Basic Medical Research

Effects of digoxin in inhibiting ACE2 and SARS-CoV-2 binding for attenuating COVID-19 in human adipocytes

Meity Ardiana[1], I Gde Rurus Suryawan[1], Hanestya Oky Hermawan[1], Primasitha Maharani Harsoyo Putri[1], Safira Rahma[2,3]

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Authors' affiliations:
[1]Department of Cardiology and Vascular Medicine, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo General Hospital, Surabaya, Indonesia, [2]Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia, [3]Dr. Iskak General Hospital, Tulungagung, Indonesia

Corresponding author:
Meity Ardiana
Department of Cardiology and Vascular Medicine, Faculty of Medicine, Universitas Airlangga, Dr. Soetomo General Hospital, Jalan Prof. DR. Moestopo No. 6–8, Surabaya 60286, Indonesia
Tel/Fax: +62-31-5023277/ +62-31-5031752
E-mail: meityardiana@fk.unair.ac.id

ABSTRACT

BACKGROUND Angiotensin-converting enzyme 2 (ACE2) has a role in SARS-CoV-2 incidence, and digoxin is a competitive inhibitor of SARS-CoV-2-ACE2 binding. This study aimed to investigate the effects of digoxin on SARS-CoV-2-ACE2 binding, proinflammatory cytokines, and prothrombotic factors in adipocytes of patients with COVID-19.

METHODS This *in vitro* study used adipocyte cultures, which were divided into negative control, positive control (SARS-CoV-2 S1 spike protein only), SARS-CoV-2 S1 spike protein with digoxin, and SARS-CoV-2 S1 spike protein with human recombinant soluble ACE2 (hrsACE2). Data were analyzed using one-way ANOVA and Pearson correlation.

RESULTS SARS-CoV-2 significantly elevated ACE2 and increased interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), tissue factor (TF), and plasminogen activator inhibitor-1 (PAI-1) compared to the negative control group (p<0.001). No SARS-CoV-2-ACE2 binding was detected in SARS-CoV-2 with digoxin and hrsACE2 groups, compared to the positive control group (0 ng/ml *versus* 0 ng/ml *versus* 36.33 [1.58] ng/ml, p<0.001). Digoxin significantly decreased IL-6 (48.94 [1.80] ng/ml *versus* 90.93 [4.29] ng/ml; p<0.001), TNF-α (87.65 [6.88] ng/ml *versus* 307.95 [57.34] ng/ml; p<0.001), TF (5.33 [0.32] ng/ml *versus* 6.85 [0.22] ng/ml; p<0.001), and PAI-1 levels (2.92 [0.168] ng/ml *versus* 4.86 [0.11] ng/ml; p<0.001), compared to positive control group. ACE2 positively correlated with IL-6 (p = 0.004, r = 0.763) and TF (p = 0.004, r = 0.768) but was not correlated with IL-1β, TNF-α, and PAI-1 levels.

CONCLUSIONS This study promoted digoxin therapy to prevent cytokine storm and thromboembolism by decreasing IL-6, TNF-α, TF, and PAI-1 in adipocyte cultured models at an early stage of COVID-19.

KEYWORDS adipocyte, digoxin, interleukin-6, SARS-CoV-2, tissue factor

The coronavirus disease 2019 (COVID-19) pandemic continues to spread worldwide, causing morbidity and mortality owing to an emerging infectious disease due to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1,2] The SARS-CoV-2 continues to spread and mutate. People can be reinfected with the same or different strains of SARS-CoV-2 during the current outbreak.[2] Moreover, COVID-19-infected patients with pre-existing cardiovascular disease (CVD), especially those with risk factors for obesity, showed poor outcomes. Approximately 25% of the obese patients with COVID-19 died, which is as high as that in patients with CVD and COVID-19. Obese individuals are prone to viral spread, increased shedding, immune activation, and amplification of cytokines, all of which contribute to COVID-19.[3]

