Letter to the Editor

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# Detection of Hemosiderin-Laden Macrophages in Bronchoalveolar Lavage Fluid of COVID-19 Patients: Is Perls Stain a Potential Indicator of Oxidative Alveolar Damage?

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Dear Editor,

An interesting special issue was recently published in your journal *Acta Cytologica* (January–February 2020) concerning the ancillary techniques in cytopathological specimens, supervised by Baloch and Gupta as guest editors [1]. In this issue, the manuscript of Zhou and Moreira stressed the importance of auxiliary techniques and special staining in pulmonary cytopathology, not only in the differential diagnosis and predictive testing of lung tumors but also in the detection of pathogenic agents of infectious disease [2].

The last few days have seen an important revelation regarding the pathophysiology of the pandemic COVID-19 infection caused by SARS-CoV-2 for severe acute respiratory syndrome coronavirus 2. This novel RNA virus is composed of RNA-dependent RNA polymerase, structural proteins (spike protein, envelope protein, membrane protein, and nucleocapsid phosphoprotein), and a set of nonstructural proteins (ORFs) [3]. The typical chest computed tomography scan features of emerging COVID-19 pneumonia included bilateral ground-glass opacities with a predominantly peripheral distribution [4].

First, a proinflammatory syndrome with notably increased levels of cytokines and chemokines (cytokine storm) or macrophage activation syndrome has been noted in hospitalized COVID-19 patients [5, 6]. More recently, bioinformatics analysis reveals that the virus causes prolonged and progressive hypoxia by binding to the heme groups of hemoglobin in red blood cells (RBCs) and inhibiting heme metabolism [7]. Consequently, the pulmonary lesions described on chest computed tomography scan are thought to be the result of the inability to exchange carbon dioxide and oxygen and the release of oxidative iron from the hemes, which overwhelm the natural defenses against pulmonary oxidative stress and may eventually result in bilateral ground-glass-like opacities in COVID-19 patients.

It is well known that RBCs carry oxygen from the lungs to other organs. They can do this with the help of hemoglobin, which is an assembly of 4 globular protein subunits called hemes. A heme group consists of an iron atom (Fe) held in a heterocyclic ring, known as porphyrin acting as its container. The Fe may be either in the ferrous ($Fe^{2+}$) or in the ferric ($Fe^{3+}$) state, but $Fe^{3+}$ cannot bind oxygen. Oxygenation changes the electronic state of the $Fe^{2+}$-heme complex. When RBCs are exposed to oxidizing agents, the heme iron in hemoglobin is oxidized from $Fe^{2+}$ to $Fe^{3+}$ state to form methemoglobin, which is unable to bind oxygen [8]. Thus, iron must exist in the $Fe^{2+}$ state to bind oxygen. In this way, the iron ion can be safely transported by hemoglobin, but used to bind to oxygen when it reaches the pulmonary alveoli, where all the gas exchanges take place, and then goes to deliver oxygen to the other organs.

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In the case of COVID-19 infection, the surface glycoprotein of the virus binds to the porphyrin of the heme. At the same time, nonstructural proteins of SARS-CoV-2 coordinate attack the heme on the 1-beta chain of hemoglobin to dissociate the iron from the porphyrin [7], and in doing so, dissociated oxidizing iron ion moves freely. Without the iron ion, hemoglobin can no longer bind to oxygen. In theory, once all hemoglobin is altered, the RBC becomes unable to carry oxygen and simply runs with the SARS-CoV-2 attached to its porphyrin. This means, on the one hand, a lack of oxygen for all the organs, and on the other hand, that released iron floats freely causing oxidative damage to these organs. This hypothesis may explain in part extrapulmonary lesions caused by COVID-19.

However, the lungs have a primary defense mechanism to maintain iron homeostasis, known as iron sequestration. The initial players in this mechanism are the alveolar macrophages that collect free radicals such as iron [9]. In COVID-19 patients, this mechanism seems to be overwhelmed by the excess of oxidizing iron and so begins the process of pulmonary oxidative stress, which leads to inflammation that is usually bilateral with COVID-19 infection.

