Hindawi
Andrologia
Volume 2023, Article ID 9059973, 13 pages
https://doi.org/10.1155/2023/9059973

Research Article

Serum from Men with the Severe Form of COVID-19 Impairs the Nitric Oxide Signaling Pathway in Isolated Corpus Cavernosum from Mice: An *In Vitro* Study

Gabriela Reolon Passos,[1] **Guilherme Ruiz Leonardi**,[1] **Mariana Gonçalves de Oliveira**,[1] **Erick de Toledo Gomes**,[1] **Ana Carolina Ghezzi**,[1] **Edson Antunes**,[1] **Fernanda Andrade Orsi**,[2,3] **Jose Luiz da Costa**,[4] **and Fabiola Zakia Mónica**[1]

[1]*Department of Translation Medicine (Pharmacology Area), University of Campinas, Campinas, Brazil*
[2]*Hematology and Hemotherapy Center, University of Campinas, Campinas, Brazil*
[3]*Department of Clinical Pathology, University of Campinas, Campinas, Brazil*
[4]*Campinas Poison Control Center, University of Campinas, Campinas, Sao Paulo, Brazil*

Correspondence should be addressed to Fabiola Zakia Mónica; fabiolataufic@gmail.com

Received 29 October 2022; Revised 23 December 2022; Accepted 14 February 2023; Published 26 April 2023

Academic Editor: Takashi Yazawa

Introduction. Erectile dysfunction (ED) is characterized by the inability to achieve and/or maintain an erection during sexual activity. Vascular-related diseases such as obesity, diabetes, aging, and hypertension are known risk factors that increase the prevalence of ED. The viral infection caused by SARS-CoV-2, the etiological agent of COVID-19, can evolve into mild to severe forms. Patients with comorbidities such as diabetes and obesity present a more severe state of the disease. The cytokine storm and reactive oxygen species associated with COVID-19 can lead to progressive systemic inflammation and vascular dysfunction. This study is aimed at assessing the *in vitro* effects of serum obtained from unvaccinated men who had the severe form of COVID-19 in isolated corpus cavernosum (CC) from healthy mice. *Methods*. Concentration-response curves to endothelium-dependent and endothelium-independent substances were carried out in isolated CC from mice after being incubated (3 hours) with serum obtained from patients with and without (control group) the severe form of COVID-19. qRT-PCR and western blotting were also carried out. *Results*. The relaxing responses induced by acetylcholine (ACh), a nitric oxide donor (sodium nitroprusside), soluble guanylate cyclase (sGC) stimulator (BAY 63-2521), and phosphodiesterase type 5 (PDE5) inhibitor (tadalafil) were significantly reduced in CC incubated with COVID-19 serum when compared with CC incubated with the control serum. Nonetheless, the relaxation induced by the sGC activator BAY 58-2667 was unaffected in CC stimulated with the COVID-19 serum. The coincubation of control or COVID-19 serum with a free radical scavenger (PEG-SOD; 150 UI/mL, 3 hours) significantly improved the relaxation induced by ACh. On the other hand, catalase did not improve ACh-induced relaxation in CC incubated with the sera. The gene expression for PDE5 and NADPH oxidase type 4 was increased in CC stimulated with COVID-19 serum in comparison to tissues stimulated with the control serum. The protein expression for sGC subunits was similar in both groups. *Conclusion*. Serum obtained from unvaccinated men who presented the severe form of COVID-19 impaired the relaxation induced by cyclic guanosine monophosphate-accumulating substances in CC from mice.

1. Introduction

Erectile dysfunction is characterized by the inability to achieve and/or maintain the erection during sexual activity. Tumescence, erection, and detumescence are the main phases involved in the erectile cycle. Substances released from nerve fibers and from the endothelial layer are involved in the contraction and relaxation of the penis. Catecholamines, endothelin-1, thromboxane A2, angiotensin II, and prostaglandin F2 contract the smooth muscle, whereas nitric

oxide (NO), adenosine, prostacyclin, and prostaglandin E2 (via EP2/EP4 receptors) relax the smooth muscle [1]. Vascular-related diseases such as obesity [2], diabetes [3], aging [4], and hypertension [5] are known risk factors that increase the prevalence of erectile dysfunction (ED). Endothelial dysfunction is characterized by an imbalance between the production of contractile and relaxing substances, while a higher expression of adhesion molecules and a proinflammatory state are the main causes of vasculogenic ED [6].

The viral infection caused by SARS-CoV-2 can evolve into mild to severe forms of COVID-19. Patients with comorbidities such as diabetes and obesity are known to present a more severe state of the disease [7]. Both the SARS-CoV-2-induced cytokine storm and the reactive oxygen species associated with COVID-19 can lead to progressive systemic inflammation and vascular dysfunction [8].

In the corpus cavernosum (CC) obtained from patients undergoing penile prosthesis surgery due to severe ED and a history of COVID-19 infection, viral RNA was observed near the vessels [9]. When compared to men without COVID-19 infection, those with COVID-19 were older and had a higher prevalence of diabetes and hypertension; however, COVID-19 diagnosis was still significantly associated with ED [10]. Since erection is a vascular event, we hypothesized that the systemic inflammation generated during the SARS-CoV-2 infection could impair the relaxing responses of the penis. Therefore, in the present study, we evaluated by means of functional and molecular assays the *in vitro* effects of serum obtained from patients with the severe form COVID-19 in CC from healthy mice.

2. Materials and Methods

2.1. Human Samples. The present study was approved by the Human Ethics Committee of the University of Campinas (UNICAMP) (Protocol Number: 30648520.6.0000.5404). Blood was collected from volunteers at Hospital das Clinicas of the University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil. None of the patients were vaccinated against COVID-19 at the time that blood was collected. Blood was collected in a vacuette tube (Greiner Bio-one) and centrifuged at 2,500 rpm (10 min, 25°C). Serum from ten participants from the "control group" and seven participants from the "COVID-19 group" were transferred to cryovials and maintained at -80°C until used.

Inclusion criteria were as follows: (i) *COVID-19 group*: men over 18 years old who were admitted to the intensive care unit (ICU) after diagnosis of SARS-CoV-2 infection determined by RT-PCR of nasopharyngeal samples. Serum was obtained from the patients with COVID-19 within 72 hours of ICU admission between April and May of 2021. (ii) *Control group:* men over 18 years old who did not present fever, cough, sore throat, headache, pain, shortness of breath, diarrhea, tiredness, or loss of appetite or taste for 15 days before blood collection. Healthy men with comorbidities such as diabetes, hypertension, and obesity were also included. Only participants who signed the informed consent were included.

Exclusion criteria were as follows: (i) *COVID-19 group*: serum from men obtained after being discharged from ICU; men who were taking any of the following drugs: tadalafil, sildenafil, vardenafil, phentolamine, alprostadil, yohimbine, papaverine, thiazide diuretic drugs, or beta-blockers, with the exception of carvedilol and nebivolol. (ii) *Control group:* men with any of the symptoms mentioned above; men with a history of erectile dysfunction; men who were taking medication for the treatment of erectile dysfunction, thiazide diuretics, or beta-blockers, with the exception of carvedilol or nebivolol. Participants who did not sign the informed consent were excluded.

2.2. Animals. A total of fifty-five male C57BL/6 mice (12-16 weeks old) were used from the animal facility of the Central Animal House Services of UNICAMP. The animals were housed in ventilated cage shelters (humidity: 55 ± 5%; temperature: 24 ± 1 ∘ C) under a 12 h light-dark cycle and received filtered water and food *ad libitum*. All experimental protocols were carried out according to the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation and were approved by the Institutional Committee for Ethics in Animal Research of the University of Campinas (CEUA/UNICAMP number: 6007-1/2022). The corpus cavernosum (CC) from mice was selected since the tissue shares similar physiological pathways to that of human CC. The innervation patterns of adrenergic, cholinergic, and nonadrenergic/noncholinergic are similar in mouse and human erectile tissue [11], as are the molecular mechanisms to induce cavernous smooth muscle contraction or relaxation (e.g., sGC/cGMP and AC/cAMP pathways; [1, 12]). A murine model was chosen due to its overall utility and technical feasibility (in terms of cost, supply, housing, and handling), its wide acceptance as a model for the study of penile erection [13], and the inability to collect human CC tissue due to the suspension of transplant surgeries during the pandemic period. Animal studies were reported to be in compliance with ARRIVE guidelines.