Angiotensin-converting enzyme 2 (ACE2) receptor protein is a critical host receptor for receptor-binding domain (RBD) on SARS-CoV-2 S1 spike protein.[2,4] ACE2 receptor is present in adipocytes, smooth muscle cells,

and endothelial cells[3] across multiple organs, such as the colon, gallbladder, heart muscle, kidney, breast, lung, prostate, and esophagus.[5,6] Increased ACE2 levels are in line with a greater spread of SARS-CoV-2.[7] SARS-CoV-2 virus infection in patients with obesity is associated with increased proinflammatory cytokines leading to a cytokine storm[3,7] and increased hemostatic complications, such as thrombosis[8] and multiple organ damage.[9]

Digoxin, a cardiac glycoside, and an inexpensive drug shows antiviral activity, such as in HIV treatment, respiratory syncytial virus (RSV), cytomegalovirus, and herpes simplex virus.[10] Digoxin is a competitive inhibitor of ACE2-RBD binding.[4] Digoxin can suppress proinflammatory cytokines in influenza, especially against COVID-19.[10] Nevertheless, further studies are needed to support these findings. This study aimed to elucidate the role of digoxin on ACE2 expression, several proinflammatory cytokines, and prothrombotic factors in adipocytes infected with SARS-CoV-2 by mimicking obesity conditions *in vitro*.

METHODS

Experiment protocol

This was an *in vitro* experimental study with a post-test-only control group. A 35-year-old healthy male donor with obesity (body mass index 33 kg/m^2) and no history of HIV, HBV, or HCV infection was included. The abdominal adipose tissue was used to harvest adipocytes. He had no history of acute myocardial infarction, peripheral arterial disease, heart failure, malignant arrhythmia, transient ischemic attack, stroke, diabetes mellitus, or renal failure. Furthermore, he had never received a COVID-19 vaccination and had no history of infection, as verified by negative results of polymerase chain reaction swab results and medical history. Echocardiography was performed to confirm the normal cardiac anatomy. The Health Research Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, approved this study (No. 198/EC/KEPK/07/2021). Using the methods described by Carswell et al,[11] the obtained adipocytes were enzymatically isolated and processed with collagenase type 1. In brief, the mixture was shaken at 100 rpm in a water bath at 37°C for 30 min and grown in alpha minimum essential medium supplemented with 10% fetal bovine serum (Brawijaya Inc., Indonesia) and 1% penicillin-streptomycin (Brawijaya Inc.). Adipocyte cultures were incubated at 37°C with 5% CO_2 in an incubator.

Administration of digoxin and human recombinant soluble ACE2 (hrsACE2)

Adipocytes were grown in a 12-well culture, divided into four groups with six samples each based on Federer sample size calculation: negative control, positive control (SARS-CoV-2 S1 spike protein only), SARS-CoV-2 S1 spike protein with digoxin, and SARS-CoV-2 S1 spike protein with hrsACE2. SARS-CoV-2 subunit S1 spike protein (10 nM) followed the protocol described by Dosch et al.[12]

After a 24-hour incubation with SARS-CoV-2 S1 spike protein, both 0.15 µM digoxin[10] and 100 µg/ml hrsACE2[2] were each added to different culture groups for comparison. All experiments were repeated 3 times to ensure the reliability of our findings.

ACE2-spike protein binding assay, inflammatory cytokines, and prothrombotic factors evaluation

The ability of digoxin and hrsACE2 to affect the binding of SARS-CoV-2 and ACE2 was measured using a binding assay kit. The reagent was fit into SARS-CoV-2

