COVID-19: ORF8 Synthesizes Nitric Oxide to Break the Blood-Brain/Testis Barrier and Damage the Reproductive System

Wenzhong Liu^{1,2,*}, Hualan Li²

¹ School of Computer Science and Engineering, Sichuan University of Science & Engineering, Zigong, 643002, China;

² School of Life Science and Food Engineering, Yibin University, Yibin, 644000, China;

* Correspondence. Wenzhong Liu, liuwz@suse.edu.cn.

Abstract

Studies have revealed that the SARS-CoV-2 virus can break through the blood-brain/testis barrier and invade the human reproductive system, causing infertility or complications in patients. Excessive nitric oxide (NO) is a fundamental reason for breaking the Blood-Brain/Testis Barrier. Nitric oxide often relates the spread or replication of many viruses in the body. The nitric oxide synthase (NOS) that synthesizes NO in the human by binding heme to complete the oxygenase reaction. This study focused on the relationship between heme, NO, and the novel coronavirus using bioinformatics methods such as domain search and molecular docking. The results showed ORF8 had three domains similar to nitric oxide synthase: oxygenase, reductase, and calmodulin (CaM). ORF6 could bind to these three domains. The dimer of ORF8 was identical to the dimer of NOS enzyme. The oxygenase domain was in the core, and the reductase domain was on both sides. ORF8 could capture Heme, H4B, L-arginine, FAD, FMN, and NADPH, and bind with CaM protein to catalyze NO production. The heme bound by ORF8 mainly came from the attacked hemoglobin. ORF8 also attached to E protein and synthesized NO through the heme hunted by E protein. We believed NO synthesized by ORF8 inhibited SARS-CoV-2 from reinfecting infected cells and controlled the virus replication speed to avoid cell collapse because of exhaustion of resources. After the SARS-CoV-2 virus combined with an extensive ORF8, the produced NO stream permeably expanded blood vessels and broke the blood-brain/testis barrier. The SARS-CoV-2 virus spread to nearby tissues through small blood vessel holes created by NO stream. It would increase the tendency to bleed and the blood clotting of the tissue: blood clotting and viral infections severely damaged organs such as the respiratory, heart, nerve, reproductive. Short NO could not open vascular permeability dilation, causing high viral load in asymptomatic patients' blood. Excessive NO stimulated the reproductive organs and generated abnormal functions. It also made abnormal hormone regulation, such as excessive secretion of luteinizing hormone (LH). Much LH would hurt the reproductive organs, leading to infertility. Extreme NO also interfered with the human NO signaling pathway and damaged the immune nerve, metabolism, cardiovascular and other systems. This theory is for academic discussion only. We hope that this discovery will help block the virus's transmission through the human circulatory system and help reproductive health management during the pandemic.

Keywords: Nitric oxide; Nitric Oxide Synthase(NOS); Luteinizing hormone (LH) ; Oxygenase; Heme; Dimer.

1. Introduction

The SARS-CoV-2 virus targets cells through the S protein binding to the human ACE2 receptor. The cellular serine protease transmembrane protease serine 2 (TMPRSS2) triggers a cascade event, leading to a fusion between the cell membrane and the viral membrane to complete the cell infection process. The testis is one of the body tissues with the highest expression of ACE2 mRNA and protein, which shows that SARS-CoV-2 infection may threaten male fertility(1). ACE2 is also widely expressed in the ovaries, uterus, vagina, and placenta, so SARS-CoV-2 may also interfere with female reproductive functions(2). Statistics indicate that the incidence and mortality of COVID-19 in men are higher than those in women(3), then the reproductive system of men is more vulnerable to damage. But there are contradictory reports in the literature about SARS-CoV-2 infection of the human reproductive system. Therefore, studying the damage mechanism of the SARS-CoV-2 virus to the reproductive system is of great significance for reproductive health management during the pandemic.

There are reports that SARS-CoV-2 is not detected in the vaginal fluid of women with severe COVID-19 infection, and the results of vaginal swabs are negative to SARS-CoV-2(4). Another report shows that women's vaginal secretion test is positive(5). There is no epidemiological investigation describing evidence that COVID-19 is a venereal disease(6). Because of viremia in SARS-CoV-2 patients, invasive operations such as vaginal oocyte retrieval increase the chance of infection(7). When assisted reproductive technology induces pregnancy, the risk of SARS-CoV-2 disease also increases. ACE2 receptors have been detected in the ovaries of childbearing women and postmenopausal women. ACE2 is abundantly expressed in ovaries and oocytes, and SARS-CoV-2 may target ovaries and oocytes(2). ACE2 is more expressed in oocytes than TMPRSS2, which suggests that the latter may be a potential limiting factor for female gamete infection, and cumulus-encapsulated oocytes are unlikely to be infected by SARS-CoV-2(8). But In novel coronavirus pneumonia patients not related to pregnancy, pelvic illness, or surgery, right ovarian vein thrombosis (OVT) also are observed(9). Ovarian vein thrombosis is a rare disease related to postpartum conditions, pelvic infection, gynecological surgery, and other thrombotic conditions. This situation highlights that the SARS-CoV-2 virus induces a hypercoagulable state in female reproductive organs. In OVT, the use of new oral anticoagulants causes premature delivery in COVID-19-positive women(10). SARS-CoV-2 was also detected in the blood samples of mothers and their newborns, and the placental SARS-CoV-2 gene was also positive(11). It represents pregnant women who were severely infected with COVID-19, meets a vertical transmission of the virus(12), then placental transmission causes neonatal viremia and inflammation of the placenta. Besides, RT-PCR's detection of breast milk samples reveals that virus particles are positive(13), which is not suitable for breastfeeding of COVID-19-positive women.

