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Changing in nasal cellularity in post-acute covid patients

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Abstract

Introduction

The sudden and storming onset of coronavirus 2 infection (severe acute respiratory syndrome coronavirus 2 [SARS-CoV-2]) was associated by severe acute respiratory syndrome. Corona virus disease 19 (COVID-19) has appeared as a pandemic throughout the world. The mutational nature of the virus, along with the different means of entering and spreading throughout the body has involved different organs. Thus, patients are faced with a wide range of symptoms and signs.
SARS-CoV-2 enters the human airway through aerosol or fomite transmission and comes into contact with the mucous membranes lining the oropharynx, nose and eyes. The nasal mucosa can be considered one of the first targets of SARS-CoV-2 and ciliated cells are primary targets for SARS-CoV-2 replication. Although the nose plays a crucial role in the transmission and multiplication of the virus and nasal symptoms such as anosmia/hyposmia may represent the first manifestation of SARS-CoV-2 infection, the changes in nasal mucosa induced by COVID-19 have not yet been well characterized.

Objective

The aim of this study was to evaluate cytological changings of the nasal mucosa caused by SARS-CoV-2, in patients who were still positive for the infection but who had passed the acute phase.

Material and Methods

The study involved the analysis of 26 nasal cytological samples taken from patients that had already overcome the acute phase of the disease but well still positive for COVID-19. Samples were evaluated after 21 days after sampling, to avoid an infective risk.

Discussion

The presence of both neutrophils and eosinophils underlines an inflammatory reaction in the nasal mucosa. In fact, neutrophils play a critical function in the clearance of bacteria with specific mechanisms to combat viruses. The role of eosinophilic inflammation in COVID-19 is still debated. Probably, these cells could directly affect the course of COVID-19 by being involved in killing bacteria and viruses and acting by producing antiviral molecules, but also by serving as antigen-presenting cells.

Conclusion

This study shows that SARS-CoV-2, not only causes cytopathic damage at the ciliated cells level, but also induces an important inflammatory response, responsible for symptoms commonly associated with COVID-19. Not only neutrophils but also eosinophils would appear to be involved in the inflammatory defense mechanisms activated to fight the infection.

Key words: Sars-Covid 19, Cytology, Ciliocytophthoria, nasal cell, eosinophils, neutrophils

All the Authors declare no conflict of interest in this work.

Authors may declare any patents related to the published work, either those pending or already obtained.

“Written informed consent has been obtained from the patients to publish this paper”

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Introduction

In December 2019 the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was identified in Wuhan, China, and quickly spread all over the world[1]. After causing an outbreak in China, coronavirus disease (COVID-19) has developed into a pandemic that rapidly spread worldwide, resulting in over 60 million cases and more 1.000.000 deaths within a few months.
Among the European countries, Italy was the first place to be hit by the COVID-19 disease[2]. COVID-19 mainly affects lower respiratory tract causing symptoms such as fever, cough, dyspnea, and chest tightness that could progress rapidly to acute respiratory distress syndrome[3]. However, the disease also causes upper respiratory tract symptoms including nasal congestion, sore throat, and olfactory dysfunction[4]. As a matter of fact, SARS-CoV-2 enters the human airway through aerosol or fomite transmission and comes into contact with the mucous membranes lining the oropharynx, nose and eyes[5]. Then the virus binds with angiotensin-converting enzyme 2 (ACE2) receptors, enters inside cell, multiply there and manifests itself. Thus, the nasal mucosa can be considered one of the first targets of SARS-CoV-2. This mucosa is characterized by a pseudostratified ciliated epithelium, with active ciliary movement, interspersed with mucus-secreting globet cells and covered with a thin layer of mucus, that traps and removes pathogens, including SARS-CoV-2, and particulate matter from inspired air. While this epithelium represents a front line in respiratory defense against lower airway infection, it can also be a target tissue for infection, multiplication, and propagation of pathogens. In particular, ciliated cells have been shown to be primary targets for SARS-CoV-2 replication, since ACE2 receptor is highly abundant in the apical surface membrane in fully differentiated ciliated cells of the nasal cavity[6]. During the first stage, the primary target cells are infected and consumed rapidly. The viral load experiences a substantial increase to the peak level. This agrees with the clinical data that onsets of ARDS are observed around this time. Following the viral peak, the viral load declines slightly and enters a plateau phase in which the viral load remains approximately unchanged or declines slowly. The source of this plateau phase is the infection of lymphocytes as a secondary target. Without the infection of lymphocytes, SARS-CoV-2 would continue to infect and deplete pneumocytes. Therefore, viral load would decline quickly, and the infected individual would lose the transmissibility. After viral load persists for a period of time (e.g. a week in some patients or month in other one), the next phase follows as the adaptive immune response emerges. Seroconversion was also detected about a week after acute respiratory disease (ARDS) onset in COVID-19 patients. During this stage, viral load declines rapidly to an undetectable