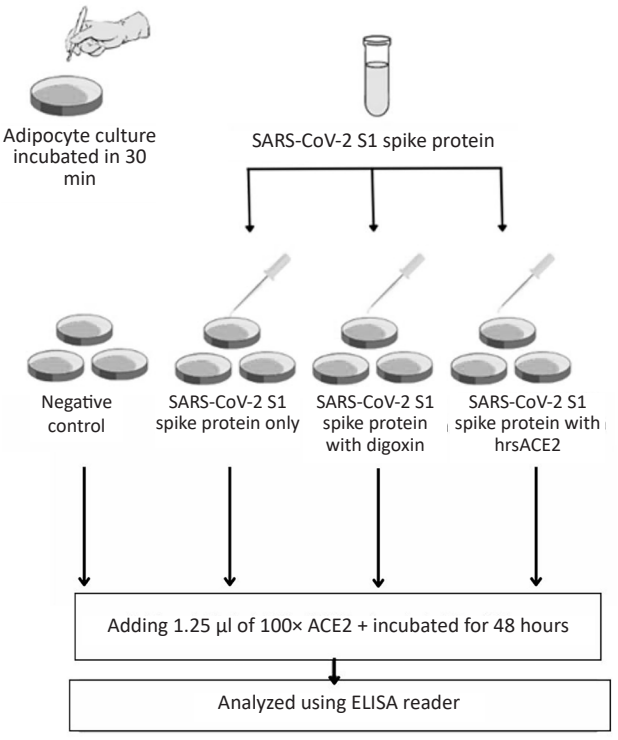

Figure 1. Flow of human donor adipocyte cultures. ACE2=angiotensin-converting enzyme 2; ELISA=enzyme-linked immunosorbent assay; hrsACE2=human recombinant soluble ACE2; SARS-CoV-2=severe acute respiratory syndrome coronavirus 2

spike protein-coated wells, added 1.25 µl of 100x ACE2 to mixing digoxin or hrsACE2 sample, then hatched overnight at 4°C with delicate shaking. The wells were washed 4 times using 300 µl wash buffer. Subsequently after extra washing, 100 µl horseradish peroxidase-conjugated anti-IgG, 100 µl tetramethylbenzidine substrate, and 50 µl halt arrangement were successively added per well. We examined the effects of digoxin and hrsACE2 on ACE2 expression, the levels of proinflammatory cytokines (interleukin (IL)-6, IL-1β, tumor necrosis factor-alpha [TNF-α]), and the levels of prothrombotic factors (tissue factor [TF] and plasminogen activator inhibitor 1 [PAI-1]). Subsequently, absorbance at 450 nm was measured using an enzyme-linked immunosorbent assay reader after 48 hours. The experimental process is shown in Figure 1.

Statistical analysis

Statistical analyses were performed using SPSS software version 22.0 (IBM Corp., USA), with a one-way analysis of variance for comparison and Pearson's correlation method to check the association. $p<0.05$ indicated statistically significant results.

RESULTS

The mean ACE2 level was significantly lower in the hrsACE2 group (11.59, $p<0.001$). In contrast, the digoxin group had higher ACE2 levels (95.75), but the difference was statistically insignificant compared to the negative control group (Table 1, Figure 2).

ACE2 levels were strongly and positively correlated with IL-6 (p = 0.004, r = 0.763) and TF (p = 0.004, r = 0.768) levels. Higher levels of IL-6 and TF correlated with higher levels of ACE2. However, the correlation between ACE2 levels and IL-1β, TNF-α, and PAI expressions was insignificant ($p>0.005$).

In the SARS-CoV-2 infected adipocytes-only-group, IL-6 levels, and TNF-α were significantly increased. The IL-1β levels were higher than the baseline, but the difference was insignificant. Digoxin significantly reduced IL-6 levels by 1.8 times, TNF-α levels by 3.5 times, and IL-1β levels by 1.3 times in the infected SARS-CoV-2 adipocytes compared to the positive control group (Table 1). The hrsACE2 groups had 4 times lower IL-6 levels than the digoxin group. The TNF-α and IL-1β levels in the hrsACE2 groups were higher than in the digoxin groups, but the differences were insignificant.

Table 1. Effects of digoxin administration on ACE2 levels, SARS-CoV-2-ACE2 binding, proinflammatory cytokines, and prothrombotic factors

