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Research Article

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Development and Validation of Antibodies Against Sars Cov-2 by Immunohistochemistry to Assess the Pathogenesis of COVID-19 and Its Role in Cytokine Storm Syndrome and Coagulopathy

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ABSTRACT

Introduction: Understand how the SARS-Cov-2 virus uses the ACE2, TMPRSS2, and CD147 receptors to infect human cells. Research and better understand "Cytokine Storm Syndrome" and coagulopathy induced by COVID-19 infection.

Objectives: To determine the expression, by means of immunohistochemistry (IHC) in FFPE tissues to evaluate the general pathological events caused by the virus.

Materials and Methods: Antibodies from Bio SB Inc were used to analyze post-mortem material from SARS-CoV-2 positive patients by immunohistochemistry.

Results: The findings confirmed the presence of viral particles, Cytokine Storm Syndrome inflammation effects, and indicators of coagulopathy.

Conclusion: We consider that this study has enough material to provide clinicians and researchers with a detailed picture of the pathogenesis of SARS-CoV-2.

Keywords: IHC, Sars-CoV-2, Cytokine Storm, Coagulopathy

Introduction

The first reports of a virus causing respiratory infection, highly contagious in humans, were in December 2019 in Wuhan, China. On February 11, 2020, it was taxonomically designated "Severe Acute Respiratory Syndrome Coronavirus 2" (SARS-CoV-2). Structurally, SARS-CoV-2 has four major structural proteins, including the spike glycoprotein (S), the small envelope glycoprotein (E), the membrane glycoprotein (M), and the nucleocapsid protein (N), and also several accessory proteins. The SARS viral spike protein uses the angiotensin II converting enzyme receptor (ACE2) to enter epithelial cells (Figure 1).

In 80% of cases, patients with SARS-CoV-2 generate a typical immune response and favorable prognosis. In a normal immune response, immune cells (lymphocytes) release chemical messengers in an intricate sequence, causing a localized mild inflammatory state. In less-favorable cases, patient condition deteriorates suddenly in the later stages of the disease or in the recovery process. Acute Respiratory Distress Syndrome (ARDS) and multi-organ failure occur rapidly, causing loss of vital functions and life. Cytokine Storm Syndrome (CSS) is considered one of the main causes of ARDS and multiple organ failure.

The first cytokines released are interleukin 1β (IL-1β) and tumor necrosis factor-α (TNFα), which attract a variety of circulating white blood cells (neutrophils, monocytes, macrophages and killer T cells (NK)) to the site of infection. These cells directly attack the invading pathogen and release additional cytokines, among which interleukin 6 (IL-6) stands out for invoking the adaptive immune response (T, B and T helper cells) at the site of infection. IL-6 also increases recruitment, proliferation, and activation of macrophages. (Figure 2).

Goal

Identify expression of SARS-CoV-2 proteins, receptors, and related immune response signals in FFPE tissues, using

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immunohistochemistry (IHC) to assess general pathological events caused by the virus. Investigate and better understand the signaling pathways of coagulation and the "Cytokine Storm Syndrome (CSS)" induced by COVID-19 infection, including the human immune cells and factors such as IL1a, IL1b, IL6, IFN-α, IFN-γ, TNFα, and Tissue Factor/CD142. The intention was to assess the pathological damage caused by COVID-19 and the downstream effects of the signaling cascades, using single and multiplex IHC in FFPE SARS-CoV-2 positive biopsies.