level.[30] Although the nose plays a crucial role in the transmission and multiplication of the virus and nasal symptoms such as anosmia/hyposmia may represent the first manifestation of SARS-CoV-2 infection, the changes in nasal mucosa induced by COVID-19 have not yet been well characterized[7]. Therefore, the aim of this study was to evaluate cytological changings of the nasal mucosa caused by SARS-CoV-2, in patients who were still positive for the infection but who had passed the acute phase[31]. Unlike viruses such as influenza, smallpox, and polio, coronaviruses have only recently been discovered to infect the human population. When they were first discovered in the 1960s, there was almost no epidemiological, genomic, or pathogenic information about these viruses – only that they contained RNA surrounded by a membrane composed of 'spike'-shaped proteins [39]. The crown-like appearance of these surface 'spike' proteins gave the virus family the name – 'corona' is Latin for the crown. Viruses with that specific shape and structure belong to the family of Coronaviridae, which are grouped into four genera using their phylogeny: alpha-CoV, beta-CoV, gamma-CoV, and delta-CoV [40]. As of 2020, the US-based Centers for Disease Control and Prevention (CDC) recognizes seven coronavirus strains that can infect humans. In general, they are classified as single-stranded, positive-sense RNA genome-bearing viruses. Their genome is estimated to be around 26–32 kilobases (for comparison, the human genome is 3 000 000 kilobases). The first identified coronaviruses in the human population were human CoV-229E (HCoV-229E) and HCoV-OC43. These viruses were found to cause upper respiratory tract diseases, such as the common cold, and infections caused by the viruses have low levels of severity. After the emergence of the first two coronavirus strains, two other strains were identified: HCoV-HKU1 and HCoV-NL63. Three other coronavirus strains that have been identified in the human population since are SARS-CoV, MERS-CoV, and SARS-CoV-2. All three of these coronavirus strains vary from the four common strains as they can cause severe illnesses that may result in death.[41]

According to the World Health Organization, COVID-19 has been confirmed in more than 113 million cases across 223 countries, leading to more than 4.1 million deaths. Recent estimates indicate that up to 98% of individuals diagnosed with COVID-19 developed forms of chemosensory disorders, most prominently smell loss. Data collected before the COVID-19 pandemic showed that up to 49% of the population report an episode of olfactory loss over their lifetime, with 5% of them reporting complete smell loss (anosmia). Population-based epidemiological studies before COVID-19 provide prevalence estimates of smell loss ranging from 2.7 to 24 and taste disorders ranging from 0.6 to 20%. Moreover, in older adults the prevalence of olfactory impairment increases. Reports to date reveal that the COVID-19 pandemic has already significantly

increased the prevalence of chemosensory disorders worldwide, especially among younger cohorts, yet the global estimates on chemosensory disorders may be markedly underestimated.

