

# Happy Hypoxia: Higher NO in red blood cells of COVID-19 patients

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## Research Article

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# Abstract

**Background:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that causes coronavirus disease 2019 (COVID-19) has spread to almost 100 countries, infected over 10M patients and resulted in 505K deaths worldwide as of 30<sup>th</sup> June 2020. The major clinical feature of severe COVID-19 requiring ventilation is acute Respiratory Distress Syndrome (ARDS) with multi-functional failure as a result of a cytokine storm with increased serum levels of cytokines. The pathogenesis of the respiratory failure in COVID-19 is yet unknown, but diffuse alveolar damage with interstitial thickening leading to compromised gas exchange is a plausible mechanism. Hypoxia has been seen in the COVID-19 patients however, patients present with a distinct phenotype. Intracellular levels of NO playing important role in the vasodilation of small vessels.

**Objectives:** To elucidate the intracellular levels of NO inside of RBCs in COVID-19 patients compared with that of healthy control subjects.

**Methods:** We recruited 14 COVID-19 infected cases who had pulmonary involvement of their disease, 4 non-COVID-19 healthy controls (without pulmonary involvement and were not hypoxic) and 2 hypoxic non-COVID-19 patients subjects who presented at the Masih Daneshvari Hospital of Tehran, Iran between March-May 2020. Whole blood samples were harvested from patients and intracellular levels of NO in 1 million red blood cells (RBC) was measured by DAF staining using flow cytometry (FACS Calibur, BD, CA, USA).

**Results:** The Mean florescent of intensity for NO was significantly enhanced in COVID-19 patients compared with healthy control subjects ( $P \leq 0.05$ ). As a further control for whether hypoxia induced this higher intracellular NO, we evaluated the levels of NO inside RBC of hypoxic patients. No significant differences in NO levels were seen between the hypoxic and non-hypoxic control group.

**Conclusions:** This pilot study demonstrates increased levels of intracellular NO in RBCs from COVID-19 patients. Future studies should examine whether intracellular NO would be increased in large number of COVID-19 patients for usage of possible NO therapy in severe patients.

## Introduction

The coronavirus SARS-CoV-19 that causes coronavirus disease 2019 (COVID-19) has spread to almost 100 countries, infected over 10 million patients and resulted in 505K deaths worldwide as of 30<sup>th</sup> June 2020 (1). The major clinical feature is acute Respiratory Distress Syndrome (ARDS) with a key complication being heart and multi-functional failure abnormal blood oxygen saturation is at least 95% in most lung diseases, such as pneumonia. Whilst decreasing oxygen saturation accompanies other change, such as stiff or oedematous lungs, increasing levels of carbon dioxide are usually seen in COVID-19 patients with pneumonia (2). Thus, many COVID-19-infected patients with pulmonary involvement have hypoxia and dyspnea as important hallmarks of disease. In COVID-19 patients, despite the

respiratory system insufficiently oxygenating the blood, these patients are often alert and feeling relatively well and can easily talk (3).

Red blood cells (RBCs) are highly adapted cells for blood gas transport. At the high oxygen tensions ( $PO_2$ ) prevailing in the pulmonary system, the blood is normally completely saturated with oxygen and hemoglobin (Hb) will form an R structure. When the blood enters the microcirculation, the  $PO_2$  is attenuated promoting oxygen dissociation from hemoglobin and a shift to the T form (4).

Clinical examination of severe cases of COVID-19 patients shows a decreased ratio of arterial oxygen partial pressure to fractional inspired oxygen ( $PaO_2:FiO_2$  ratio) with concomitant hypoxia and tachypnea in most cases (5). Nitric oxide (NO) plays a key role in controlling the vascular system by regulating vascular tone and blood flow following activation of soluble guanylate cyclase (sGC) within the vascular smooth muscle. NO also controls mitochondrial oxygen consumption by inhibiting cytochrome *c* oxidase (6). RBCs have long been considered as powerful scavengers of endothelial cell-derived NO, participating in systemic NO metabolism mainly by limiting NO bioavailability (7). RBCs passing through the microcirculation sense tissue oxygen conditions via their degree of deoxygenation and couple this information to the release of vasodilatory compounds including ATP and NO to enhance blood flow to hypoxic tissues (8). NO is a free radical and has a critical pathophysiological role in infectious diseases.