Compared with ovarian cells, testicular ACE2 expression is significantly higher, and male gonadal functions (including steroid production) are more susceptible to SARS-CoV-2 induced damage(14). The testis also showed almost the most elevated expression levels of ACE2 mRNA and protein among different body tissues(15). There are four main cell types at the testicular cells (1, 16): vas deferens, spermatogonia, stromal cells, and supporting cells. For the evidence of testicular infection, current literature reports are also inconsistent. It reports that using qRT-PCR, no SARS-CoV-2 virus is found in semen samples of healthy male COVID-19 patients(17). The

blood-testis barrier protects it, and the incidence of testicular infections in COVID-19 patients is low. All are plausible reasons(18). It also reports that the SARS-CoV-2 virus is contained in semen samples of acute and recovered male COVID-19 patients (19). Using RT-qPCR technology, it finds SARS-CoV-2 nuclei protein in infected men's testicular tissue, showing that the virus directly invaded the male reproductive system(20). SARS-CoV-2 may cause male reproductive diseases by regulating the level of autophagy in male germ cells(21). It finds seminiferous tubule damage reduces Leydig cell number and then causes lymphocytic inflammation in the testes of COVID-19 patients(22). It also reveals that COVID-19 positive cases have lymphocyte and macrophage infiltration, coronavirus-like particles, and abnormal sperm morphology(23). In SARS coronavirus-infected testes, leukocyte infiltration shows the virus disrupts the blood-testis barrier, and subsequently, the body loses immune protection(24). Like other SARS viruses, SARS-COV-2 may damage tissues and blood testicular barriers(25) and hurt high-risk testicular cells(26, 27). If research confirms viruses in semen, assisted reproductive technology will also be affected(28).

Orchitis is a complication of SARS(24). The crucial question is whether COVID-19 causes testicular damage and infertility in male patients. So far, there is no definite answer(29). In COVID-19 cases, testicular degeneration is because of the indirect effects of inflammation leading to an increase in testicular temperature(25), affecting testicular function(30, 31). Oxidative stress(OS) is widely recognized as an essential cause of male infertility(32). Testicular pathology under inflammation and oxidative stress may lead to infertility in a large proportion of COVID-19 survivors(33). SARS-CoV-2 can activate pathways sensitive to oxidants through the innate immune pathway's inflammatory response, inducing oxidative stress(34, 35). Oxidative stress by SARS-CoV-2 has long-term harmful effects on male fertility, especially on developing sperm(36). In the epididymis, sperm are more susceptible to oxidative damage(37). SARS-CoV-2 induces impaired spermatogenesis and delayed sperm maturation, which may be the PT-mediated depletion of testosterone levels(38). A recent study shows that because Leydig cells express ACE2, there may be a direct correlation between lower serum testosterone and inflammatory cytokines and poor clinical outcomes in male patients infected with SARS-CoV-2 (39).

Most viruses enter the human body through the nasal cavity and oral cavity, and virus particles may destroy the blood-brain barrier. According to reports, brain cells (glial cells and neurons) also express ACE 2 receptors, making them a target for inducing neurons' death. Notably, the central nervous system plays a crucial role in endocrine control and sperm formation (40). The central nervous system's disease in patients with COVID-19, includes increased antidiuretic hormone secretion, hypothalamic-pituitary disorders, and abnormal LH secretion rhythm(41). Although the impact of COVID-19 on the hypothalamic-pituitary-adrenal (HPA) axis abnormality has been speculated, the mechanism of SARS-CoV-2 mediated hypothalamic-pituitary-gonad (HPG) axis dysregulation remains unclear(42). SARS-CoV-2 infection may produce an active inflammatory response in the hypothalamus-pituitary-testis (HPT) axis, leading to reduced LH, stimulating follicle hormone (FSH), and testosterone levels (30, 34). However, there is a contradiction with this theory. Because it has recently been reported that compared with healthy men, patients with COVID-19 have lower serum testosterone levels, higher LH levels, and a lower ratio of testosterone to LH(43). It shows that SARS-CoV-2 has subclinical damage to male gonadal function. Another report indicates that the serum LH level of COVID-19 patients is significantly higher than that of healthy men, but testosterone/LH and FSH levels are lower,

showing potential hypogonadism(44). In the COVID-19 group, more elevated serum luteinizing hormone (LH) and lower testosterone(T) are observed, and there is no significant difference in the ratio of serum FSH, serum E2 and T:E2(45). It negatively correlated the LH ratio with the WBC count and CPR level of COVID-19 patients. The evidence reveals the complex relationship between SARS-CoV-2 infection and dysregulation of sex hormones.

The hypothalamic-pituitary-gonadal axis (HPGa) plays a vital role in reproduction. HPGa can inhibit the body's reproductive function through hormones. The activation of HPGa and subsequent changes in hormone concentration holds a key role in sperm quality. Sperm count/sperm volume/seminal volume, and a higher sperm DNA fragmentation, also induce sexual dysfunction(46). GnRH neurons in the hypothalamus secrete Gonadotropin-releasing hormone(GnRH). Then GnRH activates follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secreted by the pituitary gland. Low GnRH levels reduce FSH and LH, leading to impaired Sertoli and Leydig cell function(43). GnRH neurons' cell bodies are in the endplate microvascular apparatus (OVLT) area, and the cell bodies of NOS neurons overlap with GnRH neuron fibers (47). The NOS enzyme of NOS neurons synthesizes NO, and the released NO stimulates GnRH neurons to secrete GnRH. Both endogenous and exogenous NO can stimulate the secretion and release of GnRH (48). High and enough NO stimulates the high secretion of GnRH(49). The high secretion of GnRH causes the level of LH to increase (50).

