2.5 mM/L sodium pyrophosphate, 1 mM/L β-glycerophosphate, 1 mM/L sodium orthovanadate, 1% Triton X-100, supplemented with protease inhibitor cocktail). The cell lysates were incubated on ice for 20 min before supernatant was separated by centrifugation at 12,000 rpm for 10 min at 4°C. Then the protein concentration was determined by DC protein assay (#5000112, Bio-Rad). Twenty-five to forty μg proteins were separated in 10% or 15% SDS-PAGE, followed by standard Western blotting protocol by probing with antibodies for ACE2 (1:1000, #ab15348, Abcam), TMPRSS2 (1:1000, #ab92323, Abcam), NOX1 (1:250, #611414, BD Biosciences), NOX4 (1:1000, #NB110-58849, Novus), MCP-1 (1:500, #ab9669, Abcam), and β-actin (1:1000, #A2066, MilliporeSigma). The densities of protein bands were quantified by NIH Image J program.

For RT-PCR determination of ACE2, NOX2, and MCP-1 mRNA expression, total RNA was isolated from cultured BAECs using TRizol (†15956018, Invitrogen) according to the protocol provided by manufacturer. Reverse transcription was performed using iScript reverse transcriptase supermix (#170–8840, Bio-Rad). For real-time PCR amplification, iQ SYBR green supermix (#64102502, Bio-Rad) was used and primer sequences for PCR were summarized in the Supplementary Table 1. PCR amplifications were carried out on a CFX Connect™ Real-Time PCR detection system (Bio-Rad) with the protocol of [(95°C/3 min), (95°C/10 s, 60°C/30 s) × 39 cycles, melt curve (55°C–95°C, increment 0.5°C), (95°C/30 s)]. The PCR mix contained GAPDH primers to generate GAPDH amplicon that served as an internal control and ΔΔCq expression was used to calculate the relative expression levels over control.

2.4. Determination of superoxide production by electron spin resonance (ESR)

Superoxide production in BAECs and HAECS was determined by ESR (eScan, Bruker) as we previously published [35–39,45–51]. After indicated treatment protocols, BAECs or HAECS were collected in cold modified Krebs/HEPES (KHB) buffer (99 mmol/L of NaCl, 4.69 mmol/L of KCl, 1.03 mmol/L of KH₂PO₄, 2.50 mmol/L of CaCl₂, 1.20 mmol/L of MgSO₄, 25.0 mmol/L of NaHCO₃, 5.6 mmol/L of glucose, and 20.0 mmol/L of Na-HEPES, pH 7.35). Cell suspension was mixed with superoxide-specific spin trap CMH (1 mmol/L, #ALX-430-117-M250, Enzo Life Sciences) in nitrogen gas bubbled KH₂ buffer containing diethyldithiocarbamic acid (5 μmol/L, #D3506, MilliporeSigma) and deferoxamine (25 μmol/L, #D9533, MilliporeSigma). Then the cell mixture was loaded in glass capillaries and analyzed immediately by ESR. Superoxide signal was measured in the presence or absence of polyethylene glycol-superoxide dismutase (PEG-SOD, 20 μ/mL, #S9549, MilliporeSigma). The PEG-SOD inhabitable superoxide signal was calculated and normalized to protein concentrations. The ESR settings used were as follows: center field 3477 or 3480 G; sweep width 2.8840, Bio-Rad). For real-time PCR determination, iQ SYBR green supermix (#64102502, Bio-Rad) was used and primer sequences for PCR were summarized in the Supplementary Table 1. PCR amplifications were carried out on a CFX Connect™ Real-Time PCR detection system (Bio-Rad) with the protocol of [(95°C/3 min), (95°C/10 s, 60°C/30 s) × 39 cycles, melt curve (55°C–95°C, increment 0.5°C), (95°C/30 s)]. The PCR mix contained GAPDH primers to generate GAPDH amplicon that served as an internal control and ΔΔCq expression was used to calculate the relative expression levels over control.

2.5. Statistical analysis

All data are presented as Mean ± SEM. One-way ANOVA was used to compare the mean among multiple groups with a Newman-Keuls post hoc test. p < 0.05 was considered statistically different.