Materials and Methods

Our study involved the analysis of 26 cytological samples taken from patients admitted to a subacute COVID-19 Center for rehabilitation after an acute viral infection caused by SARS-CoV-2. All the patients had a Covid-Sars 19 infection that causes hospitalization and began oxygen therapy and steroids. Various ventilation methods were used during hospitalization, from orotracheal intubation to the use of a helmet for proper ventilation, to the use of a venturi mask with various oxygen levels. 20 patients had ventilation in intensive care and use of a helmet, and 6 patients had only high ventilation with venturi mask. The patients had all overcome the acute phase of the disease and were hospitalized to continue their rehabilitation pathway. Inclusion criteria were arranged as follows: genders: both; pathology: documented SARS-CoV-2 infection, assessed by at least one positive molecular test for SARS-CoV-2 and one positive nasopharyngeal (NP) swab, with the first positive test within the previous 8 to 21 days. Specific exclusion criteria were ongoing local or systemic corticosteroid treatments; non-authorization to perform the nasal cytological sampling. All recruited patients agreed to undergo nasal cytology and signed informed consent. No patients had an endoscopic exam of the nasal fossa due to the infection of coronavirus and the impossibility to do an exam in an isolated area. We have done only anterior rhinoscopy with Kilian to evaluate the nasal fossa and to do the nasal scarping. All the patients had an inflammation of the mucosa, only 5 patients had turbinate hypertrophy, and all the patients has consistent secretion inside the nose, due to the use of oxygen therapy.

Nasal cytology (NC) represents a useful and easy diagnostic tool to study rhinitis, because it allows to detect and measure the cell population within the nasal mucosa at a given instant, to better discriminate different pathological conditions and to evaluate the effects of various stimuli (allergens, infectious, irritants, physio-chemicals) or treatments. At the end of the 1800s, Gollash and Von Mihalkovics, firstly depicted the microscopic aspects of nasal mucosa, but this remained only an anatomical and morphological description. In 1927, Eyermann firstly identified eosinophils in the nasal secretion of patients suffering from hay fever. Although the pathogenesis of allergic reactions was still over the horizon, these authors clearly underlined the relationship between a specific cell population and a specific clinical disease. After decades of scarce interest, the study of nasal cytology had a rapid and progressive development during the 1970s, when the technique was

used to assess the effects of various drugs and stimuli [6–8]. The use of nasal scrapings was further developed, with no standardized techniques, during the last decades. The technique of NC was better systematized and investigated in depth starting from 2006. The NC approach subsequently provided relevant contributions to the knowledge of rhinitis from a pathophysiological point of view, allowing also to identify different phenotypes.

The nasal mucosa is a pseudo-stratified ciliated epithelium, containing also mucinous cells that are responsible for the continuous mucus secretion. The ciliated cell Fig. 1 is the most differentiated cell type in the nasal mucosa. Ciliated and mucinous cells both contribute to the mucociliary clearance that is part of the innate and first-line defense of airways. The normal ciliated/mucinous cell rate is around 4:1. In normal conditions (healthy individuals without nasal diseases), only four cytotypes can be identified at NC: ciliated cells, mucinous cells, basal cells/striated cells; only sparse neutrophils can be found occasionally. The perinuclear halo or hyperchromatic supranuclear stria in ciliated cells is a hallmark of normal function. On the contrary, the detection of eosinophils, mast cells, bacterial or fungal hyphae clearly identifies a pathological condition. NC is easy to perform, not invasive, cheap, and repeatable in the same subject also at short time intervals. For these reasons, it represents an affordable diagnostic technique that can be applied in all age ranges, also at the physician's office. In detail, the technique involves sampling, processing and microscope reading. Sampling requires the collection of cells from the surface of nasal mucosa. This can be made by a common sterile cotton tip or, better, with a sterile disposable curette (9Rhino-Probe, Arlington Scientific, Springville, UT, USA). Cotton tips can be used in infants when an anterior rhinoscopy may be considered more difficult to perform. Nonetheless, in our experience, due to the conformation of nostrils and accessibility, there is no special problem for the procedure even in very young children. It must be considered that this procedure does not require a biopsy (histological sample), but a simple surface cytological collection. Samples should be collected from the middle portion of the inferior turbinate where the rate ciliate/ mucinous cells are expected to be well balanced. The procedure can be easily performed under anterior rhinoscopy, with an appropriate light source. No application of anesthetic is required because the procedure is totally painless. Obviously, the operator should be well trained, to ensure a proper sampling. The presence of squamous cells usually indicates a contamination from the skin epithelium of nares, thus a not optimal sampling. When the curette is used, the sample is immediately smeared on a glass slide and air-dried. Then, the slide is stained with the common May–Grunwald–Giemsa (MGG) procedure. This staining method allows to easily identify all the cellular components (neutrophils, eosinophils, lymphocytes,