NO is also an effective vasodilator that promotes vasodilation. NO mainly acts on sGC to regulate cerebral vascular endothelial cells' permeability under physiological conditions (51). Then control the transport of ions, nutrients, and other molecules across the blood-brain barrier through cGMP. Glutamate closely related the synthesis/release of NO to the permeability changes in the blood-brain barrier(52). Angiotensin-converting enzyme 2(ACE2) is the receptor of SARS-CoV-2, which regulates vascular function by controlling the release of nitric oxide (NO) and oxidative stress. In severe SARS patients, iNO can significantly improve ARDS patients' oxygenation and reduce pulmonary vascular resistance, reducing the need for invasive mechanical ventilation(53). But Excessive NO may damage tissue and cell. NO rapidly reacts with Hb in red blood cells, inactivating NO(53). The free heme in the blood binds to nitric oxide (NO), further aggravating the organ's ischemia. Also, because nitric oxide induces blood vessel relaxation and dilation, reducing blood pressure, the increase in nitric oxide content may develop the disease into a severe bleeding. During focal cerebral ischemia, much NO is neurotoxic to hurt nerve cells(54). NO directly increases the blood's permeability and breaks the blood-brain barrier(55). The increased synthesis and release of NO during acute hypertension is essential for damaging the blood-brain barrier(56). Therefore, for COVID19 patients, excessive NO may osmotically expand blood vessels and break the blood-brain barrier.

It relates nitric oxide to many viruses' pathogenic mechanism (such as human rhinovirus, hepatitis C, dengue fever virus, herpes simplex virus 1))(57). Nitric oxide synthase in the body needs to bind heme when synthesizing NO. So heme is essential for NO synthesis. In the theory of heme (or hemoglobin), we found that ORF3a could attack hemoglobin and dissociate heme into porphyrin and iron(58). At the same time, viral proteins could bind porphyrin to inhibit heme metabolism. Why was doxyhemoglobin more vulnerable to attack? After ORF3a attacked hemoglobin, much heme was fallen off. What was the significance of this dissociated heme to the virus? The biological mechanism of which was still unknown. In the ROS damage theory, we found that E protein has the functions of cytochrome C oxidase, catalase, peroxidase, and

superoxide dismutase by binding heme or iron(59). E protein catalyzed the production of ROS free radicals to damage immune cells. The virus escaped the attack of immune cells by breaking down ROS free radicals through E protein. Is there another viral protein that bound heme to produce NO? This research tried to discover the relationship between heme, nitric oxide, and viral proteins.

This study adopted bioinformatics methods such as domain search and molecular docking to study the relationship between the novel coronavirus proteins and nitric oxide. The results showed ORF8 had three functional domains of NOS synthase: oxygenase domain, reductase domain and calmodulin (CaM) binding domain. It must combine NOS synthase into a dimer to be catalytically active. Interestingly, ORF8 also has into a dimer structure(60), a rapidly evolving protein structure may help viruses achieve immune escape(61) and interfere with signal transduction pathways(62). Therefore, the ORF8 antibody has also become the primary clinical marker of SARS-CoV-2 infection(63). We found ORF8 could capture heme, H4B, L-arginine, FAD, FMN, NADPH, combine with CaM to form a NO generator, and synthesize nitric oxide. The heme bound by ORF8 mainly came from the attacked hemoglobin. SARs-COV-2 (E protein) also could combined with ORF8 to generate NO stream to break the blood-brain (testi) barrier and damage reproductive system and immune cells.

2. Data sets and methods

2.1. Analysis flow

It shows the data analysis process of this research in Figure 1. It divides the process into seven steps: The first step is to download the protein sequence data set; The second step is homology modeling of viral proteins; The third step is to search for the conserved domains of viral proteins; The fourth step is the protein docking of ORF8 and CaM binding protein; In the fifth step, it is docked ORF8 with heme, H4B, L-arginine, FAD, FMN, NADPH molecules; The sixth step is protein docking between viral proteins; The seventh step is the analysis of dimer structure of ORF8.

Figure 1. Analysis flow chart

2.2. Data set

1. The sequences of SARS-COV-2 proteins. The SARS-COV-2 protein sequences came from the NCBI database. Including: S, E, N, M, ORF3a, ORF8, ORF7a, ORF7b, ORF6, ORF10, orflab, orfla. Among them, the orflab and orfla sequences also included corresponding subsequences. Since the sequences corresponding to these structures in PDB database are missing some fragments, homology modeling of these proteins is required.

We also downloaded the two isoforms sequences of human CaM 3.

2. Bacterial nitric oxide synthase sequence. We downloaded 3,094 protein sequences of bacterial nitric oxide synthase from the UniProt data set and searched keyword was "Bacterial + nitric oxide synthase". The nitric oxide synthase sequences were compared with the viral proteins to search for the NOS conserved domains.

3. Crystal structures of S and ORF8. we downloaded the S (PDB ID: 6xlu) protein and ORF8 (PDB ID: 7JTL) protein (SARS-COV-2) from the PDB database.

4. Crystal structures of ligand molecules. We downloaded the ligand molecular structure files of heme, H4B, L-arginine, FAD, FMN, and NADPH from the PDB database.

2.3. Molecular structure modeling

1. Robetta modeling. The modeling method is the same as that of the heme theory(58). Modeling E, ORF3a, ORF8, ORF7a, ORF7b, ORF6, ORF10, and CaM 3.

2. Discovery Studio 2016 Modeling. Before using Discovery-Studio (DS) to model homology of an unknown protein (such as S), the pdb structure file of the template protein, such as 6xlu Structure of SARS-CoV-2 spike at pH 4.0, should be downloaded from the PDB database. Next, the sequence alignment tool of Discovery-Studio is utilized to align homologous sequences between 6xlu and S. Then the spatial structure file of S was constructed based on the template protein 6xlu.

2.4. Localized MEME tool to scan for conserved domains.

The steps for using LibDock molecular docking with Discovery-Studio are same as the ROS damage theory study(59).

2.5. Docking of Discovery Studio 2016

Molecular and Protein docking technology. The steps for using LibDock molecular or protein docking with Discovery-Studio are same as the heme theory study(58).

3. Results

3.1 Homologous modeling of virus protein and CaM protein

We modeled the structural and non-structural proteins of the virus (except N, M, orflab and orfla), and the two isoforms of human CaM. The resulting files are in the URL attached at the end of the paper. Due to limitation of the paper space, the structure file pictures are not posted here.