RBC intracellular NO is derived from three sources: a) entry from the cell exterior by binding to the highly conserved  $\beta$ -globin chain cysteine 93 residue to form bioactive S-nitrosohemoglobin (SNO-Hb) (9), b) formation from nitrite entering RBC due to the reductive potential of deoxyhemoglobin (10) and c) intracellular production of NO by RBC derived from an active and functional eNOS-like enzyme (RBC NOS). This is localized in the RBC membrane and cytoplasm and has similar properties to eNOS in terms of phosphorylation sites controlling enzymatic activity and its activity dependence on intracellular calcium and L-arginine concentrations (11).

Transfer of NO from SNO-Hb to the membrane-bound anion exchanger (AE1) is required for transfer of NO out of the RBC and is dependent on both the SNO-Hb state (T or R) and the SNO-Hb concentration. Therefore, the ability of SNO-Hb to transfer NO to AE1 or other proteins (e.g., glutathione) are limiting factors in respiratory efficiency. The kinetics and allosteric regulation of Hb nitrosylation by oxygen and pH are consistent with the physiologic mechanisms that modulate tissue blood flow, namely acidosis, hypoxemia and tissue hypoxia lead to NO generation by the RBC via SNO-protein transfer of NO activity (12). In addition, insults such as cellular stress activates RBC NOS, leading to NO release and vasodilation of vessel segments under hypoxic conditions. Together, this supports a prominent role of RBC-derived NO in the regulation of local blood flow (13).

Therefore, the erythrocrine function of RBCs i.e. the release of bioactive molecules including NO, NO metabolites, and ATP are likely to be important in tissue protection and regulation of cardiovascular homeostasis by RBCs. Despite this clear role of NO in vasodilation, there is little evidence regarding the role of NO in COVID-19 particularly in 'happy hypoxic' patients. To examine the hypothesis that NO is

important in regulating vasodilation during hypoxia in these subjects we studied intracellular levels of NO in COVID-19 patients.

## Materials And Methods

We examined the 14 COVID-19 infected cases who had lung involvement of their disease, 4 non-COVID-19 healthy control (without lung involvement and not hypoxic) and 2 hypoxic-nonCOVID-19 patients subjects who presented at the Masih Daneshvari Hospital of Tehran, Iran between March-May 2020. All COVID-19 infected cases were diagnosed based on the World Health Organization (WHO) interim guidance. Patients were confirmed positive for COVID-19 nucleic acid in the respiratory samples via real-time reverse-transcriptase polymerase-chain-reaction (RT-PCR) or serum specific antibodies and chest imaging including chest X ray and CT scan. Demographic data of all participants presented in Table 1. Red blood cells were isolated from 3 ml whole blood cells with EDTA used as an anticoagulant. Whole blood was diluted 400 x with FACS buffer (BSA and PBS) and then stained with the NO-specific probe, 4-amino-5-methylamino-2', 7'-difluorofluorescein (DAF-FM DA) (BD Pharmingen, catalog 566663, USA). Intracellular NO was detected within RBCs by flow cytometry (FCM, BD FACS Calibur) using DAF-FM DA dye as fluorochrome. Following incubation of RBC cell suspensions for 30 min with the dye (10 $\mu$ M), a fluorescent signal was detected that corresponded to the level of NO.

## Results

Chest X ray and CT imaging of the lungs shows significant changes in the lungs with bilateral alveolar diffusion (Fig. 1 A and B).

After isolation of RBC, they were stained with DAF and intracellular NO determined. COVID-19 patients had a greater shift reflecting higher intracellular levels of NO (Fig. 1C). The mean fluorescent intensity (MFI) was calculated for each subject and plotted as a histogram (Fig. 1D). This showed a significant increase in intracellular NO levels in RBCs from COVID-19 subjects ( $P \leq 0.05$ ).

To determine whether hypoxia may be responsible for the increased levels of intracellular NO in COVID-19 patient's RBC, we examined intracellular NO levels of RBC from hypoxic patients without COVID-19 (due to COPD and emphysema). As depicted Fig. 1E, no significant increase of intracellular NO was seen in hypoxic patients compared to non-hypoxic controls.

## Discussion

We demonstrate increased levels of intracellular NO in RBC from COVID-19 subjects. This is not due to the presence of hypoxia *per se* but may afford protection against the hypoxia seen in COVID-19 patients. During health, constitutive NO production in RBCs is largely NOS-dependent, whereas in hypoxic conditions NO production may involve nitrite reduction by deoxyhemoglobin carbonic anhydrase and/or eNOS itself (14).