3. Results

3.1. CSE has no effects on endothelial ACE2 and TMPRSS2 expression

We first examined whether exposure of endothelial cells to CSE upregulates expression ACE2 and TMPRSS2. To validate the specificity of ACE2 antibody, Western blot analysis was conducted from cell lysates prepared from ACE2 overexpressed cells using pcDNA3.1-hACE2 plasmid, and cell lysates prepared from ACE2 silenced cells using RNA interference. As a result, ACE2 antibody we used in the present study was able to detect ACE2 protein specifically by picking up bands around 150 kDa for overexpressed ACE2 with C9 tag in pcDNA3.1-hACE2 constructed from ACE2 overexpressed cells using pcDNA3.1-hACE2 (150 kDa for overexpressed ACE2 with C9 tag in pcDNA3.1-hACE2). Western blotting results indicate that CSE had no effects on protein expression of ACE2 and TMPRSS2 in endothelial cells. It has
been shown that TMPRSS2 undergoes autoactivation to result in self-cleavage. Nonetheless, both full-length (~41 kD) and cleaved (activated) (~22/23 kD) TMPRSS2 were not altered in CSE stimulated endothelial cells (Fig. 1D). Our results seem consistent with recent findings in primary cultured human airway basal stem cells (ABSCs) from nonsmokers, where CSE failed to change ACE2 expression level [21]. These data indicate that current status of cigarette smoking might not necessarily explain more prevalent and severe disease of COVID-19 in men (or women) who smoke, at least not via regulation of ACE2 and TMPRSS2. Development of more severe disease of COVID-19 in smokers
is however likely linked to baseline respiratory pathologies induced by chronic cigarette smoking.

3.2. IL-6 specifically upregulated endothelial expression of NOX2 and NOX2-dependent ROS production

To examine whether IL-6 induces NOX-dependent oxidative stress in endothelial cells, we treated BAECs with IL-6 and examined expression of NOX isoforms (NOX1, NOX2, and NOX4) by Western blotting analysis. Interestingly, exposure of cells to IL-6 (100 ng/mL) for 24 h resulted in upregulation of NOX2, but not NOX1 or NOX4 (Fig. 2A–C). We next measured superoxide production specifically and quantitatively using electron spin resonance (ESR), in IL-6 treated BAECs transfected of NOX2 siRNA or negative control siRNA. As shown in Fig. 3, we found that IL-6 treatment resulted in marked increase in superoxide production in endothelial cells, which was completely attenuated by NOX2 siRNA but not by scrambled control siRNA, indicating that IL-6 specifically increases endothelial ROS production via activation of NOX2.

3.3. Estrogen administration alleviated IL-6-induced upregulation of NOX2 and MCP-1

To test the hypothesis that estrogen is involved in the protection of endothelial cells from IL-6-induced oxidative stress, BAECs were treated with various doses of 17β-estradiol (10 nmol/L to 10 μmol/L, 24 h) following IL-6 exposure (100 ng/mL, 30 min). Of note, estrogen receptors, ERα and ERβ, have been previously described to be functional in BAECs [52,53]. At a minimal dose of 10 nmol/L, 17β-estradiol almost completely abrogated IL-6-induced upregulation of NOX2 (Fig. 2B). Treatment of 17β-estradiol did not change protein expression of NOX1 or NOX4 in the presence of IL-6 (Fig. 2A and C). As a downstream effector of IL-6, MCP-1 was also examined (Fig. 2D), and found upregulated by IL-6, while this response was abolished by estrogen

Fig. 2. IL-6 selectively upregulated protein expression levels of NOX2, and MCP-1, which were substantially attenuated by 17β-estradiol. Bovine aortic endothelial cells (BAECs) were pre-treated with IL-6 (100 ng/mL) for 30 min prior to exposure to 17β-estradiol (E2) for 24 h at indicated concentrations. A, Representative Western blots and grouped data of NOX1 protein expression indicating no effects of IL-6 or 17β-estradiol. Data are shown as Mean ± SEM, n = 3–5. B, Representative Western blots and grouped data of NOX2 protein expression indicating upregulation of NOX2 expression by IL-6, which was substantially attenuated by 17β-estradiol treatment at concentration as low as 10 nmol/L. Data are shown as Mean ± SEM, n = 3–5. C, Representative Western blots and grouped data of NOX4 protein expression indicating no significant effect by IL-6 or 17β-estradiol. Data are shown as Mean ± SEM, n = 3–4. D, Representative Western blots and grouped data of MCP-1 protein expression indicating upregulation by IL-6 and reversal by 17β-estradiol. Data are shown as Mean ± SEM, n = 4–5. *p < 0.05, **p < 0.01 vs. Control group; #p < 0.05, ##p < 0.01 vs. IL-6 group by One-Way ANOVA.
treatment. These data indicate that estrogen administration protects endothelial cells from IL-6 induced oxidative stress via attenuation of NOX2 and MCP-1 activation, hence may be used as a potential therapy for cytokine storm mediated ARDS/multi-organ injury during the pathogenesis of COVID-19, to effectively reduce disease severity and mortality especially in men. Of note, we are proposing acute treatment of COVID-19 patients with estrogen, so the potential side effects of chronic treatment with estrogen are not relevant.