and mast cells), plus bacterial and fungal spore/hyphae. The traditional MGG staining procedure requires about 30 min, but pre-mixed compounds, the stained sample is read at optical microscopy, with a 1000X objective with oil immersion. At least 50 fields should be read, to obtain a mean value of the differential cellular count. The count of each cell type can be expressed as a percentage of the total cells (including mucinous and ciliated cells), as an absolute value, or by a semi-quantitative grading. It is obviously essential that the same count method is always used in reporting the results within clinical studies or routine activity. This aspect remains one of the major drawbacks of NC, because the reporting of cellular count varies from author to author and from a laboratory to another. Despite this limitation, the differential cell count and the microscopic appearance of nasal smears usually allow to discriminate different pathological aspects Nasal cytology samples were taken from the middle part of the inferior turbinate under anterior rhinoscopy, according to validated criteria, using a Nasal Scraping. The samples were then smeared on a glass slide and air-dried. Since SARS-CoV-2 has been shown to maintain a dangerous viral load on inanimate surfaces, such as glass, for up to 21 days, nasal cytology samples were stained with May-Grunwald-Giemsa only after 21 days[9]. Stained samples were then read at optical microscopy, with a 100x objective with oil immersion. A minimum of fifty fields is considered necessary to identify a sufficient number of cells[10]. The presence of any cytopathological alterations as well as inflammatory cells infiltrating the nasal mucosa was evaluated.[35,36,37,38]

Results

Twenty-six consecutive patients were recruited, including 15 males (58%). The patients mean age was 69 years (age range= 29-92 years). All patients were still positive for SARS-CoV-2 infection when underwent nasal cytology after written consent. Of the 26 patients, 18 (69%) were still receiving supplemental oxygen administered with nasal cannulas (flows varying from 2 l/min to 4 l/min), none with a venturi mask. 7 (27%) patients had a history of allergies: 5 (19%) had allergies to pollen and 2 (8%) to drugs (amoxicillin, ASA). The analysis of the cytological samples showed that all patients had a variable degree of neutrophilic infiltration and an abundant eosinophilic infiltrate. The presence of biofilm was found in 10 (38%) samples. Interestingly, rarefaction of the ciliary apparatus was found in all the cytological samples, since all the ciliated cells showed the disappearance of the hyperchromatic “Supranuclear Stria” (SNS), corresponding to the Golgi apparatus, which is considered a marker of wellness of ciliated cells[11]. Moreover, demonstrating cellular distress, all ciliated cells showed signs of “Ciliocytophthoria” (CCP), such as condensed nuclear chromatin,

marginalization of nucleoli, inclusion bodies, presence of intranuclear halo and cytoplasmic vacuoles[12].

Discussion

The central role played by the nose in SARS-CoV-2 infection has already been 8demonstrated, as viral entry-associated genes have been detected in nasal epithelial cells together with genes involved in innate immunity, highlighting the cells' potential role in initial viral infection, spread and clearance. As a matter of fact, SARS-CoV-2 entry receptor ACE2 and viral entry-associated protease TMPRSS2 are highly expressed in nasal goblet and ciliated cells. This finding implicates these cells as loci of original infection and possible reservoirs for dissemination within and between individuals. Moreover, the co-expression of these receptors in superficial conjunctival cells could explain the ocular symptoms observed in COVID-19 patients, in which the virus could potentially spread from the nose to the eyes through the nasolacrimal duct[13-29].