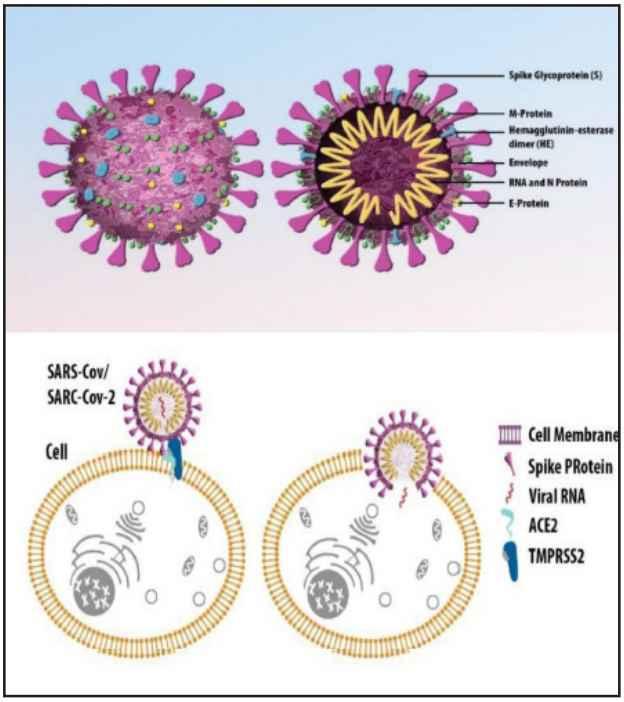

Figure 1: A- Structure of SARS-CoV-2 Virus, B- SARS-CoV-2 Receptors.

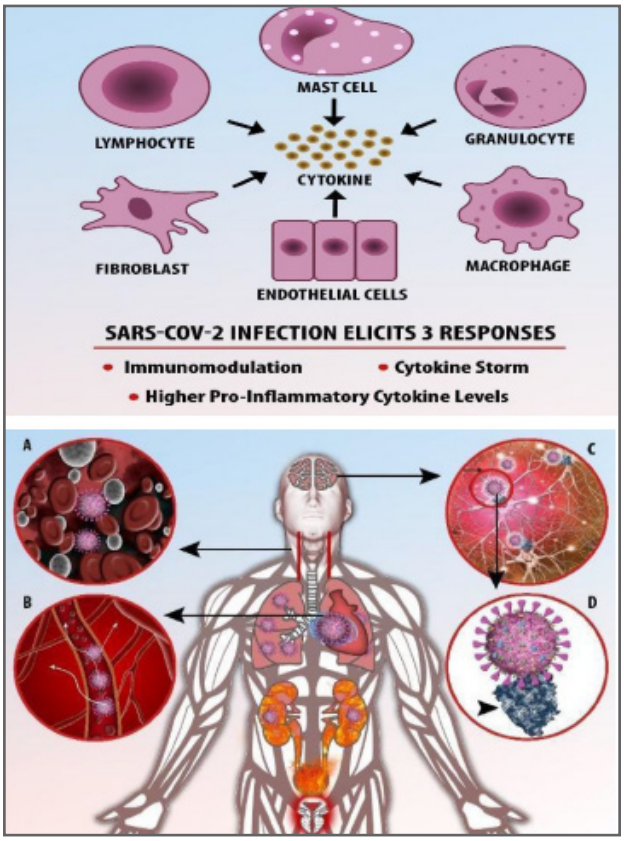

Figure 2: A- Cytokine Storm Syndrome, B- ACE2 Tissue Distribution

Materials and Methods

12 lungs, 9 bone marrows, 17 hearts, 4 brains, 2 placentas, and 1 liver from post-mortem material were analyzed and correlated with the clinical data from COVID-19 patients during the May-August period of 2020. 2 acetone-fixed nasal swabs were also analyzed from a live SARS-CoV-2 patient. The patients included 21 males and 6 females with a median age of 60 years, and 18 of unknown age/gender, and were treated at institutions in Mexico City. Monoclonal antibodies from Bio SB Inc were used to identify the SARS-CoV-2 nucleocapsid protein, the viral receptors ACE2 and CD147 and protease TMPRSS2, and signaling molecules by immunohistochemistry in FFPE biopsies. Antibodies from Bio SB Inc were also used to identify interleukins and other immune signaling molecules, clotting factors, and markers of various immune cells (B and T lymphocytes, NK cells, monocytes, macrophages).