Groups	Replication (n)	ACE2 (ng/ml)		SARS-CoV-2-ACE2 binding (ng/ml)		IL-6 (ng/ml)		IL-1β (ng/ml)		TNF-α (ng/ml)		TF (ng/ml)		PAI-1 (ng/ml)	
		Mean (SD)	Min–max	Mean (SD)	Min–max	Mean (SD)	Min–max	Mean (SD)	Min–max	Mean (SD)	Min–max	Mean (SD)	Min–max	Mean (SD)	Min–max
Negative control (baseline)	3	14.48 (2.75)	11.73–17.24	0 (0)	0–0	19.92 (0.53)	19.38–20.45	726.66 (103.00)	614.00–816.00	126.78 (52.53)	85.93–186.05	2.99 (0.64)	2.43–3.69	1.95 (0.07)	1.88–2.03
Positive control	3	80.31 (9.31)	71.00–89.62	36.33 (1.58)	34.75–37.91	90.93 (4.29)	86.64–95.23	919.00 (99.00)	820.00–1018.00	307.95 (57.34)	263.73–372.76	6.85 (0.22)	6.60–7.02	4.86 (0.11)	4.75–4.98
Digoxin	3	95.75 (8.20)	89.89–105.13	0 (0)	0–0	48.94 (1.80)	47.53–50.98	697.76 (100.83)	621.12–812.00	87.65 (6.88)	82.34–95.44	5.33 (0.32)	5.13–5.72	2.92 (0.168)	2.77–3.10
hrsACE2	3	11.59 (1.33)	10.13–12.93	0 (0)	0–0	22.61 (0.92)	21.69–23.54	811.575 (260.02)	608.18–1,104.55	128.55 (25.89)	100.64–151.78	4.12 (0.43)	3.76–4.61	3.37 (0.44)	3.10–3.89

ACE2=angiotensin-converting enzyme 2; hrsACE2=human recombinant soluble ACE2; IL-1β=interleukin-1β; IL-6=interleukin-6; PAI-1=plasminogen activator inhibitor-1; SARS-CoV-2=severe acute respiratory syndrome coronavirus 2; SD=standard deviation; TF=tissue factor; TNF-α=tumor necrosis factor alpha

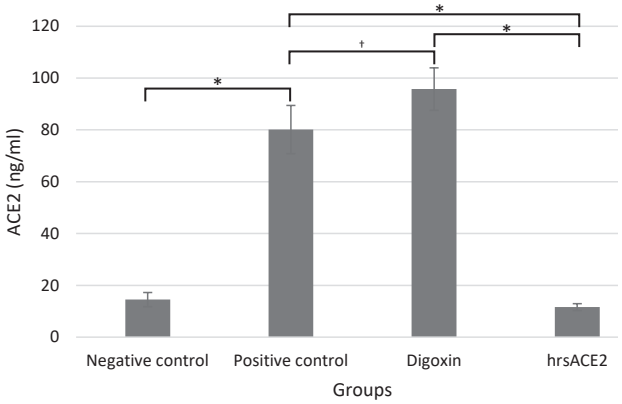

Figure 2. Comparison of ACE2 levels in each group. One way-ANOVA: **p*<0.001; †*p* = 0.0706
ACE2=angiotensin-converting enzyme 2; ANOVA=analysis of variance; hrsACE2=human recombinant soluble ACE2

Initially, the adipocytes showed lower TF and PAI-1 levels at the baseline. However, upon infection with SARS-CoV-2 S1 spike protein, there was a notable increase in procoagulation factors, with statistically significant TF and PAI-1 levels (Table 1).

Digoxin and hrsACE2 lowered TF and PAI-1 levels in infected SARS-CoV-2 adipocytes compared to those in the positive control (Table 1). The digoxin groups showed a statistically significant decrease in TF levels by 1.2 times and PAI-1 levels compared with the positive control group. The hrsACE2 group showed significantly decreased TF levels but not PAI-1 levels compared to the digoxin group. Nevertheless, the PAI-1 levels in the hrsACE2 group remained significantly lower than those in the positive control group.

DISCUSSION

Adipocyte culture as the negative control showed ACE2 receptors, proinflammatory cytokines (IL-6, TNF-α, and IL-β), and prothrombotic factors (TF and PAI-1) as the baseline, suggesting that ACE2 receptors, proinflammatory cytokines, and prothrombotic factors are usually present at low levels in healthy individuals. TNF-α, IL-1, and IL-6 are preactivated in adipose tissue in obesity.[3] In another study, the inhibition mechanism of SARS-CoV-2 by digoxin may be similar to that of RSV, wherein inhibition occurs during viral RNA synthesis by inhibiting viral mRNA expression, copy number, and viral protein expression.[10]

Similar to previous studies,[13,14] ACE2 receptor expression increases following exposure to SARS-CoV-2. Elevated soluble active ACE2 in the first week of infection due to upregulation by interferon stimulation following SARS-CoV-2 infection[13] is associated with worsened outcomes of COVID-19 and severe symptoms.[15] We also confirmed SARS-CoV-2-ACE2 binding. A previous study showed that the RBD on the SARS-CoV-2 viral spike protein binds to the host receptor ACE2 protein in organs that express it, such as adipocyte cells.[4] This study increased proinflammatory cytokines and prothrombotic factors due to SARS-CoV-2 viral infection.