2.3. Experimental Design. Isoflurane (above 5%) was used for euthanasia, followed by cervical dislocation. The animal penis was isolated, and the penile vein, corpus and bulb spongiosum, and connective tissues were removed. Two strips of corpus cavernosum (CC) were obtained for each penis and placed into a different well in sterile 96-well plate divided into two groups: "control serum" and "COVID-19 serum" (Figure 1). The wells containing CC strips were covered with 150 μL of Krebs-Henseleit solution (mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, and 5.5 glucose. Then, 50 μL of human "control serum" or "COVID-19 serum" was added, and the plate was maintained at 4°C for 3 hours. After this period, the strips were removed and used for either functional or molecular assays, as detailed below. To assess if the serum addition itself could interfere with smooth muscle reactivity, we incubated the CC strips with only Krebs-Henseleit solution (3 h, 4°C). We carried out preliminary experiments by incubating mouse CC with human serum at three different time points

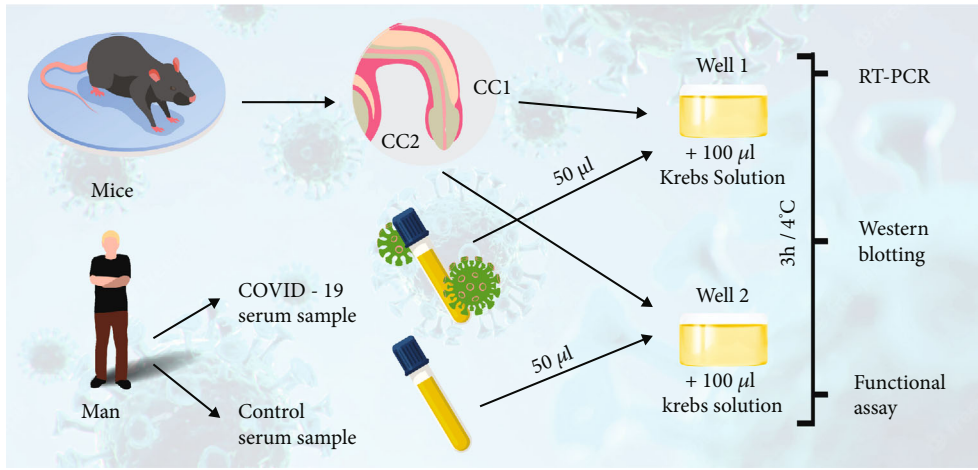

Figure 1: General workflow showing the incubation of human control and COVID-19 serum used to perform RT-PCR, functional assays, and western blotting in isolated corpus cavernosum (CC) from mice.

(0.5, 1, or 3 hours) and performed functional assays (data not shown), which indicated that three hours was the only incubation time at which impaired relaxing responses could be observed.

2.4. Functional Assays. After incubation, the CC was mounted in a 10 mL organ bath containing Krebs-Henseleit solution, continuously bubbled with a carbogenic mixture (95% O_2/5% CO_2, 37°C). The tissues were equilibrated for 45 min under 5 mN of tension, replacing fresh Krebs-Henseleit solution every 15 min. Isometric force was recorded using a PowerLab system (ADInstruments, I., Sydney, Australia). The tissues were then precontracted with the α1-adrenoceptor agonist phenylephrine (10^{-6} mol/L). Once the contraction reached a plateau, cumulative concentration-response curves (CRCs; 10^{-12} to 10^{-6} mol/L) were carried out for the NO donor sodium nitroprusside (SNP, Sigma-Aldrich, Missouri, United States), the muscarinic agonist acetylcholine (ACh, Sigma-Aldrich, Missouri, United States), and the phosphodiesterase type 5 (PDE5) inhibitor tadalafil (Biolab Sanus Pharmaceutics, Sao Paulo, Brazil), and the soluble guanylyl cyclase (sGC) stimulator (riociguat, BAY 63-2521, Selleck, Planegg, Germany) and activator (BAY 58-2667, Tocris, Bristol, United Kingdom) were carried out. In another set of experiments, superoxide dismutase-polyethylene glycol (PEG-SOD; 150 UI/mL, Sigma-Aldrich, Missouri, United States) or catalase (300 UI/mL, Sigma-Aldrich, Missouri, United States) was coincubated within the serum for 3 hours before being used for CRCs for ACh and tadalafil.

Only one concentration-response curve was carried out for each strip. The relaxation results were expressed as a percentage of the response induced by phenylephrine (10^{-6} mol/L). Nonlinear regression was used to determine the potency (pEC_{50}), carried out using GraphPad Prism (GraphPad Software, Inc., California, USA) with the constraint that $F = 0$. All concentration-response data were evaluated for a fit to a logistic function in the form: $E = E_{max}/([1 + (10c/10x)n] + F)$, where E is the effect of above basal; E_{max} is the maximum response produced by agonists; c is the logarithm of the pEC_{50}, the concentration of drug that produces a half maximal response; x is the logarithm of the concentration of the drug; the exponential term, n, is a curve-fitting parameter that defines the slope of the concentration-response line; and F is the response observed in the absence of the added drug.

2.5. Quantitative Real-Time RT-PCR (qPCR). After incubation, the CC segments were homogenized in TRIzol® reagent (Invitrogen, Mississippi, USA) to obtain total RNA, according to the manufacturer's protocol. The RNA samples were transcribed with High-Capacity Reverse Transcription Kit® (Applied Biosystems, California, USA). Spectrophotometry was used to assess RNA concentration and purity. After transcription, cDNA was quantified with a NanoDrop Lite® (Thermo Scientific, Massachusetts, USA) and 10 ng cDNA was used to perform the reactions. The reactions were performed with cDNA, SYBR Green Master Mix® (Life Technologies, California, USA), and optimal primer concentration, in a total volume of 12 μL. Real-time PCR was performed in a StepOne-Plus® Real-Time PCR System (Applied Biosystems, California, USA). Synthetic primers (Quantitec®, Qiagen, North Rhine-Westphalia, Germany) were as follows: *ACTB* (QT00095242, NM_007393), *GUCY1A1* (QT00133581, NM_021896), *GUCY1B1* (QT00103376, NM_001161796), *CYBB* (QT00139797, NM_007807), *NOX4* (QT00126042, NM_015760), *SOD1* (QT00165039, NM_011434), *NOS3* (QT00152754, NM_008713), and *PDE5A1* (QT00048167, NM_033430). The reaction was programed for 45 cycles of 95°C for 15 seconds and then 60°C for 1 minute. The threshold cycle (Ct) was defined as the point at which the fluorescence rose appreciably above the background fluorescence. Amplification specificity was verified using a dissociation curve. The results are expressed as arbitrary units, and each gene studied was normalized according to *ACTB* expression, which did not differ between groups (24.37 ± 0.55 and 24.92 ± 0.48 Ct, for the control and COVID-19 groups, respectively, $n = 6$). Careful consideration was taken to adhere to the MIQE checklist.

2.6. Western Blotting. After incubation, CC tissues were homogenized in RIPA buffer (Sigma, Minnesota, USA)

buffer using BeadBlaster™24 (Benchmark, New Jersey, USA) for disintegration. Samples were centrifuged for 20 minutes at 1500 g (4°C), the supernatant was collected, and protein concentration was determined by the Lowry assay. The samples were treated with the Laemmli buffer, heated at 100°C for 5 minutes, and protein samples were separated by electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes (BioRad, California, USA), blocked with 3% bovine serum albumin, and then incubated overnight with specific primary antibodies for GCα1, GCβ1 (Novus Biologicals, Colorado, USA), and β-actin (Sigma, Missouri, USA). Specific secondary peroxidase conjugated antibodies (Santa Cruz Biotechnology, Texas, USA) and ECL-solution (BioRad, California, USA) were used for detection. Densitometry was performed using a ChemicDoc® MP Image System (BioRad, California, USA), and GCα1 and GCβ1 protein expressions were normalized to β-actin. Careful consideration was taken to adhere to the MIQE checklist.

2.7. IL-6 and TNF-α Serum Quantification. Sera from the "control group" and "COVID-19 group" were collected as described above. Serum levels of interleukins were measured using commercial, enzyme-linked immunosorbent assay kits against IL-6 (human EIA Kits 501030, Cayman Chemical, Michigan, USA), and TNF-α (human, EIA kits DTA00C, Minnesota, USA). According to the manufacturer's product insert, the minimum detectable concentrations of IL-6 and TNF-α were 3.9 pg/mL and 15.6 pg/mL, respectively.