3.4. SARS-CoV-2 S protein dose-dependently stimulated endothelial ROS production

To examine whether S protein directly induces endothelial superoxide production, which might be the underlying mechanism of SARS-CoV-2 promoted endothelial dysfunction, BAECs were treated with S protein at doses of 0.5 μg/mL, 1 μg/mL, and 5 μg/mL for 24 h. As is clear in Fig. 4, treatment with S protein at low dose of 0.5 μg/mL induced a marked, 4.4 fold increase in endothelial superoxide production (p < 0.001). The superoxide production was aggravated in a dose dependent manner. These data for the first time demonstrate that S protein alone stimulates an excessive endothelial ROS production. These results not only validate our experimental model using S protein to examine ROS production implicated in endothelial dysfunction in vitro, but also suggest that S protein provoked endothelial superoxide production might be the underlying mechanism of endothelial dysfunction and inflammation following SARS-CoV-2 infection.

3.5. Estrogen administration alleviated SARS-CoV-2 S protein upregulation of NOX2, ACE2 and MCP-1

To examine whether infection by SARS-CoV-2 can directly induce NOX-dependent oxidative stress in endothelial cells, we treated endothelial cells with S protein. As shown in Fig. 5B, expression of NOX2 was robustly upregulated by S protein (500 ng/mL) after 24 h incubation, which was however significantly attenuated by 17β-estradiol treatment at concentration as low as 100 nmol/L. Similar to observations in IL-6 treated endothelial cells, neither NOX1 nor NOX4 was upregulated by S protein treatment (Fig. 5A and C). Of note, ACE2 was abundantly expressed in endothelial cells as shown in Fig. 6A. The protein abundance of ACE2 was significantly upregulated by S protein treatment, which was substantially attenuated by 17β-estradiol (10 μmol/L). As shown in Fig. 6B, upregulation of MCP-1 by S protein treatment was evident, which was abolished by 17β-estradiol at all doses (10 nmol/L to 10 μmol/L). Of note, S protein significantly upregulated protein expression of NOX2, ACE2, and MCP-1 in cultured human aortic endothelial cells (HAECs) as well (Fig. 7A-C), which was completely attenuated by 100 nmol/L of 17β-estradiol to control levels.

3.6. Estrogen administration alleviated ACE2-dependent upregulation of NOX2, MCP-1 and ROS production

Consistent with changes in protein levels, mRNA levels of ace2, nox2, and mcp-1 were upregulated in S protein treated endothelial cells, all of which were significantly reversed by 17β-estradiol (Fig. 8A–C). Of note, expression of nox2 and mcp-1 at mRNA levels were completely suppressed by ACE2 antibody neutralization, indicating an intermediate role of ACE2 in these responses (Fig. 8D–E). Moreover, S protein treatment induced marked increase in superoxide production in endothelial cells, which was completely alleviated by NOX2 siRNA transfection (Fig. 9A). Also, S protein induced superoxide production was abolished by treatment with 17β-estradiol in both BAECs and HAECs (Fig. 9B and C) at concentration as low as 100 nmol/L, which was effective to inhibit NOX2 activation, implicating a protective role of estrogen in attenuating S protein induced endothelial dysfunction via inhibition of NOX2-dependent superoxide production. Of interest, ACE2 antibody neutralization significantly blocked S protein induced superoxide production as shown in Fig. 9D, suggesting that S protein activation of NOX2 and ROS production is ACE2 dependent. However, acute exposure of cigarette
smoke extract (CSE) prior to S protein treatment did not affect S protein activation of superoxide production (Fig. 10), again indicating that current smoking status might not be related to more severe disease in chronic smokers. Taken together, as summarized in Graphical abstract, these data indicate that viral infection itself and consequent cytokine storm (IL-6) can both trigger NOX2-dependent ROS production, ACE2 upregulation, and inflammatory cytokine expression in endothelial cells, resulting in endothelial dysfunction and inflammation to mediate ARDS/multi-organ failure and mortality during pathogenesis of COVID-19. These responses however can be completely alleviated by estrogen treatment, indicating a robust therapeutic effect of estrogen on COVID-19, especially in men and post-menopause women.