Antibodies for IHC

Table 1: IHC Antibodies for SARS-CoV-2 and Receptors

Antibody	Clone
SARS-CoV-2	BSB-134
ACE2	BSB-135
CD147	BSB-137
TMPRSS2	BSB-136

Table 2: IHC Antibodies for Immune Cells

Antibody	Clone
CD4	RBT-CD4
CD8	C8/144B
CD56	123C3.D5
CD68	KP-1
CD163	10D6

Table 3: IHC Antibodies for Vascular & Platelets

Antibody	Clone
VEGF	RBT-VEGF
CD142/ TF / Coagulation Factor III	BSB-142
Complement Factor H	BSB-164
CD61	2f2

Table 4: IHC Antibodies for Cytokines

Antibody	Clone
IL-1a	BSB-138
IL-1b	BSB-139
IL-6	BSB-140
TNFa-IP2	BSB-141
IFN-Alpha	BSB-158
IFN-Gamma	BSB-161

The tissues were retrieved in a pressure cooker for 15 minutes in ImmunoRetriever Citrate solution, assayed with the antibodies listed in Table 1 and the PolyDetector Plus Detection System and protocol. The slides were scored on a scale of 0-4 points of IHC signal intensity, and analyzed to determine the presence of cells and signaling biomarkers related to the immune response against SARS-COV-2 and the resulting pathology.

FFPE Tissue Biopsies

- 12 lungs
- 9 bone marrows
- 17 hearts
- 2 placentas
- 4 brains
- 1 liver
- 2 nasal swabs fixed with 100% acetone for 10 min

Negative controls- normal and cancerous Tissue Microarrays (TMAs) from Bio SB Inc, containing 2-mm diameter FFPE tissue cores from various organs (pre-COVID-19):

- 23-core normal TMA
- 23-core cancer TMA
- 11-core bone marrow TMA

Results

Histopathological findings in post-mortem lung tissue from IHC analysis of COVID-19 induced pneumonia showed diffuse alveolar damage (DAD) in all cases. Patients with a shorter illness duration (≤11 days from symptom onset to death) tended towards acute phase DAD, with viral antigen located in the cytoplasm of alveolar macrophages. The SARS-CoV-2 proteins were detectable by IHC exclusively in the acute phase, but not in the organizing phase of DAD, suggesting that the virus may play an important role in the early acute lung injury, but by the time the DAD progressed to the organizing phase, the patient's immune response may have cleared the virus from the lung. The main vascular findings included thrombi in medium and large vessels, and the presence of platelet and fibrin microthrombi. Frequent thrombi and microthrombi may also present potential targets for therapeutic intervention. (Figure 3).

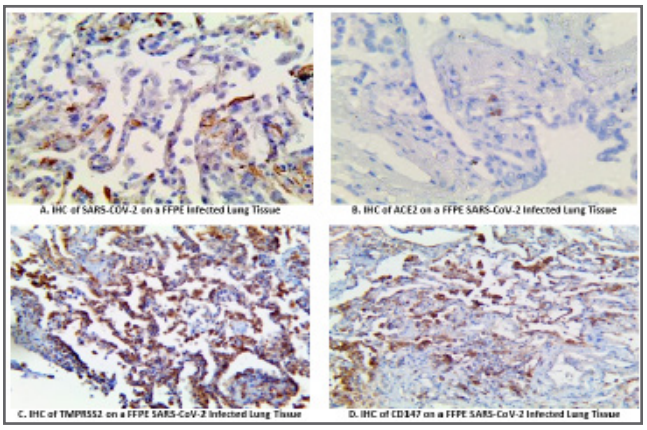

Figure 3: IHC of SARS-CoV-2 and receptors in lung tissues: SARS-CoV-2 (A), ACE2 (B), TMPRSS2 (C), CD147 (D).

The numbers of CD4+ and CD8+ T cells in the lungs were large relative to normal tissue, evidence of inflammation that becomes harmful or fatal in severe cases. T cells express TNF-α and IFN-γ in response to viral infections, and initiate their own inflammatory cascades. The presence of CD68 in the infected lung may indicate macrophages and dendritic cells, and large numbers of CD163+ macrophages were seen, which contribute to respiratory distress and the "ground-glass opacity" symptom seen in the lungs. Inflammatory macrophages secrete cytokines such as TNFα, while CD163+ macrophages could be a regulating response to the inflammation. The increased presence of these immune cells contributes to lung weight gain, and cytokine-producing cells contribute to the cycle of extreme inflammation. (Figure 4).