2.8. Statistical Analysis. GraphPad Prism (GraphPad Software, Inc.) was used to perform the statistical analyses. First, a Shapiro-Wilk test was performed to analyze normality distribution. For statistical comparisons, an unpaired Student's t-test (between two groups) or one-way ANOVA (among three groups), followed by Bonferroni's posttest, was applied. $p < 0.05$ was considered statistically significant. Chi-square tests were utilized to determine the differences between groups for selected demographic variables. The data were presented as the mean ± standard error of the mean (SEM). The n values refer to the number of animals and human serum used in each protocol. Since the study has an exploratory character, the number of mice was chosen to obtain reliable results while simultaneously maintaining the number of animals low to avoid ethical issues.

3. Results

3.1. Demographic Characteristics. Patient characteristics are described in Table 1. In both groups, there were patients with and without comorbidities. No statistical differences were observed in the ages between the groups. In the COVID-19 group, there were more diabetic men than in the control group. The C-reactive protein plasma levels were higher in the COVID-19 group. The levels of both TNF-α and IL-6 were significantly higher in the sera from the COVID-19 group than in the control group.

3.2. Effect of "Control Serum" in Isolated CC. Firstly, we compared the relaxation response of CC incubated (3 h) with serum obtained either from the control group or the vehicle (Krebs-Henseleit solution). As shown in Table 2, the control serum did not significantly alter the CC relaxation induced by SNP, ACh, tadalafil, BAY 63-2521, or BAY 58-2667, in comparison with the vehicle. The amplitude of contraction induced by phenylephrine (10^{-6} mol/L) did not differ ($p < 0.05$) in CC incubated with the vehicle (0.35 ± 0.02) or control (0.41 ± 0.05) or COVID-19 (0.40 ± 0.05) sera.

3.3. The Relaxing Responses Induced by cGMP-Increasing Substances Are Impaired in Isolated CC Stimulated with Human COVID-19 Serum. As shown in Figure 2, the relaxing responses were reduced ($p < 0.05$) by ACh (39%; Figures 2(a) and 2(b)), SNP (35%; Figures 2(c) and 2(d)), BAY 63-2521 (46%; Figures 2(e) and 2(f)), and tadalafil (51%; Figures 2(g) and 2(h)) in CC stimulated with COVID-19 serum in comparison with CC stimulated with the control serum. In contrast, the relaxation induced by the sGC activator, BAY 58-2667 (Figures 2(i) and 2(j)), was unaffected ($p = 0.17$) in CC stimulated with the COVID-19 serum when compared to tissue stimulated with the control serum. The pEC_{50} and E_{max} values are depicted in Table 2.

3.4. PEG-SOD Reversed the Impaired Relaxation Induced by ACh, but Not by Tadalafil. The presence of PEG-SOD (3 h) completely reversed the impaired response induced by ACh in CC strips incubated with serum from the COVID-19 group in comparison to CC incubated without PEG-SOD (Figures 3(a) and 3(b)). The relaxation induced by ACh was also improved by PEG-SOD in tissue incubated with the control serum (Figures 3(a) and 3(b)). However, the addition of PEG-SOD did not improve the relaxing response induced by tadalafil in CC incubated with control or COVID-19 serum (Figures 3(c) and 3(d)). Conversely, prior incubation with catalase (300 UI/mL, 3 h) did not improve the impaired relaxation induced by ACh in tissue stimulated with COVID-19 or control serum (Figures 3(e) and 3(f)).

3.5. Gene and Protein Expression. While no changes were observed in the mRNA expression of endothelial nitric oxide synthase (eNOS; *NOS3*; Figure 4(a)) or sGCα (*GUCY1A1*; Figure 4(b)) and sGCβ subunits (*GUCY1B1*; Figure 4(c)), a significant ($p < 0.05$) increase was observed in the gene expression of PDE5 (*PDE5A1*; Figure 4(d)) in CC incubated with COVID-19 serum in comparison with tissue stimulated with control serum. As the bioavailability of NO can also be controlled by its reaction with reactive oxygen species, the gene expression for the NADPH oxidases (NOX) was also assessed. The gene expression of neither *CYBB*, referred to NOX2 protein (Figure 4(e)), nor SOD1 (Figure 4(g)) was significantly altered in CC stimulated with COVID-19 serum. The gene expression for NOX4 (Figure 4(f)), however, was significantly greater ($p < 0.05$) in CC stimulated with COVID-19 than with control serum.

The protein expression for sGCα (Figure 4(h)) and sGCβ (Figure 4(i)) subunits did not differ between CC stimulated with control compared to COVID-19 serum.

Table 1: Characteristics of patients from the control and COVID-19 groups.

	Control ($n = 10$)	COVID-19 ($n = 7$)	p value
Age (years; mean ± SEM)	46.9 ± 2.7	49.6 ± 4.7	0.3
Classes of medication in use			
Hormone	10%	14.3%	0.8
Angiotensin-converting enzyme inhibitors	10%	28.6%	0.3
Angiotensin II receptor antagonists	30%	14.3%	0.6
Antihyperglycemic agents	0	14.3%	0.2
Diuretics	0	14.3%	0.2
Statins	0	14.3%	0.2
Anticoagulants	0	14.3%	0.2
Antiplatelets	0	14.3%	0.2
Antiemetics	0	14.3%	0.2
Comorbidities			
Hypertension	40%	57.1%	0.2
Overweight (BMI 25-29.9 kg/m^2)	10%	0%	0.2
Obesity (BMI > 30 kg/m^2)	10%	28.6%	0.3
Diabetes	0	28.6%	0.03*
Clinical characteristics			
Hospitalization (days, mean ± SEM)	0	23.6 ± 6.6	0.0005*
ICU hospitalization (days, mean ± SEM)	0	18 ± 6.3	0.001*
Mortality rate (%)	0	42.9	0.01*
Laboratory tests			
Creatinine (mg/dL, mean ± SEM)	0.98 ± 0.03	2.77 ± 1.15	0.09
Lymphocytes (mm^3, mean ± SEM)	1.67 ± 0.06	1.08 ± 0.22	0.008*
Platelets ($_\times 10^3$/L, mean ± SEM)	202250 ± 10552	272857 ± 32926	0.02*
C-reactive protein (mg/dL, mean ± SEM)	1.81 ± 0.78	35.9 ± 12.82	0.007*
IL-6 serum levels (pg/mL, mean ± SEM)	15.09 ± 4.3	86.41 ± 19.16	0.006*
TNF-α serum levels (pg/mL, mean ± SEM)	5.9 ± 1.21	30.45 ± 9.36	0.01*

BMI: body mass index; ICU: intensive care unit; *$p < 0.05$, control vs. COVID-19, unpaired t-test. SEM: standard error of the mean.

4. Discussion

Our results clearly show that the incubation of human serum from patients with the severe form of COVID-19 impaired the relaxations induced by the endothelium-dependent (ACh) and endothelium-independent (tadalafil, BAY 63-2521, and SNP) substances in isolated CC from healthy mice. The impairment of ACh relaxation was prevented when the serum was coincubated with PEG-SOD, but this was not observed with catalase coincubation. A significant increase in *PDE5* and *Nox4* mRNA was observed in isolated CC incubated with COVID-19 serum. The protein expression for sGC subunits did not differ between CC stimulated with the control and COVID-19 serum.

Several efforts have focused on identifying cytokine profiles that predict a poor outcome in COVID-19 infection. In this regard, several reports have identified increased circulating levels of individual cytokines such as IL-6 and TNF-α as potential biomarkers or targets for therapy [14–18]. In patients with severe COVID-19, high serum IL-6 levels represent a hallmark inflammatory signature, and IL-6 receptor blocking antibodies (e.g., tocilizumab and sarilumab) have been approved for treating patients with COVID-19 and serious pulmonary damage. TNF-α is also one of the most important proinflammatory cytokines of the innate immune response and has a direct effect on IL-6 levels [19], although anti-TNF therapy for COVID-19 is still under discussion. Additionally, impaired microvascular function is a key feature of erectile dysfunction [20], and both IL-6 and TNF-α are considered to be important contributors to endothelial dysfunction [21–23]. Our results corroborate with the previous findings, as the levels of IL-6 and TNF-α were increased in patients in the acute phase of COVID-19. More studies are needed to determine if the increased levels of IL-6 and TNF-α in COVID-19 serum were responsible for the impaired CC relaxation.