### 4. Discussion

In the present study, we have demonstrated that CSE stimulation of endothelial cells has no effects on the expression levels of ACE2 and TMPRSS2, indicating that the prevalence and severity predisposition to COVID-19 in men may not result from higher rate of current smoking status in men to induce more efficient viral entry via ACE2 and TMPRSS2. We therefore hypothesized that lower incidence and less severe disease in women might be related to estrogen mediated protection. We found that the major mediator of cytokine storm, IL-6, specifically induced upregulation of NOX2, but not that of NOX1 or NOX4, in endothelial cells. Superoxide production, determined selectively and quantitatively by ESR, was markedly increased by IL-6 but completely alleviated by transfection of endothelial cells with NOX2 siRNA. In

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**Fig. 5. SARS-CoV-2 Spike protein (S protein) selectively upregulated protein expression of NOX2, but not NOX1 or NOX4, in endothelial cells, which was substantially attenuated by 17β-estradiol.** Bovine aortic endothelial cells (BAECs) were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 17β-estradiol (E2) for 24 h at indicated concentrations. A, Representative Western blots and grouped data of NOX1 protein expression indicating no effects of S protein or 17β-estradiol. Data are shown as Mean ± SEM, n = 6. B, Representative Western blots and grouped data of NOX2 protein expression indicating upregulation of NOX2 by S protein, which was markedly attenuated by 17β-estradiol treatment at concentration as low as 100 nmol/L (effective range: 100 nmol/L to 10 μmol/L). Data are shown as Mean ± SEM, n = 5–6. C, Representative Western blots and grouped data of NOX4 protein expression indicating no significant effect by S protein. Data are shown as Mean ± SEM, n = 5. *p < 0.05, **p < 0.001 vs. Control group; #p < 0.05, ###p < 0.001 vs. S protein group by One-Way ANOVA.

**Fig. 6. SARS-CoV-2 Spike protein (S protein) upregulated protein expression levels of ACE2 and MCP-1 in endothelial cells, which was substantially attenuated by 17β-estradiol.** Bovine aortic endothelial cells (BAECs) were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 17β-estradiol (E2) for 24 h at indicated concentrations. A, Representative Western blots and grouped data of ACE2 protein expression indicating upregulation of ACE2 by S protein, which was completely attenuated by 10 μM of 17β-estradiol. Data are shown as Mean ± SEM, n = 4–6. B, Representative Western blots and grouped data of MCP-1 protein expression indicating upregulation of MCP-1 by S protein, which was substantially attenuated by 17β-estradiol at concentration as low as 10 nmol/L (effective range: 10 nmol/L to 10 μmol/L). Data are shown as Mean ± SEM, n = 4–5. *p < 0.05, **p < 0.001 vs. Control group; ##p < 0.01, ###p < 0.001 vs. S protein group by One-Way ANOVA.
Fig. 7. SARS-CoV-2 Spike protein (S protein) upregulated protein expression levels of NOX2, ACE2, and MCP-1 in human aortic endothelial cells, which was completely attenuated by 17β-estradiol. Human aortic endothelial cells (HAECs) were pretreated with S protein (500 ng/mL) for 30 min prior to exposure to 100 nmol/L of 17β-estradiol (E2) for 24 h. A, Representative Western blots and grouped data of NOX2 protein expression indicating upregulation of NOX2 by S protein, which was completely attenuated by 100 nmol/L of 17β-estradiol. Data are shown as Mean ± SEM, n = 4. B, Representative Western blots and grouped data of ACE2 protein expression indicating upregulation of ACE2 by S protein, which was completely attenuated by 100 nmol/L of 17β-estradiol. Data are shown as Mean ± SEM, n = 5. C, Representative Western blots and grouped data of MCP-1 protein expression indicating upregulation of MCP-1 by S protein, which was completely attenuated by 100 nmol/L of 17β-estradiol. Data are shown as Mean ± SEM, n = 3. *p < 0.05, **p < 0.01, ***p < 0.001 by One-Way ANOVA.

