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Coronavirus SARS-CoV-2: Hypotheses of Impact on the Circulatory System, Prospects for the Use of Perfluorocarbon Emulsion, and Feasibility of Biophysical Research Methods

(Editorial)

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Summary

This paper highlights published hypotheses on the possibility of coronavirus SARS-CoV-2 entry into the bloodstream, its interaction with vascular endothelium, red blood cells, hemoglobin and its fragments. As a result of such interaction, iron ions may be released into the bloodstream and, subsequently, a cytokine storm may occur. In this context, it is important to find a cytoprotective agent capable of blocking such processes. The perfluorocarbon emulsion could be a candidate for this role.

The aim of the paper is to show the feasibility of biophysical methods to study the molecular mechanisms of action of SARS-CoV-2 on human red blood cells and hemoglobin as well as the restorative and cytoprotective effect of the perfluorocarbon emulsion during Fe^{2+} oxidation in heme.

Materials and methods. High resolution spectroscopy, atomic force microscopy, atomic force spectroscopy, electroporation were used. Blood was exposed to oxidizing agents of different nature. Perfluorocarbon emulsion was added in various concentrations and its effect at various incubation times was studied. Concentration of hemoglobin derivatives was calculated considering multicollinearity, and statistical analysis of the results was performed.

Results. The perfluorocarbon emulsion was shown to have an effective restorative and cytoprotective action in iron ion oxidation in the heme: Fe^{3+} was restored to Fe^{2+}. The degree of MetHb reduction to HbO_2 and Hb depended on the concentration of the oxidizing agent and incubation time. We observed a change in MetHb content from 80-90% to 5-12%. The perfluorocarbon emulsion in clinical concentrations helped eliminate local membrane defects and restored normal erythrocyte morphology.

Conclusion. In the light of the studied hypotheses, the use of perfluorocarbon emulsion can become an effective method for blocking the consequences of coronavirus effect on the blood cells and restoring a normal gas exchange.

Keywords*: erythrocyte; membranes; hemoglobin derivatives; perftoran; cytoprotective action*

Introduction (Hypotheses)

The COVID-19 pandemic has set new challenges for science which, for obvious reasons (short time interval, lack of initial data), cannot be solved today. Data on the origin of the new coronavirus modification are still limited and continue to be updated. Many questions arise about the pattern of its existence in the human bloodstream, molecular mechanisms of interaction with the endothelium of blood vessels, blood cells, hemoglobin. The knowledge in this field is mostly represented by isolated hypotheses, which so far have practically no systematic scientific rationale.

Coronavirus SARS-CoV-2

The new coronavirus SARS-CoV-2 is a member of the Coronaviridae family, which as of January 2020 includes more than 40 species of RNA-containing viruses affecting humans and animals. In February 2020, Chinese scientists isolated the coronavirus strain and demonstrated SARS-CoV-2 electronic microscope images for the first time. The images were obtained from the national repository of pathogens and published on the agency's website, presented to WHO and are widely used by mass media. Other virus images from the electron microscope have appeared in the literature [1]. Researchers from the University of Hong Kong received an image of coronavirus exiting from an infected cell. The obtained images suggest that the size of SARS-CoV-2 practically does not differ from the other coronaviruses and is 100 - 125 nm [2], and its peplomers (club-shaped "spikes") have a height of about 10 nm. While passing through the cell membrane, the virus "stretches out" and changes its shape.

SARS-CoV-2, erythrocyte, and hemoglobin

Various hypotheses on the interaction of SARS-CoV-2 with erythrocytes and its effect on hems and porphyrin have been suggested. Two possible ways of interaction of virus proteins with hemoglobin are considered [3].

The first one is related to hemoglobin exit from erythrocyte and its entry into bloodstream. This may occur as a result of erythrocyte hemolysis, e.g. immunological. The events occurring after hem and Fe+2 ion enter the bloodstream and further oxidative stress development have been previously described [4, 5].

The second way assumes penetration of the virus or its proteins through plasma membrane into erythrocyte and further interaction of viral proteins with hem and porphyrin. Deoxyhemoglobin has been noted to be more susceptible to viral attacks than the oxygenated hemoglobin HbO2 [3]. The onslaught of viral proteins causes a decrease in the content of HbO2, a major contributor to gas exchange in the body, and results into respiratory failure.