3.2 ORF8 protein had NOS conserved domains

The human has three kinds of NOS isoenzymes, which catalyze L-arginine's oxidation to produce endogenous NO. One is nNOS in nerve cells, the other is eNOS in endothelial cells, and the third is inducible iNOS in platelets, macrophages, and glial cells. The three NOS isoenzymes have the exact three conserved domains(64, 65). The first is the N-terminal oxygenase domain (66). The domain combines heme(67), tetrahydrobiopterin (H4B), and L-arginine. The second is the C-terminal reductase domain. The domain binds to flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and reduced nicotinamide adenine dinucleotide phosphate (NADPH). The third is the calmodulin (CaM) binding area. The domain is between the oxygenase and reductase domains(68). The third domain regulates the electron transfer from the reductase domain to oxygenase domain. NADPH provides electrons, and the electron transport route is: NADPH->FAD->FMN->Heme. If the viral protein has NOS enzyme function, it needs to have three positional domains simultaneously. And the three domains should be on the same protein and not on different proteins.

We downloaded all sequences of bacterial NOS synthase from the UniProt database. We then employed the local MEME's domain search tool to find the NOS conserved domains of all SARS-COV-2 viral proteins (Table 1). Table 1 shows that among all SARS-COV-2 proteins, only ORF8 has NOS domains in three directions: N-terminal, middle, and C-terminal. The three domains are AAFHQECSLQSQCTQHQPYPY(14-31), CTQHQPYPYVDDPCPIHFYYSKWY(25-46), QYIDIGNYTVSCLPFTINCQ(72-91), respectively. CTQHQPYPY(25-31) belongs to both N-terminal and middle domains. ORF7b, N, and M proteins have no NOS domain. Through the analysis of the crystal structure of the ORF8 modeling, we found that CTQHQPYPYVDDPCPIHFYYSKWY (25-46) and QYIDIGNYTVSCLPFTINCQ(72-91) can form functional pockets, while AAFHQECSLQS(14-24) cannot form functional pockets. The N-terminal NOS domain is a region that binds to the L-Arg etc. substrates and generates NO, so it is impossible to develop an active pocket that can stabilize the binding molecules. After the pocket formed near the middle NOS domain hunts the substrate (ligand) molecule, ORF8 rapidly changes its conformation and sends the substrate to the N-terminus or C-terminus. And the molecular substrate is unstable on N-terminal domain, so the bound substrate such as L-Arg will fall off after the NO synthesis reaction.

We tried to use a similar method of the heme theory to determine the heme iron site of ORF8. We only found that the NCBI protein WP_008179633.1 corresponding to A0A1H8NYM7 (UniProt id) has "Related Structures (Summary)". But the URL of the corresponding structure file could not be opened. The ORF8's motif corresponding to A0A1H8NYM7 is "HQPYPYVDDPCPIHFYYSKW", which is in the N-terminus and middle NOS domain. Therefore, the existing data and methods cannot determine the iron linked site of ORF8.

Other viral proteins only have NOS domains in one or two directions. For orflab, "CCREHEHEIAWYTERSE" is in the middle of "YP_009742609.1 nsp2" protein, and "RRLISMMGFKMNYQVNGYPNMFITREEAIRH" is in the middle of "YP_009725309.1 3'-to-5' exonuclease" protein too. Therefore, only ORF8 protein had NOS synthase function, and other proteins had no NOS synthase function.

Table 1. NOS Domains of SARS-COV-2 proteins

Protein	NOS Domains	Count	Direction
ORF8		25	
	AAFHQECSLQSCTQHQPYP	1	N-terminal
	CPIHFYSK	1	middle
	CTQHQPYPVDDPCPIHFY	6	middle
	CTQHQPYPVDDPCPIHFYSKW	5	middle
	HQPYPVDDPCPIHFYSKW	1	middle
	HQPYPVDDPCPIHFYSKWY	10	middle
	QYIDIGNYTVSCLPFTINCQ	1	C-terminal
ORF7a		7	
	FHPLADNKFALTCFSTQ	4	middle
	FHPLADNKFALTCFSTQFAFACPD	2	middle
	YHYQECV	1	N-terminal
ORF7b	-	-	-
ORF3a		9	
	CWHTNC	1	middle
	CWHTNCYDYC	1	middle
	CWKCRSKNPLLYDANYFLCWHTNC	1	middle
	MRLWLCWKCR	2	middle
	MRLWLCWKCRSKNPLLYDANYFLCWHTNCYDYCIPY	3	middle
	MRLWLCWKCRSKNPLLYDANYFLCWHTNCYDYCIPYN	1	middle
ORF10		51	
	CRMNSRN	3	C-terminal
	CRMNSRN YIAQ	47	C-terminal
	CRMNSRN YIAQVDVNF	1	C-terminal
ORF6		4	
	IIMRTFKVSIWNLDYIINLIKNLSKSLTENKYSQ	1	middle
	MRTFKVSIWNLDYIINLIKNLSKS	1	middle
	MRTFKVSIWNLDYIINLIKNLSKSLTENKYSQLDEEQP	1	middle
	MRTFKVSIWNLDYIINLIKNLSKSLTENKYSQLDEEQPM	1	middle
orf1ab		2	
	CCREHEHEIAWYTERSE	1	N-terminal
	RRLISMMGFKMNYQVNGYPNMFITREEAIRH	1	middle
S		14	
	EQYIKWPWYIW	7	C-terminal
	EQYIKWPWYIWLGFIAGLIAIVMTIMLCCM	1	C-terminal
	KYEYIKWPWYIWLGFIAGLIAIVMTIMLCCMTSCCSCLKGCC	1	C-terminal
	WPWYIW	4	C-terminal
	YEYIKWPWYIW	1	C-terminal
E	CAYCCNIVNVSLVKPSFYVYSRVK	1	middle
N	-	-	-
M	-	-	-

3.3 ORF8 protein could bind human CaM protein

We first determined whether the NOS domain in the middle of ORF8 had the function of binding CaM. We download the human CaM protein from NCBI. It had two isoforms (NP_005175.2 calmodulin-3 isoform 1 and NP_001316854.1 calmodulin-3 isoform 2). Since the CaM structure files on the PDB database were mostly combined with other molecules, we used Robetta to model the two isoforms separately. Then adopted the DS 's protein docking tool (ZDOCK) to dock the two isoforms with ORF8, respectively.