Tissue Factor/CD142, part of the coagulation signaling pathways, is expressed on the surface of activated macrophages, monocytes, and platelets, and was found on the alveolar surface of thickened SARS-CoV-2 positive lung tissue, suggesting an environment of increasing coagulation. This vascular damage leads to complex formation with the Zymogen Factor VII and Activating Factor IX or X to initiate a coagulation cascade involving fibrin and platelets at the site of injury. (Figure 5).

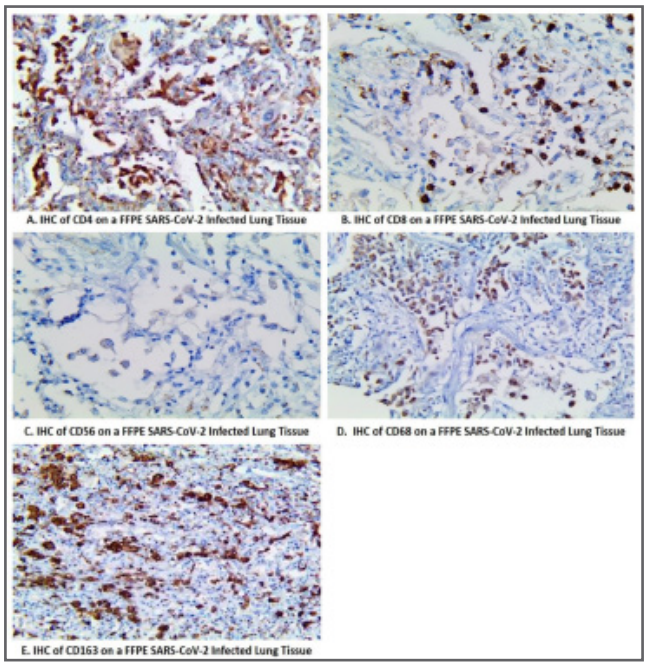

Figure 4: IHC of SARS-CoV-2 positive lung tissue with antibodies against immune cell markers (T cells, NK cells, and macrophages): CD4 (A), CD8 (B), CD56 (C), CD68 (D), CD163 (E).

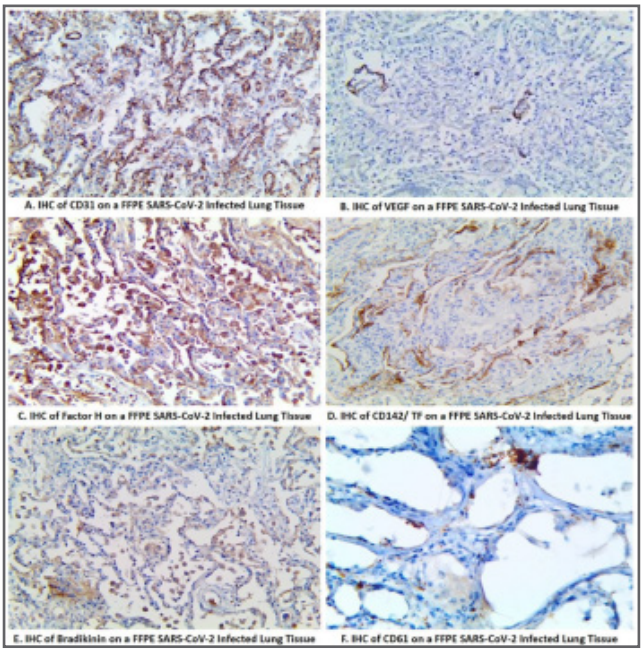

Figure 5: IHC of SARS-CoV-2 positive lung tissue with antibodies against vascular markers: CD31 (A), VEGF (B), Factor H (C), CD142/TF (D), Bradykinin (E), CD61 (F).