SARS-CoV-2 and circulatory system

Other mechanisms are also under consideration. In particular, the binding of SARS-CoV-2 to endothelial cells of human circulation system may be relevant [1]. Such interaction is highly likely and was confirmed by thrombosis occurrence in COVID-19 patients [6]. The virus may interact not only with the vascular wall, but also with RBC membranes in microvasculature. The viral proteins penetrating the cell interact with 1-beta hemoglobin chain [3], cause the screening effect of part of hemoglobin molecules, which reduces the oxygen flow from erythrocyte to tissues through the capillary wall and, accordingly, from alveoli to capillaries.

In some works, SARS-CoV-2 binding to ACE2 and CD 147 cell receptors was noted to enable viral penetration into the cell and destruction of cell membranes during virion exit [7, 8]. The destruction of membrane integrity may cause intravascular hemolysis and release of the cell-free Hb into the blood system [9,10]. Release of Hb into plasma significantly reduces the bioavailability of nitrogen oxide (NO) in the vessel, promoting vasoconstriction, endothelial dysfunction and platelet activation. Vasoconstriction (including that in micro vessels) and thrombosis are typical for Covid-19 patients [6].

Filtration-reabsorption equilibrium in the microvasculature and surrounding tissues may be disturbed if the cell structure of capillary walls is impaired [11]. As a

result, interstitial edema, alveolar hemorrhage and microvascular lung thrombosis occur [12]. Progressive endothelial thrombo-inflammatory syndrome may also include microvasculature of brain and other vital organs, resulting in multi-organ failure observed in patients with Covid-19 [1, 2].

The SARS-CoV-2 interaction with bone marrow cells is dangerous. A paper [13] describes a case of leukoerythroblastosis in Covid-19 associated with bone marrow damage and release of modified red blood cells.

The described hypotheses suggest that the coronavirus or its proteins interact with the walls of blood vessels, erythrocytes, and hemoglobin. They consider the interaction of SARS-CoV-2 proteins with heme and porphyrin, the entry of Fe+2 ions into the bloodstream and their further oxidation to Fe^{3+}. Such pathological processes can be blocked and/or restored with antioxidants and cytoprotectors [14]. The use of drugs of this class may help develop methods to control Covid-19.

The aim of the paper is to show the feasibility of biophysical methods to study the molecular mechanisms of impact of SARS-CoV-2 on human red blood cells and hemoglobin and the restorative and cytoprotective effect of perfluorocarbon emulsion during the iron oxidation in heme.

Materials and Methods

Scheme of experiments

Blood was drawn in EDTA microvettes (Sarstedt AG and Co., Germany) during a routine screening of donors. In accordance with the requirements of the ethics committee of the V.A.Negovsky Scientific Research Institute of General Reanimatology the consent of all donors for the study participation was obtained. The scheme of experiments is shown in Fig.1. Blood (150 μl) was exposed to an oxidizing agent which was either $NaNO_2$ solution or ultraviolet radiation (UV). The PBS tablets buffer (MP Biomedicals, USA), (pH 7,4) was used in experiments for cell washing and suspension dilution. Micro-22R centrifuge (Hettich-Zentrifugen GMBH&Co.KG, Germany) was used for cell separation and sedimentation. The UNICO 2800 (USA) digital automatic spectrophotometer was used to record optical spectra. The method of calculation of hemoglobin derivatives concentrations, the solution of multicollinearity problem, and relevant statistical analysis were described elsewhere [15, 16].

Atomic force microscopy and spectroscopy

Images of RBC morphology were obtained using the atomic force microscope (AFM) NTEGRA Prima (NT-MDT, Russian Federation) in a resonance mode on monolayers prepared using the sedimentation method. Cantilevers NSG01 (R=10 nm, coefficient of elasticity K = 5 N/m) were used. The number of scanning points was 512, 1024. Scanning fields were 100×100 μm^2, 30×30 μm^2. 2D and 3D images were obtained using AFM software [15]. Statistical analysis of the obtained results was performed using the Origin Lab software (USA).