Fig. 8. SARS-CoV-2 Spike protein (S protein) upregulated ace2 mRNA expression, and ACE2 dependent upregulation of nox2 and mcp-1 mRNA in endothelial cells, which were completely attenuated by 17β-estradiol. Bovine aortic endothelial cells (BAECs) were pre-treated with S protein (500 ng/mL) for 30 min prior to exposure to 17β-estradiol (E2) for 24 h at indicated concentrations. A, Grouped data of ace2 mRNA expression indicating upregulation of ace2 by S protein, which was completely attenuated by 17β-estradiol. Data are shown as Mean ± SEM, n = 3–4. B, Grouped data of nox2 mRNA expression indicating upregulation of nox2 by S protein, which was substantially attenuated by 17β-estradiol. Data are shown as Mean ± SEM, n = 5–6. C, Grouped data of mcp-1 mRNA expression indicating upregulation of mcp-1 by S protein, which was substantially attenuated by 17β-estradiol. Data are shown as Mean ± SEM, n = 3–4. BAECs were pretreated with 1 μg/mL of ACE2 antibody to neutralize membrane receptor of ACE2 for S protein binding prior to exposure of endothelial cells to S protein. This was followed by treatment with 17β-estradiol (E2) for 30 min at 100 nmol/L. D, Grouped data of nox2 mRNA expression, indicating ACE2 dependent upregulation of nox2 mRNA by S protein and its reversal by estrogen treatment. Data are shown as Mean ± SEM, n = 4. E, Grouped data of mcp-1 mRNA expression, indicating ACE2 dependent upregulation of mcp-1 mRNA expression by S protein and its reversal by estrogen treatment. Data are shown as Mean ± SEM, n = 5. *p < 0.05, **p < 0.01, ***p < 0.001 vs. Control group; #p < 0.05, ##p < 0.01, ###p < 0.001 vs. S protein group by One-Way ANOVA.
Bovine aortic endothelial cells (BAECs) were incubated with CSE at indicated NOX2 activation and hence NOX2-derived ROS production, as well as estrogen administration substantially attenuated IL-6-induced 
expression were not changed by IL-6 or estrogen. Similar results were 
found in endothelial cells treated with SARS-CoV-2 Spike protein (S protein). We found that endothelial cell NOX2 expression, ACE2 expression, and ROS production were all markedly upregulated by exposure to S protein. The S protein stimulated ROS production is 
attributed to ACE2 dependent NOX2 activation since it was completely 
attenuated by transfection with NOX2 siRNA and neutralization of ACE2 
with anti-ACE2 antibody. ACE2 antibody neutralization also attenuated 
NOX2 and MCP-1 mRNA expression. These data indicate that activation of 
NOX2 and induction of MCP-1 mediate oxidative stress and endothelial 
dysfunction triggered by SARS-CoV-2 infection upon its S protein 
binding to ACE2, and by infection related cytokine storm, which can be 
substantially alleviated by estrogen treatment. Therefore, estrogen may 
be used as a robust therapy for COVID-19, especially in men, both at the 
early stage of viral infection and the later stage of cytokine storm and 
ARDS/multi-organ failures. It is anticipated to effectively reduce disease 
severity and mortality. 