Some pharmacological classes, such as thiazide diuretics and beta-blockers, with the exception of carvedilol and nebivolol, have been shown to interfere negatively in erectile function [24]; however, a recent meta-analysis showed that all antihypertensive classes seem to exert a neutral or insignificant effect in erectile function [25]. As the literature is

Table 2: Potency (pEC_{50}) and maximal response (E_{max}, %) values obtained from concentration-response curves for sodium nitroprusside (SNP), acetylcholine (ACh), tadalafil, sGC stimulator BAY 63-2521, and activator BAY 58-2667 in corpus cavernosum strips from healthy mice incubated (3 h, 4°C) with human serum from control and COVID-19 patients.

		pEC_{50}	E_{max} (%)	n
SNP	Vehicle	6.35 ± 0.10	107.08 ± 6.92	9
	Control serum	6.27 ± 0.22	124.22 ± 9.20	6
	COVID-19 serum	6.04 ± 0.13	81.44 ± 9.21*	7
ACh	Vehicle	6.21 ± 0.17	50.32 ± 6.15	5
	Control serum	6.51 ± 0.19	53.68 ± 8.24	6
	COVID-19 serum	6.64 ± 0.23	28.70 ± 5.11*	6
Tadalafil	Vehicle	6.37 ± 0.19	70.09 ± 9.88	5
	Control serum	6.96 ± 0.20	100.74 ± 15.64	5
	COVID-19 serum	6.42 ± 0.21	46.78 ± 6.10*	7
BAY 58-2667	Vehicle	7.42 ± 0.21	124.19 ± 1.13	3
	Control serum	7.50 ± 0.28	103.55 ± 6.65	5
	COVID-19 serum	7.41 ± 0.17	85.91 ± 3.86	5
BAY 63-2561	Vehicle	6.54 ± 0.16	128.65 ± 13.50	4
	Control serum	7.10 ± 0.24	114.96 ± 9.29	5
	COVID-19 serum	6.34 ± 0.33	67.85 ± 10.47*	5

Vehicle: Krebs-Henseleit solution. Data represents mean ± SEM. *$p < 0.05$, control serum vs. COVID-19 serum.

controversial on this point, our exclusion criteria included not using serum from patients using thiazides and/or beta-blockers. No statistical difference was observed between the groups regarding the class of drugs used. With respect to comorbidities, vascular-related diseases such as hypertension, diabetes, and obesity affect erectile function. Participants with comorbidities were present in both groups, though without any difference between groups with the exception of diabetes, which was more prevalent in the COVID-19 group. Although our sample size was small, removing the two diabetic patients from the analysis did not affect the results, showing that these samples did not respond to COVID-19 serum-induced impaired relaxation responses.

The consequences of SARS-Cov-2 infection in the urinary system have been much less studied than, for example, in the cardiovascular and neurological systems. The majority of the studies have focused on case reports of penile ischemia in patients who had COVID-19 [26–28]. A recent case-control study revealed that severe COVID-19 can be associated with ED in healthy individuals as a complication, even after their recovery [29]. The long-term consequences on erectile function in men with COVID-19 are not yet well understood [30]. To the best of our knowledge, only one study showed the presence of viral particles in perivascular erectile tissue, as well as lower protein expression for eNOS in comparison to specimens from men without COVID-19. No functional or biochemical assays were carried out in this study [9].

The use of blood, plasma, or serum from patients with COVID-19 and their role in other cells were explored in several studies. For instance, in microvascular endothelial cells from human brain infected with SARS-CoV-2, a significant increase in cytokines, chemokines, and adhesion molecules was observed [31]. Plasma obtained from patients with the severe form of COVID-19 incubated for 30 minutes with human umbilical vein endothelial cells produced lower concentrations of hydrogen peroxide (H_2O_2) and NO, accompanied by glycocalyx degradation. No alterations in the superoxide anion (O_2^-) were observed after COVID-19 plasma incubation [32]. Healthy monocytes expressed higher levels of tissue factor after being incubated with platelets obtained from patients with the severe form of COVID-19. Additionally, plasma from patients with the severe form of COVID-19 incubated with healthy platelets produced a significant increase in the expression of P-selectin and CD63, which are known markers for platelet activation [33].

In the penis, NO is formed from the nitrergic nerves or from the endothelial layer and stimulates its intracellular receptor, sGC, to produce cyclic guanosine monophosphate (cGMP). The action of cGMP is terminated mainly by phosphodiesterase type 5 (PDE-5) and type 9 (PDE-9) [34] or by the multidrug resistance protein types 4 and 5, which pump cyclic nucleotides out of the cytosol [35]. sGC is a heterodimeric enzyme consisting of two subunits known as alpha (α) and beta (β), with $\alpha_1\beta_1$being the most studied isoform. Nitric oxide binds to the heme group of sGC, which can be found in the reduced (Fe^{2+}) and oxidized (Fe^{3+}) states. The activation of sGC can also be achieved by sGC stimulators (BAY 41-2272, BAY 41-8543, and BAY 63-2521) and sGC activators (BAY 58-2667, BAY 60-2770, and HMR 1766) [36]. The sGC stimulators work by coacting with NO and are dependent on the presence of Fe^{2+}, while sGC activators preferentially activate sGC when Fe^{3+} is present [37]. To

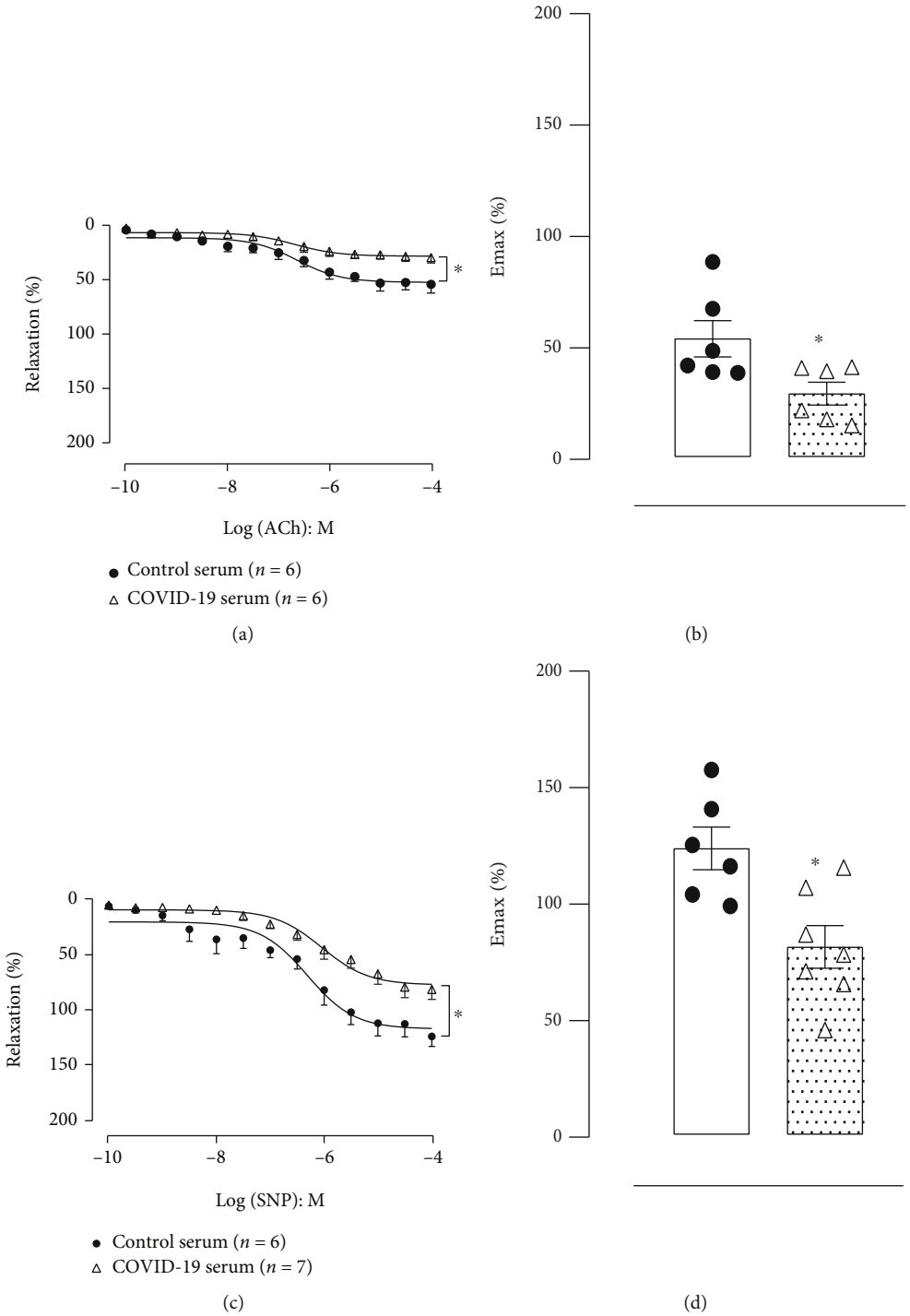

Figure 2: Continued.