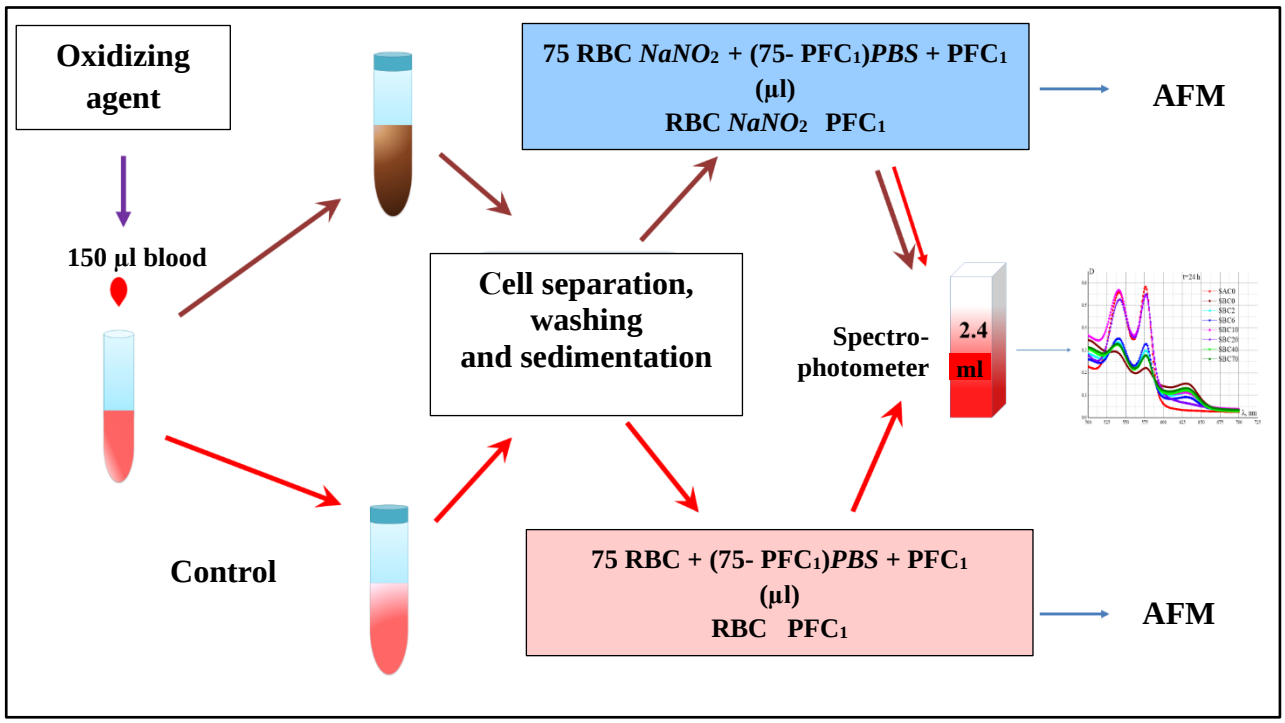

Fig. 1. Stages of the experiments: blood exposure to the oxidizing agent, addition of PFC or cytoflavin in various concentrations, measurement of optical spectra, calculation of concentrations of hemoglobin derivatives, obtaining an AFM image of cells and membrane nano-surface.

Electroporation (USA)

Laboratory electroporator provides pulse duration 3-5 ms, pulse amplitude 3×10^3 V. A cuvette 30x30 mm was equipped with flat titanium electrodes mounted on opposite walls. The intensity of the generated field was 1040 V/cm [17].

Results and Discussion

The development of oxidation processes in blood caused by $NaNO_2$ and ultraviolet radiation (λ=254 nm) was simulated using in vitro experiments. Fe^{2+} turned into an oxidized form (Fe^{3+}) in the molecules of hemoglobin, which was evidenced by the transformation of oxyhemoglobin HbO_2 into methemoglobin $MetHbO_2$. The concentration and dose dependence of these processes was established. Fe^{3+} is known to prevent gas exchange between blood and tissues. Therefore, the appearance of MetHb in the blood leads to tissue hypoxia, and in high concentrations can even be fatal.

Perfluorocarbon compound "Perftoran" (Russian Federation) [18,19] and cytoflavin (Polysan, Russian Federation) [15] were used to reduce Fe^{3+} to Fe^{2+} and, accordingly, to transform MetHb to HbO_2. At the same time, methemoglobin was reduced to oxyhemoglobin depending on the concentrations of these substances and incubation time. Concentrations of perftoran (PFC) were recorded later as indices (μl PFC per 200 μl of erythrocyte suspension). Cytoflavin concentrations were recorded as volume of cytoflavin (μl) per 150 μl of cell suspension. Fig. 2 shows MetHb concentrations on UV exposure and after introduction of Perftoran emulsion, the optical spectra, as well as the AFM field erythrocyte images under the same conditions.