RBC-derived NO causes the vasodilation of small vessels in tissues allowing oxygen to be readily released to tissues. In our study, intracellular RBC NO of COVID-19 patients is significantly higher than in healthy controls and this may enable the release of oxygen to tissues resulting in the clinical manifestation of happy hypoxia in these patients. Since NO is a pulmonary vasodilator and also as antiviral activity against coronavirus strains it is likely that NO treatment may be effective in COVID-19 subjects. There is no evidence that direct oxygen therapy is beneficial in the management of breathlessness in severe COVID-19 patients but our data suggests that NO therapy may be beneficial in COVID-19 patients with hypoxia (15).

In summary, COVID-19 patients show higher levels of NO inside RBC compared to non-COVID-19 hypoxemic patients. Whether higher levels of intracellular NO inside RBC of COVID-19 infected patients drive the unexpected silent hypoxia phenotype needs to be examined in future clinical studies using NO donors in hypoxemic COVID-19 patients.

## Conclusions

This pilot study shows that elevated levels of intracellular NO may mask the effects of hypoxia in COVID-19 patients which present as a 'happy' hypoxic state. Further studies are required to confirm this but the data suggests that trials of NO therapy or NO donors may be useful in treating severe COVID-19 patients with hypoxia.

## List Of Abbreviations

**ARDS;** Acute Respiratory Distress Syndrome (ARDS)

**COVID-19;** coronavirus disease 2019 (COVID-19)

**FACS;** Fluorescence-activated cell sorting

**MFI;** mean florescent of intensity

**NO;** Nitric oxide

**RBC;** Red blood cells

## Declarations

**Ethics approval and consent to participate:** The study was approved by Ethical committee of Masih Daneshvari Hospital IR.SBMU.NRITLD.REC.1399.123 and consent obtained from study participants was written.

**Consent for publication:** All authors approved the submission.

**Availability of data and materials:** The data will be available upon written request.

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**Competing Interests:** The authors have no conflicts of interest to declare.

**Authors' contributions:** EM performed the experiments and initial data analysis. MM, HRJ, PANN, SMRH, PT, MV and HZ provided the patients and samples. EGC, GF and IMA critically reviewed and revised the manuscript. All authors reviewed the final version and approved submission.

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## Table

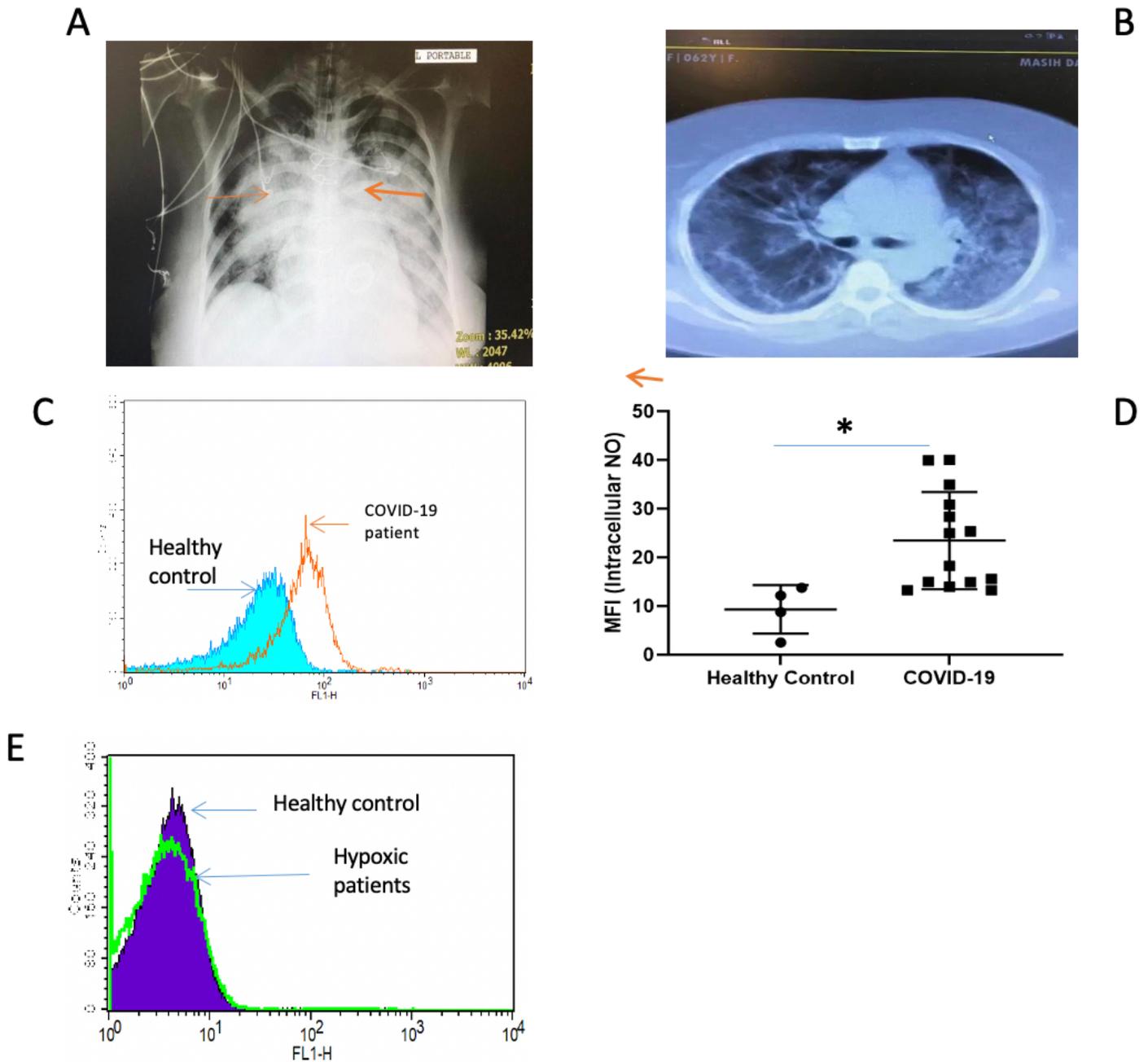
**Table.1. Demographic information of all participants (COVID-19 and control groups)**