In practice, ferric iron could be easily identified in cells and tissue samples in cytologpathology and histopathology laboratories using the routine Perls Prussian blue stain under light microscope [10]. Perls Prussian blue stain, also called as Perls stain, was described in 1867 by the pathologist Max Perls. It allows detecting the presence of iron in cells by conversion of iron to Prussian blue as shown in the following chemical formula [11]:

Ferric chloride + Potassium ferrocyanide →
Ferric ferrocyanide + Potassium chloride (Prussian blue),

$$4FeCl_3 + 3K_4Fe(CN)_6 \rightarrow Fe_4[Fe(CN)_6]_3 + 12KCl.$$

Perls stain is used to color cellular nonheme iron such as ferritin and hemosiderin but does not stain iron that is bound to porphyrin such as hemoglobin and myoglobin [12]. A combined Perls-hematoxylin-eosin stain was also proposed to easily check the presence of ferric iron in tissue sections [13]. Moreover, an immunohistochemical technique can be used to detect the presence of ferritin in tissue samples with results equivalent to Perls stain [14]. Therefore, Perls stain may be used to identify excess iron deposits caused by oxidative damage mechanism in COVID-19 patients.

The bronchoalveolar lavage (BAL) fluid is a good technique to explore alveolar macrophages, allowing for determining their percentage, size and shape, and their cytoplasm content. It also allows for making a cellular formula and searching for pathogens. Thus, LBA is considered as an important tool in the diagnosis of inflammatory, autoimmune, and infectious diseases.

In BAL fluids, hemosiderin-laden alveolar macrophages can be scored by the cytopathologist according to the hemosiderin content and the semi-quantitative method described by Golde (Golde score). Initially, the Golde score is established to assess alveolar hemorrhage in the event of capillary bed hyperpressure or alveolar wall injury; RBCs pass from the capillaries into the alveolar lumen, resulting in erythrophagocytosis. The Golde score requires a count of 100 macrophages and the establishment of a value from 0 to 4, depending on the iron density (in blue) in their cytoplasm (0 = no color in the cytoplasm, 1 = weak blue in a minor portion of cytoplasm, 2 = dark blue in a minor portion of cytoplasm or intermediate color throughout the cytoplasm, 3 = dark blue in most areas of cytoplasm, and 4 = dark blue throughout the macrophages). The result of this score depends on the sum of the number of macrophages X the value corresponding to the iron load (if counting 100 macrophages) to obtain a numerical score (Golde score: 0–20 normal, 20–70 intermediate resorption, >70 high resorption, and >100 occult alveolar hemorrhage) [15].

According to the hypothesis developed above, the alveolar macrophages collect free iron ions following heme attack by COVID-19 which separates iron from porphyrin. Consistent with this hypothesis, it can be assumed that the higher the Golde score, the more severe the hypoxia and oxidative damage.

In addition, cytological examination of BAL may help prove the alveolar damage. For example, some signs of pulmonary parenchyma aggression causing early alveolar damage such as hyaline membranes, inflammation, and desquamation of bronchiolar pneumocytes can be seen by cytological examination using standard staining protocols (Papanicolaou and May-Grünwald Giemsa stains). These pathological findings were recently described in COVID-19 patients' tissue and autopsy reports [16, 17]. BAL fluids may reveal possible viral cytopathogenic effect not obviously shown so far for COVID-19. Moreover, the BAL when clinically indicated allows looking for coinfection by the presence of second pathogen using special stains (Gram, Giemsa, Grocott-Gomori, periodic acid-Schiff, and Ziehl-Neelsen satins) [2], which could worsen the health status of COVID-19 patients, especially in higher infectious risk patients such as HIV and diabetes patients [18, 19].

It is evident that confirmation of the utility of Golde score using Perls stain or immunocytochemical technique to detect ferric iron as an indicator of pulmonary damage in COVID-19 patients requires validation by a series of cytological examination of BAL, while taking the necessary technical precautions as fresh BAL of COVID-19 patients is considered a high-risk infectious fluid for the laboratory team. If this hypothesis is confirmed in practice, the score may need to be adapted at a later date to assess the severity of COVID-19's damage. Obviously, careful examination of cytological specimens by using routine and special staining or ancillary technique can provide important diagnostic and prognostic information that may impact the management of COVID-19 patients.

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