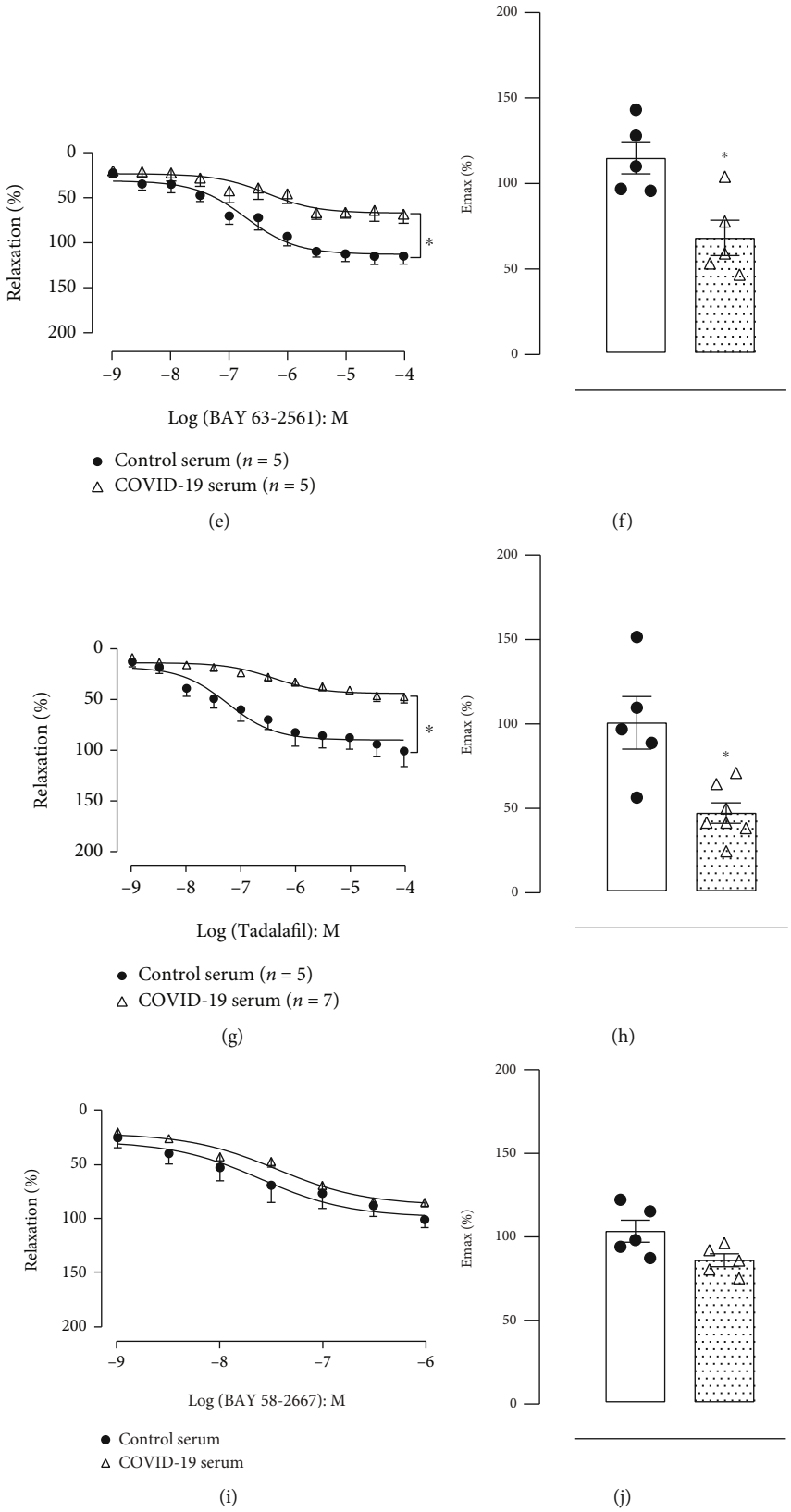

Figure 2: Relaxation responses to cumulative addition of the muscarinic agonist acetylcholine (ACh; a, b), nitric oxide donor sodium nitroprusside (SNP; c, d), soluble guanylate cyclase (sGC) stimulator (BAY 63-2521; e, f), phosphodiesterase type 5 (PDE5) inhibitor (tadalafil; g, h), and sGC activator (BAY 58-2667; i, j) in isolated corpus cavernosum (CC) from mice in the presence of control (●) or COVID-19 (△) serum, precontracted with phenylephrine (10^{-6} mol/L). Data represent the mean ± SEM. The *n* between the parentheses refer to the number of patients and animals used for each protocol. $^{*}p < 0.05$, control serum vs. COVID-19 serum.