Abbreviation used: MV: Mechanical Ventilation; NIV: Non-invasive Ventilation

In radiology: -, negative; +, Unilateral Ground glass opacity (GGO)/Consolidation, ++, Bilateral GGO/Consolidation; +++ , ARDS

3	IgM	RT-PCR COVID-19	Mortality	Radiology	Ventilation	PCO2	O2/S	Sex	AGE	
	-	-	-	-	-	41	92/97	M	39	Control
	-	-	-	-	-	40	98/97	M	65	Control
	-	-	-	-	-	40	97/98	F	44	Control
	-	-	-	-	-	42	94/98	F	54	Control
		+	+	++	MV	56	69/86	F	80	Patient 1
		+	+	+++	MV	28	34/69	M	77	Patient 2
		+	-	++	-	47	61/87	M	34	Patient 3
		+	+	++	MV	44	26/51	F	62	Patient 4
		+	+	++	MV	47	43/65	M	72	Patient 5
	+	-	+	++	-	63	36/63	M	69	Patient 6
	+	-	+	++	MV	47.5	47/82	M	60	Patient 7
	+	-	-	++	-	55	48/80	M	41	Patient 8
	+	-	-	++	MV	58.6	36/69	M	66	Patient 9
	+	-	-	++	-	45	98/98	M	61	Patient 10
	+	-	+	++	NIV	41.4	81/82.5	F	74	Patient 11
	+	-	-	++	-	41	40.5/74.6	F	58	Patient 12
	+	-	-	++	-	44	84/97	F	81	Patient 13
	-	+	+	++	MV	49	44/78	F	21	Patient 14

## Figures



**Figure 1**

(A) Representative chest X ray of a COVID-19 patient on mechanical ventilation showing bilateral consolidations (red arrows). (B) Spiral CT scan of a representative COVID-19 patient indicating multiple bilateral patchy ground glass infiltration. (C) Red blood cells were washed and preincubated with 5 mM of DAF for 20 min at 37°C in PBS containing 1% BSA, in the dark. The generation of intracellular NO was determined by FACS analysis as described in the Materials and Methods section. A representative histogram from one out of 14 COVID-19 positive patients and 4 healthy controls is shown. (D) The mean fluorescent intensity (MFI) of all the subjects in each group is presented (\*p<0.05 using Student's t-test). (E) Representative histogram of intracellular NO from RBCs of a single hypoxic non-COVID-19 patient.