Initially, 90-98% of erythrocytes were discocytes (Fig.2a). After UV exposure, the number of discocytes decreased, and stomatocytes, echinocytes, sphero-echinocytes, ghost cells appeared (Fig. 2b). As a result of subsequent PFC exposure, the percentage of discocytes recovered to 80-90%. The change in erythrocyte shapes associated with changes in concentrations of hemoglobin derivatives (Fig. 2 b, c). The initial content of MetHb was about 0.5%. After UV exposure it increased to 35-40%, and after PFC exposure it was 2-5%. Thus, in vitro experiments showed that perftoran can be effectively used to restore MetHb to HbO_2.

These experimental data demonstrate the restorative effect of PFC on erythrocytes both in damage to membranes and cell structure, and in abnormal hemoglobin molecules.

The effect of agents on the system can be demonstrated by time and concentration dependence. Absorption spectra of hemoglobin derivatives solutions for different incubation times with PFC are shown in Fig. 2c. With increasing incubation time, peak at wavelength 630 nm dropped, and peaks at 542 nm and 577 nm grew, which indicates a decrease in MetHb content and, consequently, an increase in HbO_2+Hb.

The concentration dependence of the PFC exposure is shown in Fig. 2a (left). As early as at the concentration of PFC 5-10 µl/ml of the suspension there was a significant reduction of Fe^{3+} to Fe^{2+}, i.e., MetHb to HbO_2 + Hb.

The qualitative result of transformation of methemoglobin into oxyhemoglobin is presented in the photo (Fig. 2a, right). The dark brown suspension after UV exposure changed its color to red after 24 hours of incubation with PFC.