Figure 2 shows the docking result of NP_005175.2 calmodulin-3 isoform 1 (CaM3 isoform 1, the same below) and ORF8. Ser24 (S) of ORF8 is the intersection of two proteins. The middle NOS domain, 'CTQHQPYYVDDPCPIHFYSKY' (marked yellow area), behind Ser24 is close to calmodulin-3 isoform 1. It indicates that the NOS domain in the middle of ORF8 ('CTQHQPYYVDDPCPIHFYSKY') can bind CaM protein.

Figure 2. Docking result between calmodulin-3 isoform 1 and ORF8. calmodulin-3 isoform 1 is embedded in ORF8. Ser24 of ORF8 is the crossover point. The middle NOS domain, 'CTQHQPYYVDDPCPIHFYSKWY' (marked yellow area),behind Ser24 is close to calmodulin-3 isoform 1.

Figure 3 shows the docking result of NP_001316854.1 calmodulin-3 isoform 2 (CaM3 isoform 2, the same below) and ORF8. Asp35 (D) and Pro36 (P) of ORF8 is the intersection of two proteins. The Asp35 (D) and Pro36 (P) are in sites 'CTQHQPYYVDDPCPIHFYSKQY' (middle NOS domain, marked yellow area). It indicates that the NOS domain in the middle of ORF8 ('CTQHQPYYVDDPCPIHFYSKQY') can bind CaM protein too.

Figure 3. Docking result between calmodulin-3 isoform 2 and ORF8. calmodulin-3 isoform 2 is embedded in ORF8. Asp35 and Pro36 of ORF8 are the crossover point. The Asp35 and Pro36 are in sites 'CTQHQPYYVDDPCPIHFYSKWY' (middle NOS domain, marked yellow area).

3.4 ORF8 could capture heme, H4B, L-arginine

We determined whether ORF8 could capture HEME, H4B, and L-arginine. We downloaded the corresponding structure files of the three ligands of HEM, H4B, and ARG from the PDB database. Then adopted DS's molecular docking technology (LibDock) to dock these three ligands to ORF8, respectively.

According to the technical documentation requirements of DS, FE(iron ions) should be deleted before ORF8 and heme were docked. There are three docking results between ORF8 and heme. The binding energy of SITE 1 and SITE 2 are both -88.5114 kcal/mol (selected), and the positions are the same. The binding energy of SITE3 is both -73.9493 kcal/mol, which is slightly higher than the binding energy of SITE 1 and SITE2. The docking results of SITE2 and SITE 1 are shown in Figure 4. TYR73, ILE74, ASP75, ILE76, GLY77, TYR79, GLN91 amino acids are in the C-terminal NOS domain. Therefore, ORF8 captures heme through the active pocket generated by the C-terminal NOS domain and rapidly transfers heme to the N-terminal NOS domain by changing the conformation. The N-terminal NOS domain does not form an active pocket for molecular binding. Therefore, heme may drop off after participating in the NO synthesis reaction.

Figure 4. The results of molecular docking between ORF8 and heme. A. Schematic diagram of the docking result. B. Amino acids of ORF8 that bind heme. TYR73, ILE74, ASP75, ILE76, GLY77, TYR79, GLN91 amino acids are in the C-terminal NOS domain.

ORF8 and H4B also have three docking results. The binding energy of SITE3 and SITE2 is both $-69.9598 \text{ kcal/mol}$ (selected), and the binding position is the same. The binding energy of SITE1 is -6.7802 kcal/mol , which is higher than the binding energy of SITE 3 and SITE2. The docking result of SITE3 and SITE2 is shown in Figure 5. The LYS44, TRP45, and TYR46 of binding pockets are in the middle NOS domain of ORF8. Therefore, ORF8 directly captures H4B through the middle NOS domain. The N-terminal NOS domain does not form an active pocket for molecular binding. Thus, H4B may also drop off after participating in the NO synthesis reaction.

Figure 5. Molecular docking results of ORF8 and H4B. A. Schematic diagram of the docking result. B. Amino acids of ORF8 that bind H4B. LYS44, TRP45, TYR46 are in the middle NOS domain of ORF8

There are also three docking results for ORF8 and L-Arg. The binding energy of SITE1,

SITE2, and SITE3 are all -124.315 kcal/mol, and the binding position is the same. The docking result is shown in Figure 6. ASP35, SER43, LYS44, and TRP45 are in the middle NOS domain of ORF8. CYS83, PH86, and LEU84 are in the C-terminal NOS domain of ORF8. Therefore, ORF8 captures L-Arg through the middle NOS domain and the C-terminal NOS domain. Then quickly change the conformation and move L-Arg to the N-terminal NOS domain. The N-terminal NOS domain does not form an active pocket for molecular binding. Therefore, L-Arg may fall off quickly after participating in the NO synthesis reaction.

Figure 6. The results of molecular docking between ORF8 and L-Arg. A. Schematic diagram of the docking result. B. The amino acid of ORF8 binds L-Arg. ASP35, SER43, LYS44, TRP45 are in the middle NOS domain ORF8, CYS83, PH86, and LEU84 are in the C-terminal NOS domain of ORF8.

3.5 ORF8 could capture NADPH, FAD and FMN

We determine whether ORF8 can capture NADPH, FAD and FMN. We downloaded the corresponding NAP, FAD and FMN three ligand structure files from the PDB database. Then employed DS's molecular docking technology (LibDock) to dock these three ligands to ORF8, respectively.