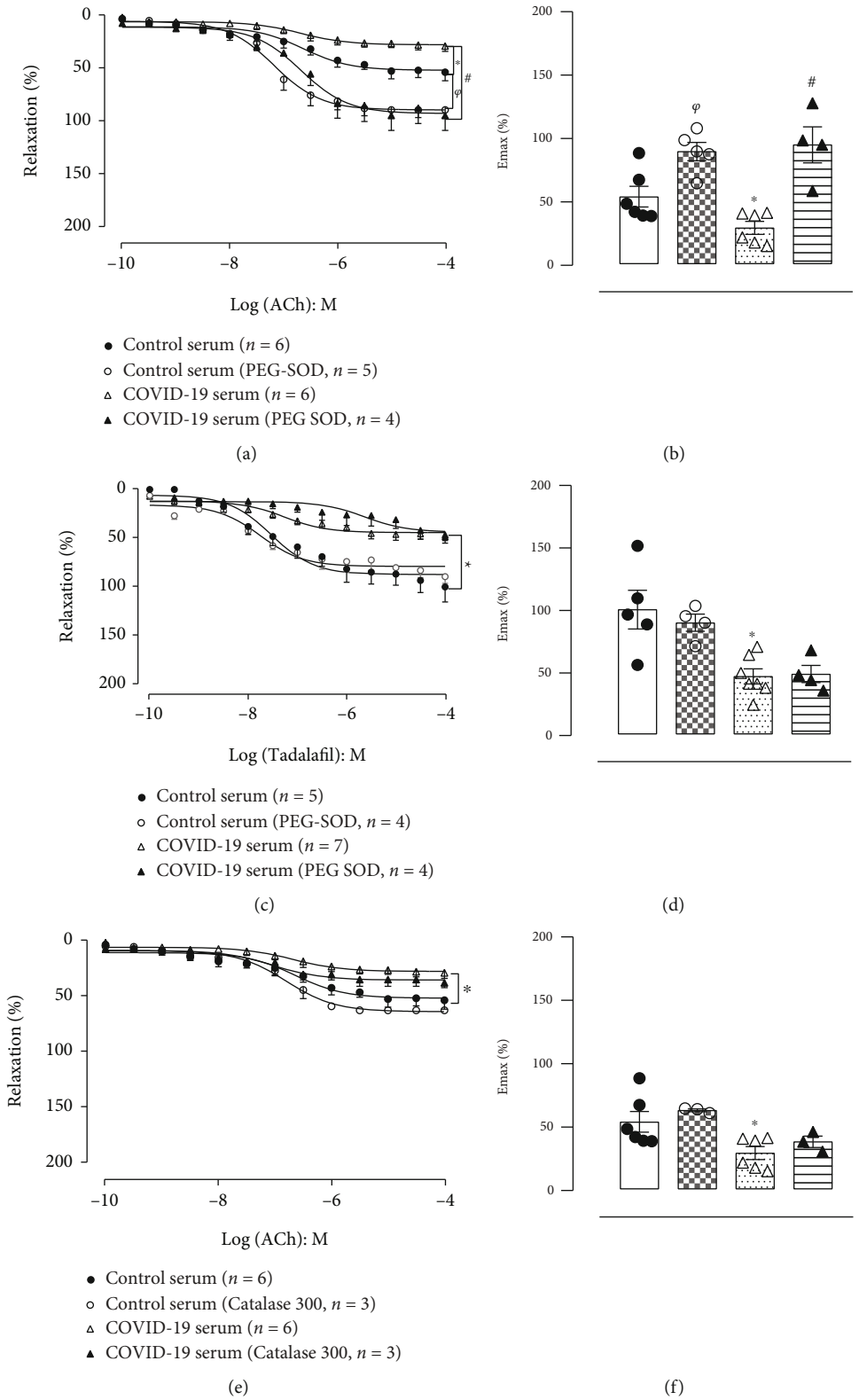

Figure 3: Relaxation responses to cumulative addition of the muscarinic agonist acetylcholine (ACh; a, b) and phosphodiesterase type 5 (PDE5) inhibitor (tadalafil; c, d) in isolated corpus cavernosum (CC) from mice in the presence of control serum (●), control serum with PEG-SOD (○; 150 UI/mL), COVID-19 serum (▲), and COVID serum with PEG-SOD (△). Relaxation responses to ACh (e, f) performed in mice in the presence of control serum (●), control serum with catalase (○; 300 UI/mL), COVID-19 serum (△), and COVID serum with catalase (▲). Data represent the mean ± SEM. The *n* between the parentheses refers to the number of patients and animals used for each protocol. $p < 0.05$ marked for control vs. COVID-19 serum (*), control vs. control PEG-SOD serum (Φ), and COVID-19 vs. COVID-19 PEG-SOD serum ($^{\#}$).

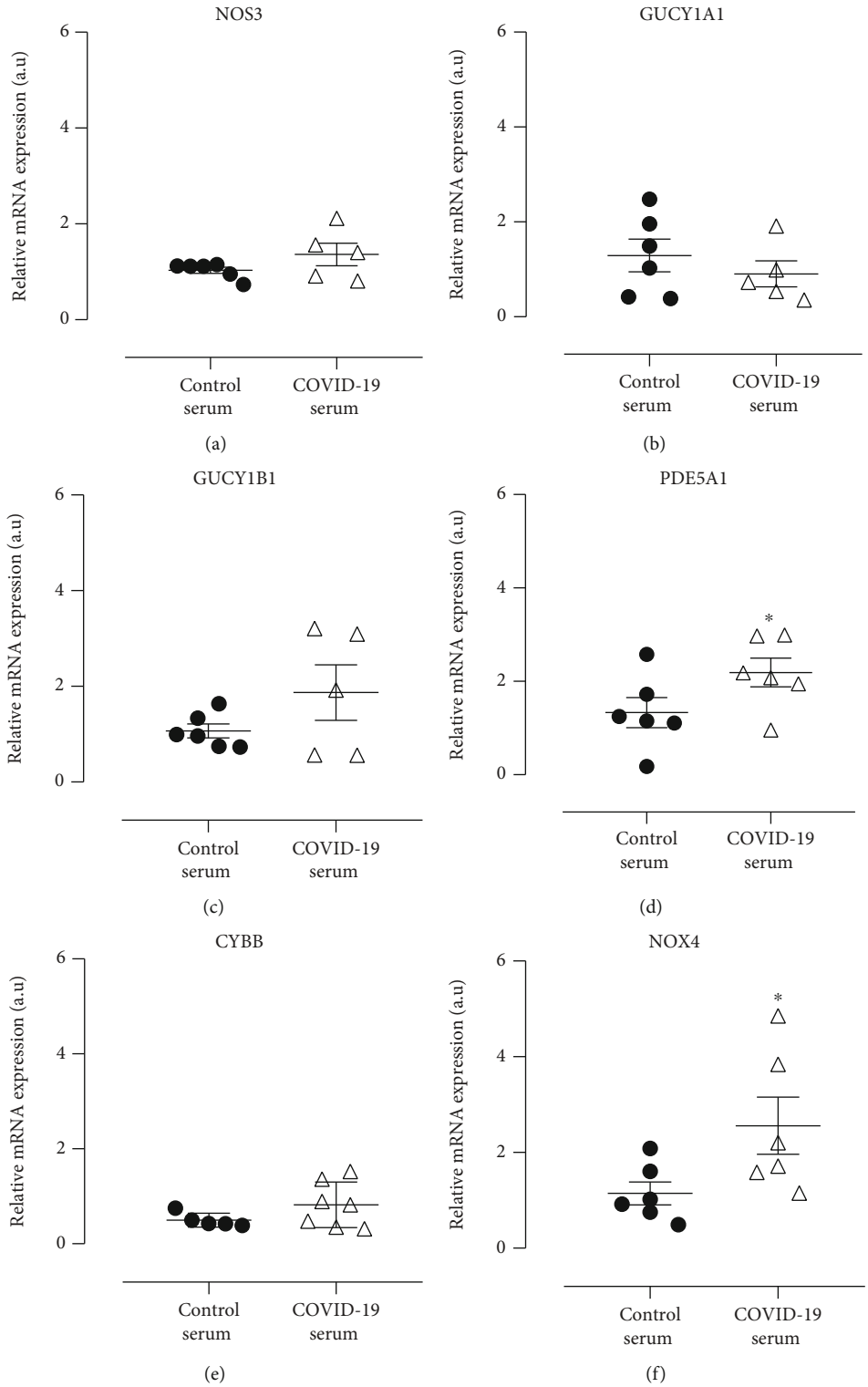

Figure 4: Continued.

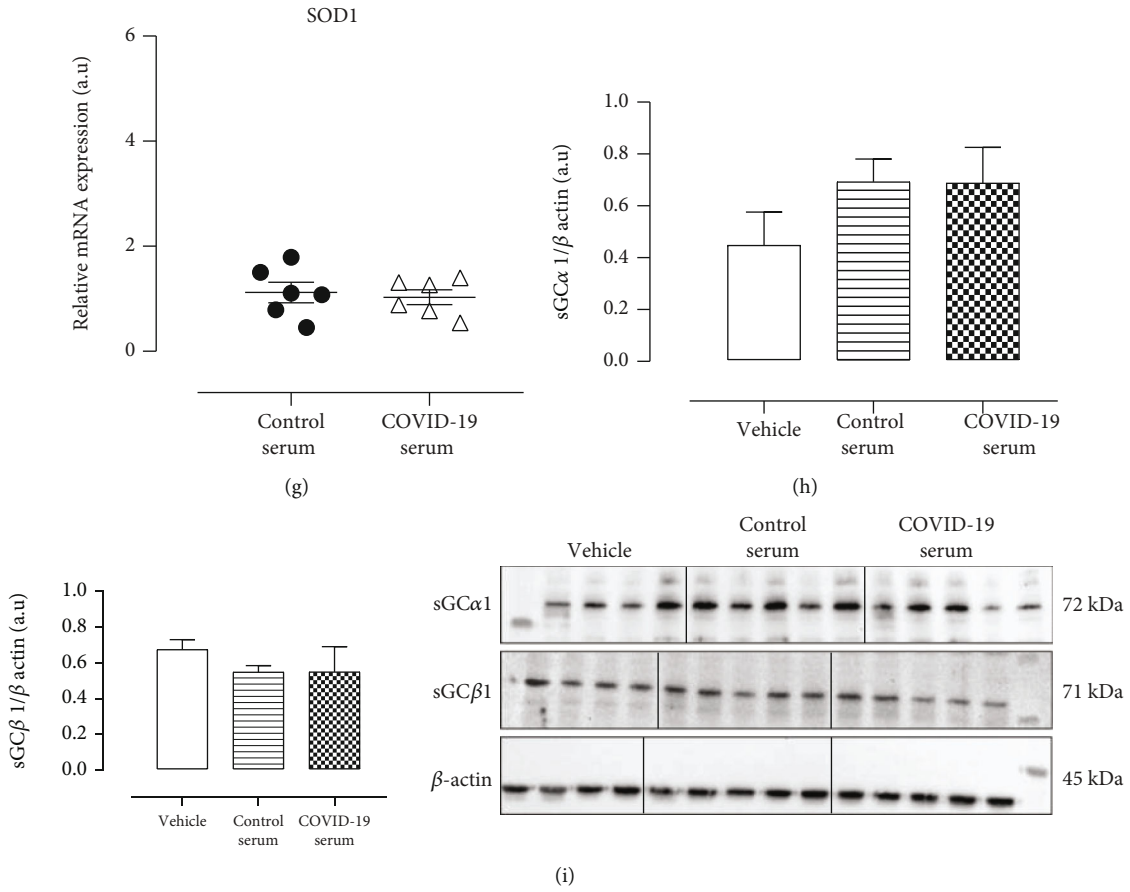

FIGURE 4: Effects of control (●) and COVID-19 (△) serum on the mRNA expression of *NOS3* (a), *GUCY1A1* (b), *GUCY1B1* (c), *PDE5A1* (d), *CYBB* (e), *NOX4* (f), and *SOD1* (g) in isolated corpus cavernosum (CC) strips of control mice. Each gene was normalized to β-actin mRNA expression levels, and the values are expressed in arbitrary units (a.u.). Effects of control (●) and COVID-19 (△) serum on the protein expression of sGCα1 (h) and sGCβ1 (i) in isolated corpus cavernosum (CC) strips of control mice. Each gene was normalized to β-actin protein expression levels, and the values are expressed in arbitrary units (a.u.). Data represent mean ± SEM. The *n* between the parentheses refer to the number of patients and animals used for each protocol. $^{*}p < 0.05$, control serum vs. COVID-19 serum.

date, riociguat (BAY 63-2521) and, more recently, vericiguat are the only sGC stimulators approved on the market to treat patients with pulmonary hypertension [38] or acute heart failure [39].