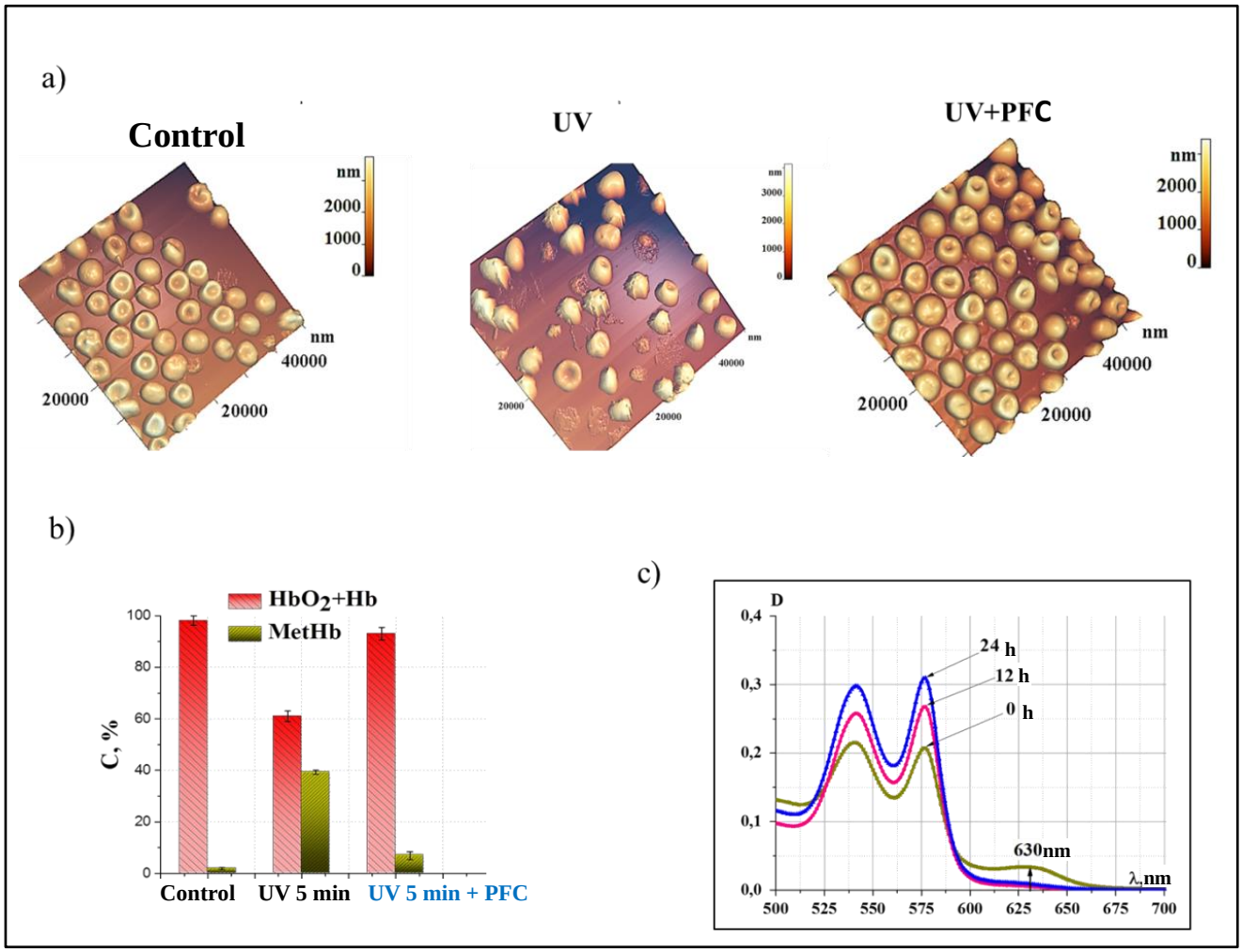

Fig. 2. The reducing effect of PFC in erythrocyte suspensions.
Note. a) AFM 3D cell images: initial (control cells) on the left, the cells damaged by the UV exposure are in the middle, on the right are the cells damaged by UV and subsequently exposed to PFC for 24 hours; b) percentage of MetHb, HbO_2, Hb in the control, after UV exposure and subsequent PFC exposure, PFC incubation time was 24 hours; c) absorption spectrum changes after UV exposure and subsequent PFC action (t=0, 12, 24 hours).

Cytoflavin was also used as a reducing agent (Fig. 3b). In these cases the dependence of effect on concentration was also observed. Cytoflavin allowed reducing MetHb concentration from 80-90% to 20-25%.

Exposure of RBCs to oxidizing agents can produce two effects:1) oxidation of iron, Fe^{2+} to Fe^{3+}, and, accordingly, increase of MetHb concentration in them; 2) erythrocyte hemolysis.