There are also three docking results for ORF8 and NADPH. The binding energy of SITE1, SITE2, and SITE3 is all -256.329 kcal/mol, and the binding position is the same. It shows the docking result in Figure 7. SER43, LYS44, TRP45 are in the middle NOS domain of ORF8. LEU84 and PHE86 are in the C-terminal NOS domain of ORF8. Therefore, ORF8 captures NADPH through the middle NOS domain and the C-terminal NOS domain. Then it quickly changes the conformation and moves NADPH to the C-terminal NOS domain.

Docking results for ORF8 and FAD is as shown in Figure 8. The binding energy of SITE1, SITE2, and SITE3 is all -144.458 kcal/mol, and the binding position is the same. LYS44, TYR46 are in the middle NOS domain of ORF8. So, ORF8 captures FAD through the middle NOS domain. Then it quickly changes the conformation and sends FAD to the C-terminal NOS domain.

Figure 7. The results of molecular docking between ORF8 and NADPH. A. Schematic diagram of the docking result. B. Amino acids of ORF8 that bind NADPH. SER43, LYS44, TRP45 are in the middle NOS domain of ORF8. LEU84 and PHE86 are in the C-terminal NOS domain of ORF8.

Figure 8. The results of molecular docking between ORF8 and FAD. A. Schematic diagram of the docking result. B. Amino acids of ORF8 that bind FAD. LYS44, TYR46 are in the middle NOS domain of ORF8.

Docking results for ORF8 and FMN is as shown in Figure 9. The binding energy of SITE1, SITE2 is all -227.695 kcal/mol (selected), and the binding position is the same. The binding energy of SITE3 is -125.086 kcal/mol. TYR73, ILE74, ASP75, ILE76, GLY77, TYR79, GLN91 are in the C-terminal NOS domain of ORF8. So, ORF8 captures FMN through C-terminal NOS domain.

Figure 9. The results of molecular docking between ORF8 and FMN. A. Schematic diagram of the docking result. B. Amino acids of ORF8 that bind FMN. TYR73, ILE74, ASP75, ILE76, GLY77, TYR79, GLN91 are in the C-terminal NOS domain of ORF8.

3.6 ORF8 could capture heme through ORF3a and E protein

Molecular docking shows that ORF8 captures heme through the C-terminal NOS domain and then sends it to the N-terminal NOS domain. It may be just one of the less efficient ways. In Table 1, ORF7a, ORF3a, ORF10, ORF6, orf1ab, E, S have partial NOS domains. In the heme theory study, we found that ORF3a and ORF10 can attack hemoglobin, while ORF3a and E could bind heme. Then we applied protein molecular docking technology of DS to study whether ORF8 interacted with these proteins and determined whether there was the possibility of heme transmission.

According to Table 1 and the heme theory, the heme linked site “CWKCR” overlaps with the NOS domain “MRLWLCWKCRSKNPLLYDANYFLCWHTNCYDYCIPYN” of ORF3a. It showed the docking result of ORF8 and ORF3a in Figure 10. **Ser24, Tyr42, and Ser43** of ORF8 are the three intersections with ORF3a. Tyr42 and Ser43 belong to the middle NOS domain of ORF8 and Ser24 belong to the N-terminal NOS domain of ORF8. From the heme theory, we know ORF3a can attack hemoglobin and hunt heme. Therefore, the heme hunted by ORF3a may be transferred to the N-terminal NOS domain of ORF8 through the middle NOS domain of ORF8.

From the heme theory, ORF10 also assisted ORF3a in attacking hemoglobin. But the docking result of ORF8 and ORF10 (Figure 11) shows that the two proteins are not embedded together. It indicates that ORF10 will not directly send ligand molecules such as heme to ORF8.

The existing literature shows that ORF8 and ORF6 have an interference effect on the immune response. Docking result of ORF8 and ORF6 (Figure 12) reveals that it embedded the two proteins in each other. It connected the NOS domain of ORF6 to the three NOS domains of ORF8. Perhaps ORF6 has a combined effect on the three structures of ORF8, and is an essential auxiliary protein in the process of NO synthesis.

From the heme theory, E protein has a heme-binding site, “CAYCC”. The site is in the NOS domain of the E protein “CAYCCNIVNVSLVKPSFYVYSRVK”. The docking result of ORF8 and E protein (Figure 13) represents that ORF8 and E are embedded in each other. The heme attachment site of E is close to the N-terminal NOS domain of ORF8. It indicates that the N-terminal NOS domain of ORF8 can use the heme hunted by E in the NOS synthesis reaction.

Docking results on ORF8 and S protein, ORF8 and ORF7a protein show that ORF8 can not be embedded with S and ORF7a. Orflab is composed of a series of sub-proteins; there is no heme-binding point found in heme theory. Therefore, ORF8 does not directly cooperate with S, ORF7a, and orflab proteins to synthesize NO.

Figure 12. Docking results of ORF8 and ORF6. ORF8 and ORF6 are embedded together. The NOS domain of ORF6 is connected to the three NOS domains of ORF8.

Figure 13. Docking result of ORF8 and E. ORF8 and E are embedded together. The NOS domain of E (heme connection site) is close to the N-terminal NOS domain of ORF8.

3.7 Dimer structure of ORF8 was similar to NOS

Two nitric oxide synthase form a dimer to have the activity of catalyzing the production of NO. On both sides of the dimer is a reductase structure, and the center of the dimer is a dimer core synthesized by two oxygenase domains(66). Interestingly, ORF8 also exhibits a unique dimer structure(60) and has a similar dimer core. We downloaded the ORF8 structure file 7JTL from the PDB database. The sequence corresponding to this structure is missing the N-terminal fragment “MKFLVFLGIITTVAAFH”. But the N-terminal NOS domain of ORF8 still exists. One ORF8 is “QECSLQSQCTQHQPYPY” and the other ORF8 is “ECSLQSQCTQHQPYPY”. As shown in Figure 14, the two N-terminal NOS domains (oxygenase) are both in the core of the ORF8 dimer, forming a paired posture. It may be the role of clamping substrates such as heme and ARG and catalyzing NO production. The two C-terminal NOS domains (reductase) are on both sides of the ORF8 dimer. The crystal structure diagram also shows that the N-terminal NOS domain nears the C-terminal NOS domain in the space structure. Therefore, it is possible that ORF8 hunts heme through the C-terminal NOS domain and sends heme to the N-terminal NOS domain through conformational changes.