IL-1b is secreted after activation of pattern recognition receptors on cells that detect damage or pathogen-induced signals and works with IL-1a to stimulate fibroblast proliferation and collagen production. IL-1 recruits CD4+ cells at sites of infection and may participate in inflammatory networks with colony-stimulating factors that recruit macrophages and granulocytes. The presence of TNFα may indicate the progression of

inflammatory pathways that induce cell proliferation and migration. (Figure 6).

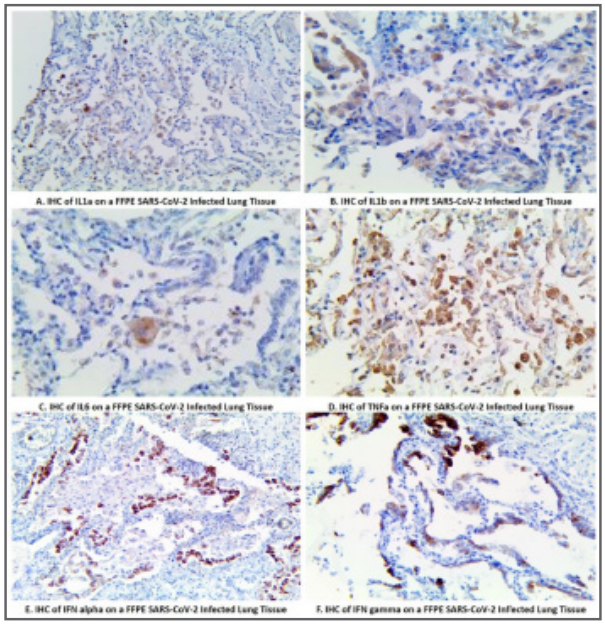

Figure 6: IHC of SARS-CoV-2 positive lung tissue with antibodies against various cytokines: IL-1a (A), IL-1b (B), IL-6 (C), TNFα (D), IFN-α (E), IFN-γ (F).

SARS-CoV-2 antigens were detected in glial cells from the infected brain, present in the cytoplasm as expected for an RNA virus. Glia and microglia are the immune or neuroinflammatory cells in the brain, which produce cytokines and chemokines to induce immune reactions in the brain. These microglial cells expressed proinflammatory cytokines similar to those expressed in the lung: weak IHC signals of IL-1b and IL-6, and stronger signals of TNFα which can lead to neurotoxicity and chronic inflammation. (Figure 7).

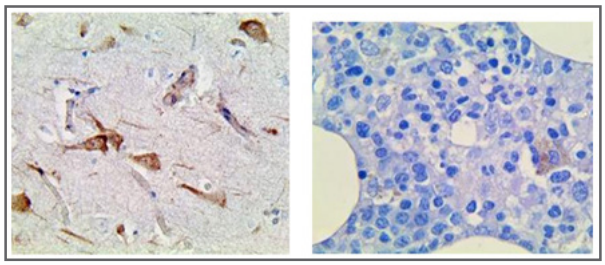

Figure 7: IHC of SARS-CoV-2 infected FFPE brain (A) and bone marrow (B) tissues analyzed with SARS-CoV-2.

In dual IHC of SARS-CoV-2 and CD163 in lung and bone marrow samples, we were able to identify SARS-CoV-2 in macrophages and Type II Alveolar cells in lung tissue, as well as megakaryocytes in bone marrow. In both cases, there were large numbers of macrophages present, some of which were infected with the virus. (Figure 8).

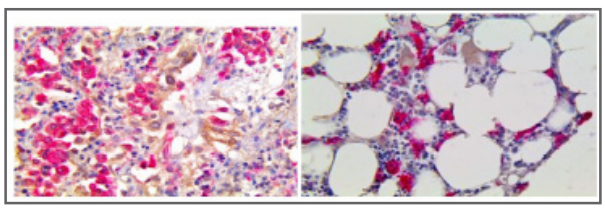

Figure 8: Dual IHC of SARS-COV-2 (DAB) and CD163 (Alk Scarlet) in SARS-COV-2 infected FFPE lung (A) and bone marrow (B) tissues.