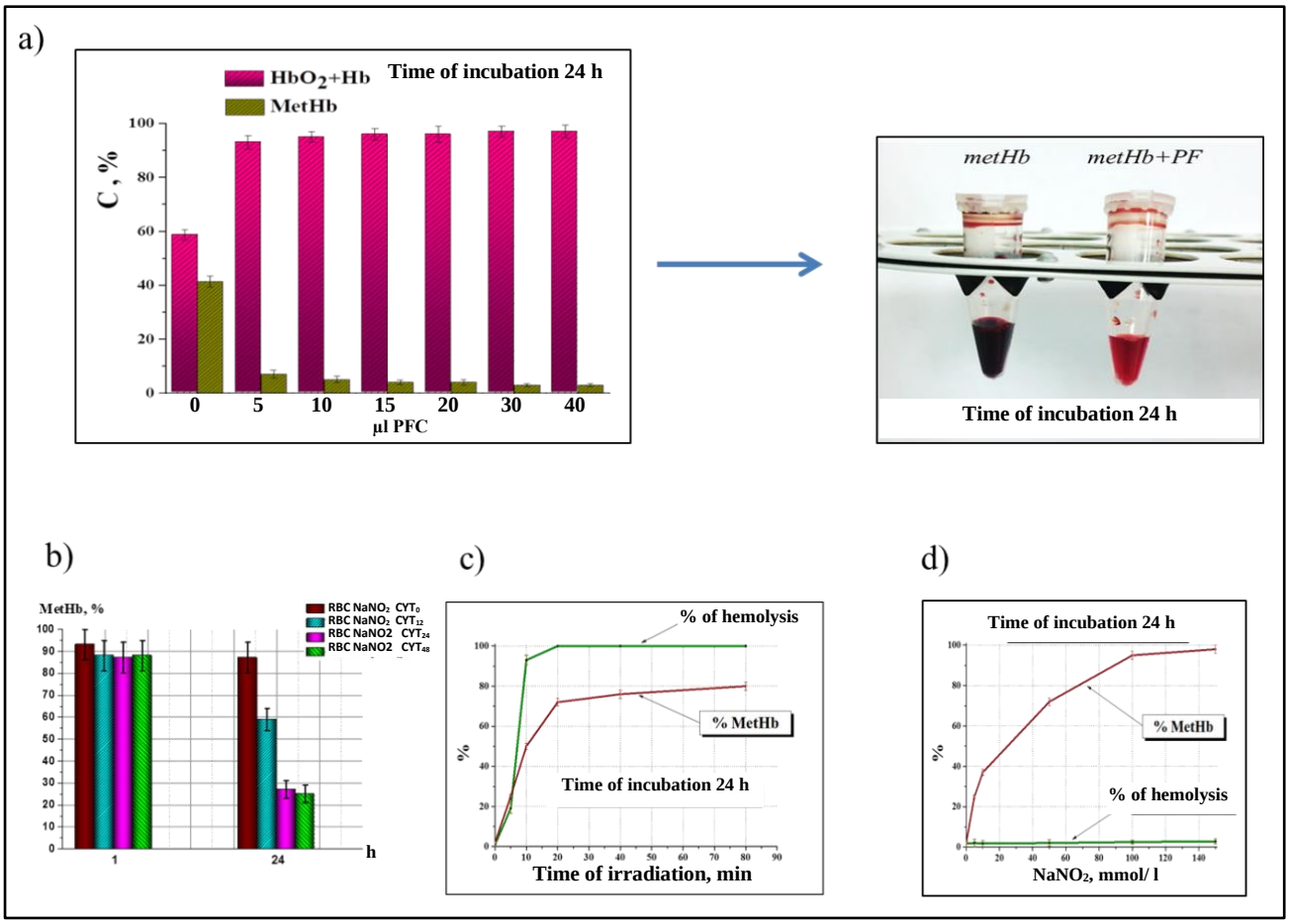

Fig. 3. Hemoglobin derivatives.
Note. (a) Suspension after UV irradiation and subsequent PFC exposure in various concentrations (concentration dependence) on the left, photos of suspension after UV irradiation and subsequent incubation with PFC for 24 hours on the right; (b) MetHb formation as a result of $NaNO_2$ exposure and reduction of Fe^{3+} to Fe^{2+} after exposure to cytoflavin in different concentrations (concentration dependence) ; (c) and (d) correlation of biological effects (iron oxidation and hemolysis) after exposure to different agents: (c) UV irradiation, (d) addition of $NaNO_2$.

Depending on the nature of the agent, the ratio of these biophysical processes was different (Fig. 3c, d). Thus, if the oxidizing agent was ionizing radiation, UV radiation, both processes occurred with the same intensity, and the dose dependence and irradiation time dependence was observed (Fig. 3c). When using the $NaNO_2$ agent (depending on the concentration), the content of MetHb in erythrocytes was intensively increased, but their hemolysis was practically absent (Fig. 3d). This interesting fact is

important to consider when analyzing the effect of factors of different nature, including viruses and bacteria, on red blood cells.

In a number of experiments, the protective effect of the Perftoran emulsion on erythrocyte membranes was shown using AFM method (Fig. 4). Hemin, $ZnSO_4$ and furosemide were used as membrane damaging agents. As a result of blood exposure to these agents, topological nanodefects of cell membranes in the range of 20-200 nm were found. Subsequent exposure to PFC led to a reduction in the size of membrane defects or to their complete "healing".