Similar to a previous study, we found a significant correlation between ACE2, IL-6 and TF levels.[16] Higher ACE2 levels activate cellular immunity predominantly by IL-6, which is linked to severe symptom manifestations of COVID-19 and changes in coagulation (lower platelet count, elevated D-dimer, and fibrinogen levels).[17] Human adipose tissue is a major source of IL-6 and IL-6 receptors. IL-6 is a strong independent predictor for severity and mortality in the early stage of COVID-19.[3] ACE2 increases angiotensin II (ANG II), and the binding of ANG II and ANG II type 1 receptors[6] increases TF and PAI-1 levels.[18] Proinflammatory or procoagulant stimuli, aggregation, and activated platelets induce TF expression by monocytes during COVID-19.[1] TF causes fibrin disposal disruption, leading to disseminated intravascular coagulation and an indirect decrease in platelet counts.[16] In the present study, IL-1β, TNF-α, and PAI-1 levels increased but were not correlated with ACE2 in the adipocyte culture. Another study revealed an association between increased PAI-1 levels, clot formation, and thrombosis in severe COVID-19.[8]

In the digoxin group, ACE2 levels increased but were not statistically significant. SARS-CoV-2-ACE2 binding after adding digoxin or hrsACE2 was not statistically significant. Digoxin has a high affinity for blocking viral penetration and is a competitive inhibitor of ACE2-RBD binding.[4] Digoxin inhibits mRNA expression, copy number, and viral protein expression and suppresses proinflammatory cytokines at the post-entry stage.[4,10] Digoxin is not effectively administered in the early stages of COVID-19 infection.[10]

In the first 48 hours, the positive control showed significantly increased IL-6 and TNF-α levels and prothrombotic factor expression, including TF and PAI-1. Viral infection amplifies the pre-existing prime organ cytokine in adipose tissue.[3] IL-6 increases TNF-α and IL-1β expressions.[3] TNF-α can cause endothelial cell damage,[19] endothelial dysfunction, and release

of endothelial PAI-1 that causes thromboembolism following COVID-19 infection.[20,21] Cytokine storm includes high IL-6, TNF-α, and C-reactive protein expressions.[8]

IL-6 and TNF-α were significantly decreased in the first 48 hours in the digoxin group. hrsACE2 significantly decreased IL-6 levels. The digoxin-treated groups showed a statistically significant decrease in TF and PAI-1 levels. However, only hrsACE2 expression was significantly associated with decreased PAI-1 levels. Digoxin experiments are rarely discussed, but they play a role in managing COVID-19 infection with hypercytokinemia.[10]

ACE2 was correlated to IL-6 and TF levels, but our results suggest that digoxin has a wider-ranging effect on inhibiting SARS-CoV-2 and ACE2 binding, reducing IL-6 and TNF-α, reducing TF and PAI-1 in adipocyte cultured models during the first 48 hours compared to hrsACE2. These results indicate that digoxin can prevent severe COVID-19 symptoms, cytokine storms, thromboembolism, and critical illness in patients, leading to death in patients with obesity and COVID-19. The role of digoxin in managing other cardiac risk factors, advanced stages, complications of COVID-19, and critically ill patients with COVID-19 should be investigated in future studies.

The limitation of this study was using only one dosage of digoxin at 0.15 µM. Further research should test various digoxin doses to determine the optimum dose for inhibiting SARS-CoV-2 infection. In conclusion, digoxin decreased IL-6, TNF-α, TF, and PAI-1 levels in adipocyte culture models during the early stage of COVID-19. Our findings provide a potential basis for promoting *in vivo* studies for digoxin therapy to prevent cytokine storms and thromboembolism in patients with COVID-19.

Conflict of Interest

The authors affirm no conflict of interest in this study.

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