Our current study investigated the underlying molecular mechanisms 
of sex difference in the morbidity and severity of COVID-19 patients, indicating that estrogen mediates the protection against COVID-19 in females. In addition, our data support the hypothesis that estrogen may be used to alleviate viral infection and cytokine storm-induced endothelial dysfunction, resulting in therapeutic effects to attenuate disease progression, severity and mortality. In an earlier report we described sex difference in patients with COVID-19 for the first time [9]. Indeed, studies of additional patient cohorts worldwide confirmed the observation of male predisposition to higher morbidity and mortality [10-16,54,55]. In addition, male gender predisposition was also seen in subgroups of COVID-19 patients with different conditions. For example, in 928 patients with cancer who were infected with SARS-CoV-2, male gender was significantly associated with increased 30-day all-cause mortality after adjustment for age, smoking status, and obesity (odds ratio 1.63; 95% CI 1.07–2.48) [56]. Of note, male gender-related higher infection incidence for SARS-CoV-2 has also been reported in pre-symptomatic patients with COVID-19 [57,58]. In the study of 196,738 COVID-19 patients from Mexico, 25,520 cases were presymptomatic [57]. In a study that analyzed 194,349,591 males and 201,715,364 females from the onset of the pandemic until June 21st by Bhopal et al., information of gender, age, and mortality in COVID-19 patients was collected by the National Institute for Demographic Studies from national statistical agencies of different countries, including England and Wales, France, Germany, Italy, Netherlands, Portugal, Korea, and Spain. The gender ratios (males vs. females) for mortality from COVID-10
have shown that S protein treatment of endothelial cells induces NOX2 baseline respiratory diseases derived from chronic smoking. More. Rather, the more severe disease in smokers might be related to NOX2 isoform, but not that of NOX1 or NOX4, to result in increased oxidative stress to result in endothelial dysfunction, need to be generate oxidative stress to result in endothelial dysfunction, need to be targeted mediator of cytokine storm during the development of COVID-19, in addition, estrogen treatment may be considered as an effective strategy to alleviate cytokine storm-induced endothelial ROS production and endothelial dysfunction/injury in patients with COVID-19. It is noteworthy that active form of S protein was used in this study to mimic the infection of SARS-CoV-2 in endothelial cells. Recombinant S protein treatment alone was found capable of activating MEK pathway, which is required for viral replication in host cells, indicating that S protein itself can trigger similar signaling mechanisms of viral infection [64]. Likewise, another study has demonstrated that S protein treatment elicits upregulation of genes related to cell growth signaling such as MAK2K and JAK2 in human umbilical vein endothelial cell (HUVEC), and upregulated genes related to signaling of IL-6, NF-kB, and chemokines in brain endothelial cells [34]. Given that S protein has been shown to bind to neuropilin-1 (NRP-1) to inhibit VEGF-A signaling in neurons, S protein binding to NRP-1 in endothelial cells might result in impaired angiogenesis [65]. Moreover, prolonged treatment with recombinant S1 subunit for 48 h resulted in endothelial cell degeneration, indicating the deleterious effect of recombinant S1 subunit on vascular cells [66]. Collectively, these studies seem to implicate that S protein treatment of ACE2 expressing endothelial cells is a highly valuable tool for studying underlying mechanisms of SARS-CoV-2 infection mediated endothelial dysfunction. In a recent study that conducted functional assessment of ACE2 using ACE2 orthologs, the internalization of SARS-CoV-2 nucleocapsid (N) protein was confirmed in bovine ACE2 expressing cell, indicating the successful infectivity of SARS-CoV-2 via interaction with bovine ACE2 [67]. In addition, specificity of the antibody used to detect ACE2 was confirmed by either overexpression of ACE2 with pcDNA3.1-hACE2 plasmid or knockdown of ACE2 using RNA interference, indicating clearly that endothelial cells used in the present study expresses abundant ACE2 and that antibody neutralization experiments using ACE2 antibody is a valid approach to examine ACE2 dependency of NOX2 activation and ROS production in response to S protein exposure. S protein treatment resulted in upregulation of ACE2, which was completely attenuated by estrogen administration. By exposure of endothelial cells to S protein, we demonstrated for the first time that S protein itself can cause an excessive ACE2 dependent endothelial oxidative stress, a crucial mediator of COVID-19 related endothelial dysfunction and inflammation. Moreover, estrogen treatment following S protein exposure abolished excessive superoxide production and upregulated expression of NOX2 and MCP-1 protein and mRNA, indicating that estrogen can be used as a therapeutic intervention to reduce subsequent ARDS and multi-organ injury resulted from endothelial dysfunction and vascular inflammation. Given that ACE2 upregulation by renin-angiotensin system (RAS) antagonist paradoxically favors SARS-CoV-2 binding to endothelial cells [68,69], downregulation of ACE2 expression by estrogen is beneficial in alleviating viral infection induced endothelial dysfunction especially in patients who need to take RAS antagonists for co-existing hypertension. Furthermore, considering that S protein increased endothelial ROS production is stronger and more robust than that of the response from IL-6 treatment, as soon as S protein binds to ACE2 on endothelial cells, it will prime excessive ROS production to contribute to endothelialitis. Of note, estrogen treatment showed reversal effects on NOX2 activation, ROS production, ACE2 upregulation and MCP-1 induction following 30 min of S protein pre-stimulation, further validating therapeutic applicability of estrogen treatment. Therefore, the protective effects of estrogen on both S protein and IL-6 induced NOX2 activation and oxidative stress in endothelial cells [30], as well as the remarkable potential of estrogen to alleviate as a robust therapy for endothelial dysfunction/inflammation that is a critical mediator of ARDS/multi-organ failure and mortality in patients with COVID-19. In conclusion, our data demonstrate for the first time that estrogen-mediated attenuation of NOX2 activation, ROS production and MCP-1 upregulation in response to S protein/IL-6 exposure of endothelial cells underlie protection against COVID-19 in females. These data therefore indicate that estrogen administration can be used as a robust
treatment option for COVID-19 to effectively reduce disease severity and improve survival, which is readily transferrable into clinical practice to treat patients with COVID-19.

Declaration of competing interest

The authors declare no conflicts of interests for this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2021.102099.

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