Given the central role of the nasal mucosa in the transmission and spread of SARS-CoV-2, research has focused on the possibility of using administered drugs/vaccines to limit the spread of the virus[14,15]. However, cytological changes in nasal mucosa of patients affected by COVID-19 have not been clearly defined. Before us, Gelardi and colleagues were the only ones to have performed Nasal Cytobrush Cytology to evaluate possible alterations of the nasal mucosa of 18 COVID-19 patients. This study showed that all patients had a mild lymphocytic infiltrate and a rarefaction of the SNS, while none of the patients had Ciliocytophthoria or other signs of cell injury[16]. Thus, our study is the first to describe the cytopathic effects of the virus on nasal cells. Indeed, as shown in the results, all patients examined showed signs of cellular injuries, such as the rarefaction of SNS and Ciliocytophthoria. Interestingly, we also found a mixed neutrophilic-eosinophilic inflammatory infiltrate. The presence of both neutrophils and eosinophils underlines an inflammatory reaction in the nasal mucosa. In fact, polymorphonuclear neutrophil granulocytes are the immune system's first responders to threats by invading microorganisms, as they combat the intruders by phagocytosis and externalization of granules containing lytic and microbicidal factors[17]. Since neutrophils play a critical function in the clearance of bacteria with specific mechanisms to combat viruses, the role of neutrophils in SARS-CoV-2 infection has been widely investigated. In this context, a rising neutrophil count and a falling lymphocyte count during the severe phase of the infection have been correlated with a worse prognosis and treatments using neutrophil extracellular traps (NETs)-targeting approaches have been considered to decrease the damage caused by hyperinflammation[18]. In

support of this, in influenza pneumonia neutrophils have already been shown to induce NET generation and increased endothelial damage, causing acute lung injury and alveolar-capillary damage[19]. Therefore, the neutrophilic nasal and subsequently pulmonary infiltrate in COVID-19 could be related to the severity of the disease and the associated symptoms. While the finding of neutrophils in cytological samples can be explained by the role intrinsically played by neutrophils, the reasons for the presence of eosinophils in the nasal inflammatory infiltrates appear less clear, as they are typically involved in parasitic infections, allergies and in several Type-2 diseases including asthma, chronic rhinosinusitis with nasal polyps, eosinophilic gastrointestinal disorders, and hyper eosinophilic syndromes[20]. The role of eosinophils in SARS-CoV-2 infection is still debated since several studies have shown that decreased eosinophil and lymphocyte count and increased neutrophil count are associated with a worse clinical outcome of COVID-19[21,22]. Nevertheless, other studies have shown that the lungs of COVID-19 patients can exhibit eosinophil-mediated inflammation, together with an elevated NKT cell response, which is associated with COVID-19 pneumonia[23]. Indeed, although eosinophil blood count could be reduced in COVID-19 patients, a more detailed analysis of eosinophils in primary care patients with COVID-19 has revealed increased eosinophilic expression of CD11, indicating baseline eosinophilic activation. These data suggest that the reactive eosinophils disappear from the blood during severe COVID-19 disease, leaving behind the resident eosinophils, but these cells show increased responsiveness and higher sensitivity or priming for (ex vivo) activation. [33] A hyper-responsive eosinophilic compartment could hint toward a more immune-regulatory function of these cells in primary care patients with COVID-19 disease. [32]Thus, eosinophils could directly affect the course of COVID-19 as they have also been described to be involved in killing bacteria and (respiratory) viruses and act by producing antiviral molecules, but also by serving as antigen-presenting cells [24,26,26]. The nasal mucosa has traditionally been considered a mere physical barrier between the host and its environment. Nowadays, it is becoming increasingly clear that nasal mucosa is metabolically active and plays a crucial role in maintaining the immunological barrier, producing various inhibitory substances and secretory IgA. While histological samples allow to directly detect any defects in the integrity of the nasal mucosal barrier, nasal cytology samples allow to indirectly detect such alterations. In particular, the Hyperchromatic Supranuclear Stria (SNS) is considered a specific cytological marker for the anatomic and functional integrity of ciliated cells and, therefore, for the mucosal barrier integrity. Indeed, since diseases affecting nasal mucosa epithelium determine its rearrangement, ciliated cells manifest distress phenomena including the disappearance of the hyperchromatic SNS. Therefore, the absence of the