Figure 14. Dimer structure of ORF8 (PDB ID: 7JTL). The two N-terminal NOS domains (oxygenase) are in the core of the ORF8 dimer, and the two C-terminal NOS domains (reductase) are on both sides of the ORF8 dimer. The ORF8 dimer structure conforms to the dimer structure characteristics of the NOS enzyme.

4. Discussion

4.1 SARs-COV-2 combined with ORF8 to generate NO to break the blood-brain (testi) barrier and damage immune cells

From the above results, the N-terminal NOS domain of ORF8 directly binds heme with low efficiency. This study found that ORF8 could bound ORF3a and E protein. The heme theory found that ORF3a attacked hemoglobin, and deoxyhemoglobin was more likely to be attacked. Therefore, heme (divalent iron) shed from attacking deoxyhemoglobin became the direct source of ORF8's heme. The conjugation of E protein to ORF8 may not be good news. This study found that E protein's close position to the N-terminal NOS domain of ORF8 is exactly the heme linked site "CAYCC" (the last C is the iron linked site). It meant that ORF8 used the heme hunted by the E protein to produce NO. In the heme theory and ROS damage theory, we found that E protein had the functions of cytochrome C oxidase, catalase, peroxidase, and superoxide dismutase. The heme iron bound by the E protein was oxidized and reduced through these enzymes' processes. After ORF8 bound to E protein, ORF8 did not need to solve the source of heme. But it is not clear that ORF8 also needed CaM, FAD, FMN, NADPH, and ORF6.

Confusingly, ORF8 is highlighted by structural plasticity and high diversity, and its gene transcripts are expressed in higher amounts(69, 70). Although ORF8 catalyzed NO production, it was necessary to continuously produce NO to expand blood vessels or open the blood-brain (testi) barrier. NO had a short lifespan and was easily bound to the iron on the heme of hemoglobin. Therefore, NO was not suitable for continuous long-distance transmission in the body. It was also

impossible for the SARS-COV-2 virus to passively expand blood vessels through NO produced by ORF8 of a long distance. E protein was the structural protein of SARS-COV-2. When E protein was combined with ORF8, the SARS-COV-2 virus was equivalent to have a weapon that continuously produces NO. Many E proteins of the SARS-COV-2 virus could bound ORF8 so that the virus could constantly create a tremendous amount of NO. The SARS-COV-2 virus achieved an osmotic expansion of blood vessels or opened the blood-brain (testi) barrier through a steady NO stream. Then the SARS-CoV-2 virus spread to nearby tissues through small blood vessel holes created by NO stream.

Of course, these NO interfered with immune cells. The free radicals converted from these NO also damaged immune cells. After the cell synthesized ORF8, ORF8 directly bound E protein. The membrane vesicle transport system transported the ORF8-E complex to the cell membrane surface. Therefore, through the ORF8-E complex, infected cells also had abnormal NO synthesis function. For immune cells (such as NK cells, lymphocytes) in contact with infected cells, the NO stream interfered with the immune cells' NO signaling pathway. Besides, the NO rapidly decayed and converted into nitrogen free radicals, which damaged the cell membrane of immune cells, making immune cells death or abnormal function.

4.2 NO made the virus effectively spread and replicate in the body

Existing evidence shows that endogenous and exogenous NO can inhibit SARS-CoV-2 virus replication(71).The target proteins of NO antiviral activity include protease, reverse transcriptase, ribonucleotide reductase, zinc fingers, and related domains(72). One mechanism is to reduce the spike (S) protein's palmitoylation, which is crucial in anchoring the S protein to the lipid bilayer(57). So nitric oxide interferes with the interaction between S protein and its cognate host receptor ACE-2. Another mechanism is the nitrosation of viral proteins. S protein and 3CL cysteine protease may be targets of nitrosation(73). Because of the potential of NO in the treatment of COVID-19 and the anti-coronavirus infection, medical regulatory authorities in some countries have approved inhaled NO as a safe treatment(74). We believed ORF8 synthesized NO to inhibit 3C-Like protein was the virus's rate-limiting behavior on its replication, avoiding the cell collapse of insufficient replication resources. ORF8 synthesized NO to inhibit S protein, preventing the SARS-COV-2 virus from re-infecting already infected cells to make the infection more efficient.

4.3 The high viral load in asymptomatic patients was associated with insufficient NO to open the vascular permeability

An enormous amount of NO also expanded the permeability of blood vessels and opened blood-brain barriers. It promoted the spread of virus particles to tissue cells near blood vessels. For the asymptomatic patient, the virus was lurking in the phagocyte vesicles or lysosomes(59), and the viral protein did not attack the heme proteins on a large scale. Therefore, there was less heme in the blood , and then the ORF8 proteins that bound heme to catalyze NO were less too. The small amount of NO combined with its brief life span made it challenging to open the vascular permeability. It created a strange phenomenon: the virus particles spread through phagocytes are in the incubation period. However, more virus particles in the blood were difficult to spread in the tissue cells because the vascular permeability was not opened. Therefore, Viral loads of asymptomatic patients were found to be significantly higher when compared with

symptomatic patients(75). So, the high viral load in asymptomatic patients was associated with insufficient NO to open the vascular permeability.

4.4 Excessive NO hiddenly damaged the female reproductive system

The inflammatory factors produced by the cytokine storm will stimulate inflammatory cells, phagocytes, and neutrophils to produce oxygen free radicals and NO. There have been reports in the literature that large amounts of NO metabolites (nitrite and nitrate) are found in the plasma and urine of COVID-19 patients. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are by-products of various cellular processes, including aerobic metabolism. These reactive oxygen/nitrogen species (RONS), namely hydroxyl, superoxide anion, nitric oxide, and nitrosyl anion, are highly reactive molecules because of their unpaired valence electrons. RONS plays a critical biological role in cell signaling (redox signaling pathway): thiol switch, regulation of inflammatory cytokines, growth factors, etc. When the redox balance is disrupted, these powerful oxidants (free radicals) have harmful effects.