The effectiveness of cGMP-increasing substances is markedly affected when endogenous NO is low or when sGC/PDE5 is dysregulated. In human corpus cavernosum, the relaxations induced by sildenafil, vardenafil, tadalafil [12], and BAY 41-2272 [40] were reduced in the presence of NO synthases (L-NAME) or sGC (ODQ) inhibitors [12]. Our results clearly show that COVID-19 serum impaired the relaxations induced by SNP, ACh, tadalafil, and BAY 63-2521 (riociguat). Despite not having quantified NO or O_2^- levels in CC incubated with the serum, our results strongly suggested that NO bioavailability might be reduced due to higher levels of reactive oxygen species in tissues incubated with COVID-19 serum. PEG-SOD is a PEG conjugate of superoxide dismutase (SOD) and powerful free radical scavenger, disproportioning O_2^-. We found that incubation of COVID-19 group serum with PEG-SOD augmented by 4-fold the relaxation induced by ACh, leading the response to similar levels of that of CC incubated with control serum+PEG-SOD. Additionally, PEG-SOD also improved the relaxation induced by ACh, thus reinforcing the increase in NO bioavailability in the presence of PEG-SOD. Similar findings were observed in iliac arteries from middle-aged (10-month-old) rats, where the *in vitro* incubation of PEG-SOD (150 UI/mL, 30 minutes) attenuated the impaired SNP relaxation. O_2^- levels were higher in iliac arteries from middle-aged compared to young rats [41]. As the gene expression for NOX4 was increased in CC incubated with COVID-19 serum, and since this isoform produces only H_2O_2, catalase (300 UI/mL, 3 h) was preincubated. This, however, did not improve the relaxation induced by ACh. We did not determine the protein expression for NOX2 or NOX4 isoforms because, although there is a range of commercially available antibodies, the majority is not validated with positive and negative controls, or there is a lack of information regarding about isoform specificity and are, therefore, not recommended by experts in the field [42, 43]. Validated anti-NOX antibodies are often generated by academic laboratories, but often with a limited supply.

At first, we hypothesized that PEG-SOD could further improve the relaxation induced by tadalafil due to an increase in NO bioavailability. However, no improvement was observed. Another possibility for the lower effectiveness of tadalafil in tissues stimulated with the COVID-19 serum was the upregulation of *PDE51A* gene. COVID-19 serum did not reduce the gene or protein expression of sGC subunits. Additionally, the relaxation induced by the sGC activator BAY 58-2667 was not shifted to the left, which could suggest oxidation of the heme portion of sGC. Conversely to sGC stimulators, the oxidation of the heme portion of sGC improves the relaxing response induced by the sGC activator BAY 60-2770 in rabbit corpus cavernosum [44]. Therefore, these results suggest that COVID-19 serum interfered in NO generation and/or cGMP degradation.

Our study presents some limitations: serum samples from patients with the mild or moderate form of COVID-19 were not used due to the difficulties in obtaining these samples during the worst periods of COVID-19 pandemic. Our laboratory does not have a license to manipulate the SARS-CoV-2 virus, and therefore, we did not assess the direct effect of the virus on the corpus cavernosum reactivity and could only assess the effect of serum obtained from patients with the acute phase of the disease. As the serum samples were from unvaccinated participants, the ability of vaccination to reverse or relieve the impaired relaxing responses observed in the present study remains unknown.

In conclusion, our results show that serum obtained from patients with the acute severe form of COVID-19 (1 to 3 days after discharge from the ICU) impaired the relaxations induced by cGMP-increasing substances. More functional and molecular studies are required to assess the long-term effects of COVID-19 on erectile function and to determine if vaccinated men may have fewer consequences in erectile function.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Ethical Approval

The present study was approved by the Human Ethics Committee of the University of Campinas (UNICAMP) (Protocol Number: 30648520.6.0000.5404). Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation and were approved by the Institutional Committee for Ethics in Animal Research of the University of Campinas (CEUA/UNICAMP number: 6007-1/2022).

Conflicts of Interest

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by CAPES (88881.504454/2020-01 and CAPES-EPIDEMIAS688/2020) and Sao Paulo Research Foundation (FAPESP, 2017/15175-1). We thank Angélica R. Cucolo for the illustration.

References

[1] S. M. MacDonald and A. L. Burnett, "Physiology of erection and pathophysiology of erectile dysfunction," *The Urologic Clinics of North America*, vol. 48, no. 4, pp. 513–525, 2021.

[2] H. A. Toque, F. H. da Silva, M. C. Calixto et al., "High-fat diet associated with obesity induces impairment of mouse corpus cavernosum responses," *BJU International*, vol. 107, no. 10, pp. 1628–1634, 2011.

[3] L. Boeri, P. Capogrosso, F. Pederzoli et al., "Unrecognized prediabetes is highly prevalent in men with erectile dysfunction-results from a cross-sectional study," *The Journal of Sexual Medicine*, vol. 15, no. 8, pp. 1117–1124, 2018.

[4] F. H. Silva, F. Z. Mónica, F. R. Báu et al., "Superoxide anion production by NADPH oxidase plays a major role in erectile dysfunction in middle-aged rats: prevention by antioxidant therapy," *The Journal of Sexual Medicine*, vol. 10, no. 4, pp. 960–971, 2013.

[5] Q. W. Liu, Z. H. Yang, J. Jiang, and R. Jiang, "Icariin modulates eNOS activity via effect on post-translational protein-protein interactions to improve erectile function of spontaneously hypertensive rats," *Andrology*, vol. 9, no. 1, pp. 342–351, 2021.

[6] F. Z. Mónica and E. Antunes, "Stimulators and activators of soluble guanylate cyclase for urogenital disorders," *Nature Reviews. Urology*, vol. 15, no. 1, pp. 42–54, 2018.

[7] A. C. Codo, G. G. Davanzo, L. B. Monteiro et al., "Elevated glucose levels favor SARS-CoV-2 infection and monocyte response through a HIF-1α/glycolysis-dependent axis," *Cell Metabolism*, vol. 32, no. 3, pp. 437–446.e5, 2020.

[8] P. Libby and T. Lüscher, "COVID-19 is, in the end, an endothelial disease," *European Heart Journal*, vol. 41, no. 32, pp. 3038–3044, 2020.

[9] E. Kresch, J. Achua, R. Saltzman et al., "COVID-19 endothelial dysfunction can cause erectile dysfunction: histopathological, immunohistochemical, and ultrastructural study of the human penis," *World Journal of Men's Health*, vol. 39, no. 3, pp. 466–469, 2021.

[10] K. Y. Chu, S. Nackeeran, L. Horodyski, T. A. Masterson, and R. Ramasamy, "COVID-19 infection is associated with new onset erectile dysfunction: insights from a national registry," *Sexual Medicine*, vol. 10, no. 1, p. 100478, 2022.

[11] H. Mizusawa, P. Hedlund, A. Håkansson, P. Alm, and K. E. Andersson, "Morphological and functional in vitro and in vivo characterization of the mouse corpus cavernosum," *British Journal of Pharmacology*, vol. 132, no. 6, pp. 1333–1341, 2001.

[12] H. A. Toque, F. B. Priviero, C. E. Teixeira et al., "Comparative relaxing effects of sildenafil, vardenafil, and tadalafil in human corpus cavernosum: contribution of endogenous nitric oxide release," *Urology*, vol. 74, no. 1, pp. 216–221, 2009.