SNS is considered a useful prognostic sign of nasal disorders. Furthermore, among the mechanisms responsible for epithelial toxicity during allergic inflammation, the predominantly eosinophilic inflammatory infiltrate has been shown to be responsible for barrier damage. As a matter of fact, eosinophils secrete several cationic proteins, including major basic protein (MBP), which induces a marked decrease in the number of desmosomes and exfoliation of epithelial cells. The noteworthy role that eosinophils play in epithelial barrier damage has been clearly described in asthmatic patients, in which a strong correlation has been found between the eosinophilic infiltrate, MBP and the presence of "Creola bodies," defined as clusters of apoptotic epithelial cells resulting from MPB-induced exfoliation. In AR, the increased permeability of the epithelium, due to damage of the cell junctions, as well as the exfoliation of the epithelial cells allows allergens and other noxious substances to penetrate the barrier and cause the main symptoms of Sino-nasal disease.

Conclusion

This study shows that SARS-CoV-2, which is a particular tropism for hair cells, not only causes cytopathic damage at the ciliated cells level but also induces an important inflammatory response, responsible for symptoms commonly associated with COVID-19, such as rhinorrhea and nasal obstruction. In this context, not only neutrophils but also eosinophils would appear to be involved in the inflammatory defence mechanisms activated to fight the infection. In particular, SARS-CoV-2 infection may drive specific innate immune responses, including eosinophil-mediated inflammation, and subsequent pulmonary pathogenesis via enhanced Th2-biased immune responses, which might be crucial drivers of critical disease in COVID-19 patients[26]. Further studies are needed to better define the characteristics of the cellular damage induced by the virus as well as to understand the inflammatory pathways activated by SARS-CoV-2 infection, with the aim of identifying new preventive and therapeutic strategies.

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Figure 1. Ciliar Cell with SIS. Magnification 100x objective with oil immersion. Stained with May Grundwald Giemsa

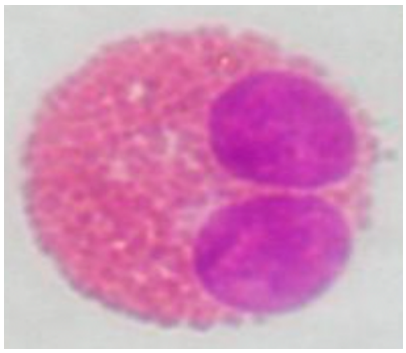

Figure 2. Eosinophilic cell. Magnification 100X objective with oil immersion Stained with May Grunwald Giemsa

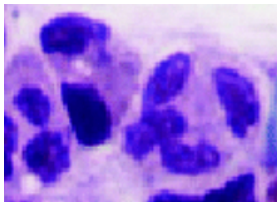

Figure 3 Neutrophil cell. Magnification 100X objective with oil immersion Stained with May Grundwald Giemsa

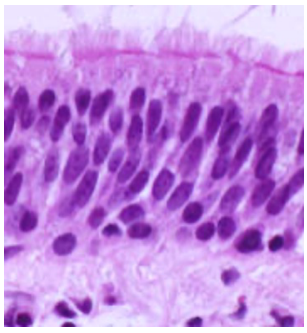

Figure 4 Pseudostratified epithelium ciliated cell objective with oil immersion Stained with May Grundwald Giemsa. Magnification 100x with optical microscope