It synthesizes nitric oxide in the breast and triggers human lactation(76). Nitric oxide metabolites and adrenomedullin are detected in breast milk(77). Luteinizing hormone (LH) is a type of gonadotropin secreted by the pituitary gland's anterior lobe. LH acts on the corpus luteum or corpus luteum cells of the ovary to promote progesterone's secretion. High luteinizing hormone is a kind of endocrine disorder that quickly affects normal follicular development, causing abnormal ovulation, and unnatural conception. The high luteinizing hormone also produces more obvious premature ovarian failure or polycystic ovary syndrome in the human body. We found ORF8 synthesized an enormous amount of NO, and these NO could trigger the abnormal secretion of milk and LH, which hiddenly damaged the breast and ovaries.

4.5 ORF8 protein interfered with the NO signaling pathway

Nitric oxide (NO) is a free gas molecule, which plays a vital role as a signal molecule in human physiology. In KEGG's signal pathway database, after inputting "Human" and "Nitric Oxide", 33 relevant and important signal pathways are retrieved (Table 2). NO has essential effects on the immune system, cardiovascular system, metabolic system, nervous system, motor system, hormone control, rhythm control, cancer occurrence, and other signaling pathways. So, ORF8 protein could catalyze the synthesis of NO, interfering with signaling pathways and damaging immune, nervous, cardiovascular, metabolic, and reproductive systems.

5. Conclusion

It relates nitric oxide to many viruses' pathogenic mechanisms. We believe that the novel coronavirus is also closely related to nitric oxide. This study adopted bioinformatics methods such as domain search and molecular docking to study the relationship between the novel coronavirus proteins and nitric oxide. The results showed ORF8 had three functional domains of NOS synthase: oxygenase domain, reductase domain and calmodulin (CaM) binding domain. ORF8 also is integrated into a dimer structure to be catalytically active. ORF8 could capture heme, H4B, L-arginine, FAD, FMN, NADPH molecules, combine with CaM to form a NO generator, and synthesize nitric oxide. The heme bound by ORF8 mainly came from the attacked hemoglobin. The high viral load in asymptomatic patients was associated with insufficient NO to open the

vascular permeability as insufficient heme. SARs-COV-2 (E protein) also could combined with ORF8 to generate NO stream to break the blood-brain (testi) barrier and damage reproductive, nervous and immune system. Then the SARS-CoV-2 virus spread to nearby tissues through small blood vessel holes created by NO stream.

Table 2. Nitric oxide is involved in human signaling pathways

Entry	Pathway Name	Entry	Pathway Name
hsa04022	cGMP-PKG signaling pathway	hsa04940	Type I diabetes mellitus
hsa04066	HIF-1 signaling pathway	hsa00330	Arginine and proline metabolism
hsa04926	Relaxin signaling pathway	hsa04071	Sphingolipid signaling pathway
hsa05332	Graft-versus-host disease	hsa04151	PI3K-Akt signaling pathway
hsa04062	Chemokine signaling pathway	hsa04270	Vascular smooth muscle contraction
hsa01100	Metabolic pathways	hsa04370	VEGF signaling pathway
hsa05014	Amyotrophic lateral sclerosis	hsa04540	Gap junction
hsa05022	Pathways of neurodegeneration	hsa04611	Platelet activation
hsa05415	Diabetic cardiomyopathy	hsa04621	NOD-like receptor signaling pathway
hsa00910	Nitrogen metabolism	hsa04730	Long-term depression
hsa05418	Fluid shear stress and atherosclerosis	hsa04915	Estrogen signaling pathway
hsa04371	Apelin signaling pathway	hsa04921	Oxytocin signaling pathway
hsa04713	Circadian entrainment	hsa04924	Renin secretion
hsa04931	Insulin resistance	hsa04970	Salivary secretion
hsa05010	Alzheimer disease	hsa05330	Allograft rejection
hsa05200	Pathways in cancer	hsa04115	p53 signaling pathway
hsa05225	Hepatocellular carcinoma		

The life cycle of NO is short. If the NO content was low, it could not break the blood-brain (testis) barrier of some patients. So the virus could not infect the reproductive organs. Much NO dilates blood vessels in the reproductive system for a long time, causing severe congestion and abnormal function. Much NO also stimulated the gonads to secrete hormones abnormally and affects sperm and ovum production quality. Extreme NO osmotically expanded blood vessels and aggravate bleeding tendency. Bleeding caused tissue congestion or coagulation. Excessive NO destroyed the blood-brain (testis) barrier, allowing the SARS-COV-2 virus to penetrate the tissues and cells around the blood vessels, and infect the nerves, reproductive and gonadal organs.

The NO produced could protect infected cells and weaken attack NK cells or lymphocytes. It also inhibited S protein not to reinfect infected cells by SARS-CoV-2 viruses. NO inhibited 3C-like protein from limiting the virus replication process's speed and preventing the host cell from crashing due to the insufficient replication resources. Viruses may hijack the cell's nitric oxide signal pathways and cause diseases such as immunity, nerves, metabolism, and cardiovascular diseases. In short, this research has important guiding significance for disease prevention and treatment, medicine, and vaccine research.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets and results supporting the conclusions of this article are available at <https://pan.baidu.com/s/1e-pPwWH86HMOglznzNS7mQ> , code: 9zza.
Or: <https://mega.nz/folder/gjgzTa7T#6J9n8CqHd70QUNSm3wXM6w>

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

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Author details

¹ School of Computer Science and Engineering, Sichuan University of Science & Engineering, Zigong, 643002, China.

² School of Life Science and Food Engineering, Yibin University, Yibin, 644000, China.

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