[13] E. Chung, L. De Young, and G. B. Brock, "Investigative models in erectile dysfunction: a state-of-the-art review of current animal models," *The Journal of Sexual Medicine*, vol. 8, no. 12, pp. 3291–3305, 2011.

[14] E. A. Coomes and H. Haghbayan, "Interleukin-6 in Covid-19: a systematic review and meta-analysis," *Reviews in Medical Virology*, vol. 30, no. 6, pp. 1–9, 2020.

[15] J. Hadjadj, N. Yatim, L. Barnabei et al., "Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients," *Science*, vol. 369, no. 6504, pp. 718–724, 2020.

[16] C. Halim, A. F. Mirza, and M. I. Sari, "The association between TNF-α, IL-6, and vitamin D levels and COVID-19 severity and mortality: a systematic review and meta-analysis," *Pathogens*, vol. 11, no. 2, p. 195, 2022.

[17] Y. Palacios and L. Chavez-Galan, "Immunosuppressant therapies in COVID-19: is the TNF axis an alternative?," *Pharmaceuticals*, vol. 15, no. 5, p. 616, 2022.

[18] Y. Que, C. Hu, K. Wan et al., "Cytokine release syndrome in COVID-19: a major mechanism of morbidity and mortality," *International Reviews of Immunology*, vol. 41, no. 2, pp. 217–230, 2022.

[19] I. Macías, S. García-Pérez, M. Ruiz-Tudela, F. Medina, N. Chozas, and J. A. Girón-González, "Modification of pro- and antiinflammatory cytokines and vascular-related molecules by tumor necrosis factor-a blockade in patients with rheumatoid arthritis," *The Journal of Rheumatology*, vol. 32, no. 11, pp. 2102–2108, 2005.

[20] M. Emfietzoglou, D. Terentes-Printzios, R. A. Kotronias et al., "The spectrum and systemic associations of microvascular dysfunction in the heart and other organs," *Nature Cardiovascular Research*, vol. 1, no. 4, pp. 298–311, 2022.

[21] S. Kang and T. Kishimoto, "Interplay between interleukin-6 signaling and the vascular endothelium in cytokine storms," *Experimental & Molecular Medicine*, vol. 53, no. 7, pp. 1116–1123, 2021.

[22] J. Lee, S. Lee, H. Zhang, M. A. Hill, C. Zhang, and Y. Park, "Interaction of IL-6 and TNF-α contributes to endothelial dysfunction in type 2 diabetic mouse hearts," *PLoS One*, vol. 12, no. 11, article e0187189, 2017.

[23] S. Pons, S. Fodil, E. Azoulay, and L. Zafrani, "The vascular endothelium: the cornerstone of organ dysfunction in severe SARS-CoV-2 infection," *Critical Care*, vol. 24, no. 1, p. 353, 2020.

[24] D. Terentes-Printzios, N. Ioakeimidis, K. Rokkas, and C. Vlachopoulos, "Interactions between erectile dysfunction, cardiovascular disease and cardiovascular drugs," *Nature Reviews. Cardiology*, vol. 19, no. 1, pp. 59–74, 2022.

[25] I. T. Farmakis, N. Pyrgidis, I. Doundoulakis, I. Mykoniatis, E. Akrivos, and G. Giannakoulas, "Effects of major antihypertensive drug classes on erectile function: a network meta-analysis," *Cardiovascular Drugs and Therapy*, vol. 36, no. 5, pp. 903–914, 2022.

[26] S. M. Alsaedi, R. M. Alsarwani, A. I. Ali, and S. A. Aladhrai, "Ischemic priapism progressing to penile gangrene in a patient with COVID-19 infection: a case report with literature review," *Case Reports in Medicine*, vol. 2022, Article ID 8408216, 5 pages, 2022.

[27] C. Kappel, J. Piticaru, G. Jones et al., "A case of possible Fournier's gangrene associated with proning in COVID-19 ARDS," *Canadian Journal of Anaesthesia*, vol. 67, no. 11, pp. 1697-1698, 2020.

[28] P. Sarkis, J. Sarkis, M. Alkassis, J. Assaf, and K. El Gharib, "Penile ischaemia secondary to COVID-19: why should the dermatologist be concerned?," *Journal of the European Academy of Dermatology and Venereology*, vol. 35, no. 8, pp. e487–e489, 2021.

[29] H. M. Saad, S. F. GamalEl Din, O. M. Elbokl, and A. Adel, "Predictive factors of erectile dysfunction in Egyptian individuals after contracting COVID-19: a prospective case-control study," *Andrologia*, vol. 54, no. 1, article e14308, 2022.

[30] M. C. Hsieh, P. J. Hsiao, M. T. Liao et al., "The role of vitamin D in SARS-CoV-2 infection and acute kidney injury," *International Journal of Molecular Sciences*, vol. 23, no. 13, p. 7368, 2022.

[31] S. Yang and X. Xu, "Mental health in postoperative thyroid patients during the COVID-19 pandemic," *Frontiers in Endocrinology*, vol. 13, article 875325, 2022.

[32] S. R. Potje, T. J. Costa, T. F. C. Fraga-Silva et al., "Heparin prevents in vitro glycocalyx shedding induced by plasma from COVID-19 patients," *Life Sciences*, vol. 276, p. 119376, 2021.

[33] E. D. Hottz, I. G. Azevedo-Quintanilha, L. Palhinha et al., "Platelet activation and platelet-monocyte aggregate formation trigger tissue factor expression in patients with severe COVID-19," *Blood*, vol. 136, no. 11, pp. 1330–1341, 2020.

[34] F. Z. Mónica and G. De Nucci, "Tadalafil for the treatment of benign prostatic hyperplasia," *Expert Opinion on Pharmacotherapy*, vol. 20, no. 8, pp. 929–937, 2019.

[35] C. Boydens, B. Pauwels, L. Vanden Daele, and J. Van de Voorde, "Inhibition of cyclic GMP export by multidrug resistance protein 4: a new strategy to treat erectile dysfunction?," *The Journal of Sexual Medicine*, vol. 14, no. 4, pp. 502–509, 2017.

[36] M. Follmann, N. Griebenow, M. G. Hahn et al., "The chemistry and biology of soluble guanylate cyclase stimulators and activators," *Angewandte Chemie (International Ed. in English)*, vol. 52, no. 36, pp. 9442–9462, 2013.

[37] M. G. de Oliveira, W. Nadruz, and F. Z. Mónica, "Endothelial and vascular smooth muscle dysfunction in hypertension," *Biochemical Pharmacology*, vol. 205, p. 115263, 2022.

[38] S. Wu, H. B. Hoang, J. Yang et al., "Drug-drug interactions in the management of patients with pulmonary arterial hypertension," *Chest*, vol. 162, no. 6, pp. 1360–1372, 2022.

[39] M. Boettcher, H. D. Düngen, F. Donath et al., "Vericiguat in combination with short-acting nitroglycerin in patients with chronic coronary syndromes: the randomized, phase Ib, VENICE Study," *Clinical Pharmacology and Therapeutics*, vol. 111, no. 6, pp. 1239–1247, 2022.

[40] J. S. Baracat, C. E. Teixeira, C. E. Okuyama et al., "Relaxing effects induced by the soluble guanylyl cyclase stimulator BAY 41-2272 in human and rabbit corpus cavernosum," *European Journal of Pharmacology*, vol. 477, no. 2, pp. 163–169, 2003.

[41] A. F. O. Justo, M. G. de Oliveira, F. B. Calmasini et al., "Preserved activity of soluble guanylate cyclase (sGC) in iliac artery from middle-aged rats: role of sGC modulators," *Nitric Oxide*, vol. 106, pp. 9–16, 2021.

[42] F. Augsburger, A. Filippova, D. Rasti et al., "Pharmacological characterization of the seven human NOX isoforms and their inhibitors," *Redox Biology*, vol. 26, p. 101272, 2019.

[43] B. A. Diebold, S. G. Wilder, X. De Deken et al., "Guidelines for the detection of NADPH oxidases by immunoblot and RT-qPCR," *Methods in Molecular Biology*, vol. 1982, pp. 191–229, 2019.

[44] C. S. Estancial, R. L. Rodrigues, G. De Nucci, E. Antunes, and F. Z. Mónica, "Pharmacological characterisation of the relaxation induced by the soluble guanylate cyclase activator, BAY 60-2770 in rabbit corpus cavernosum," *BJU International*, vol. 116, no. 4, pp. 657–664, 2015.