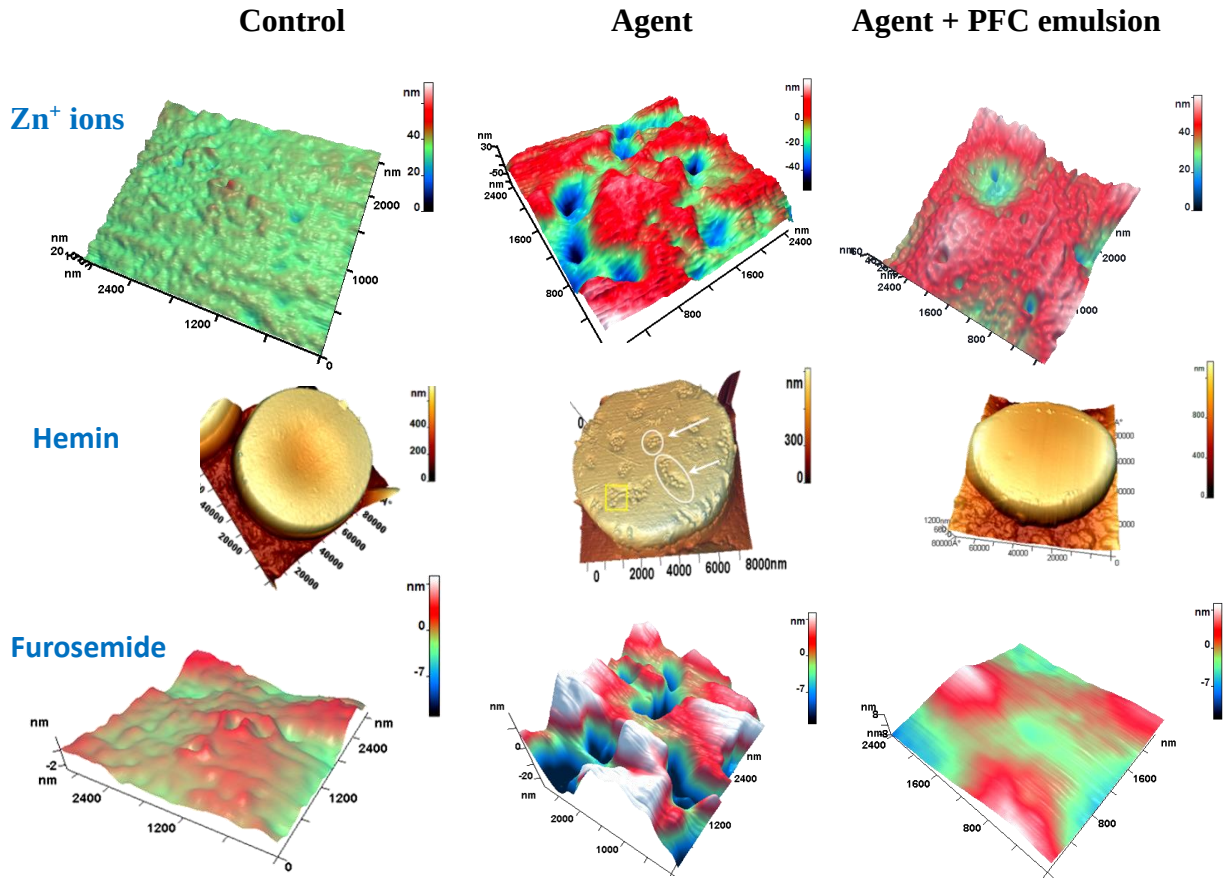

Fig. 4. Defects of erythrocyte membranes in the field of atomic force microscope after exposure to various chemical agents and the protective effect of the Perftoran perfluorocarbon emulsion.

If the virus causes structural disorders and local cell defects, Perftoran is very likely to have a protective effect and prevent damage to both cells and cell membranes.

A major problem with the Covid-19 pandemic is the high mortality rate of the elderly, with less age-dependent infection susceptibility. The high mortality in older persons is probably due to the initial condition of blood vessels and red blood cell membranes.

Using a soft electroporation method (reversible membrane perforation), we have previously shown that membranes of older persons (65-74 years old) are indeed more vulnerable and have more potential defects than those of young and middle-aged people [17]. This method can help identify patients with increased risk of SARS-CoV-2 adverse effect on blood cells.

Conclusion

Perfluorocarbon emulsions, in particular Perftoran, have a cytoprotective effect, provide additional capacity for the transfer of O_2 by blood, and contribute to the reduction of Fe^{3+} to Fe^{2+} in the heme. These emulsions are promising compounds that promote gas exchange in patients in critical conditions and can be used to protect blood cells. In the light of the hypotheses presented, perfluorocarbon emulsions (Perftoran) can be effectively used to control Covid-19. Atomic force microscopy, high-resolution optical spectroscopy and electroporation methods can be successfully used to study the molecular mechanisms of SARS-CoV-2 action on blood cells. Studies using such methods can provide a scientific rationale for the hypotheses currently presented.

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