In the liver tissue, the presence of SARS-CoV-2 viral particles was associated with mild pathological changes, including mild focal chronic inflammation of the portal tract and mild focal lobular activity. In the heart, we observed SARS-CoV-2 infection in the cytoplasm of myocardial cells. (Figure 9).

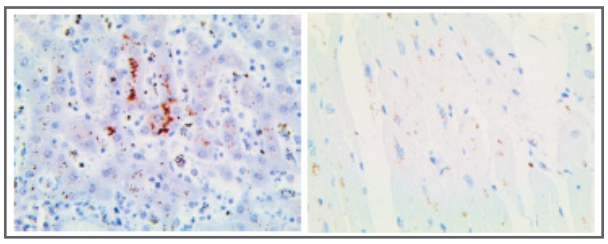

Figure 9: IHC of FFPE liver (A) and heart (B) tissues tested with SARS-CoV-2.

The placenta tissues show outstanding syncytiotrophoblast positivity by SARS-CoV-2 IHC, adding to the published evidence for transplacental viral transmission. (Figure 10)

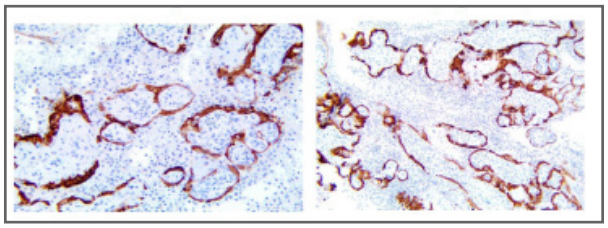

Figure 10: IHC of FFPE placental tissues tested with SARS-CoV-2.

We also observed punctiform signals on the nasal swabs fixed in 100% Acetone and assayed for SARS-CoV-2 nucleocapsid. (Figure 11).

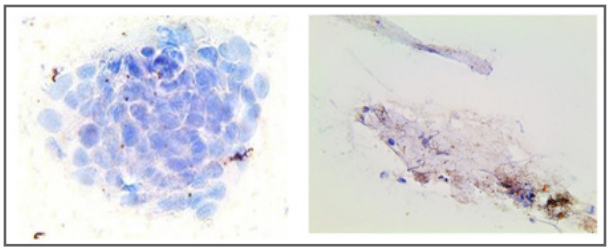

Figure 11: ICC of FFPE nasal scrapings and swabs tested with SARS-CoV-2.

Discussion

Histologically, biopsies from patients with COVID-19 showed diffuse alveolar damage corresponding to the phase of the disease (acute to fibrotic), divided into 3 main injury patterns: epithelial, vascular, and fibrotic. We found evidence that the severity of COVID-19 contributing to severe pneumonia, ARDS, and other end-organ damage, is attributed to a severe inflammatory response. This severe response or Cytokine Storm Syndrome is triggered by dysregulated expression of cytokines, in particular ILb, IL6, and TNFα. High levels of IL6 and TNFα in the bloodstream have been reported to predict worse outcomes from COVID-19. These innovations suggest a promising future in which cytokine biomarkers may aid in clinical decision-making, but may also be a target for pharmaceutical and interventional therapies. SARS-CoV-2 is a respiratory virus that primarily targets lung tissue, however experts now agree that the virus attacks multiple critical organs and multiple cell types in the cardiovascular system. Studies related to inflammation in COVID-19 patients have strong links to CSS, Macrophage Activation Syndrome, and subsequent immune overreaction or depletion.

Most of the cases presented hypercellularity of the Bone Marrow, but all cases presented an increase in the number of morphological abnormalities in megakaryocytes, varying between small, hypolobulated and polysegmented or atypical nuclei. An increase in histiocytes with hemophagocytosis and CD8+ T lymphocytes was also observed. The bone marrow cases were positive for ACE2 (9/9), CD147 (9/9) and TMPRSS2 (6/9), as well as CD8, CD68, and CD163. We found no positivity for CD4 in bone marrow samples. Interestingly, the TMPRSS2 serine protease was always absent in bone marrow of SARS-CoV-2 negative cases, suggesting a possible relevant role of this protease in the integration of the virus into the cell. In addition, a relevant hemophagocytic histiocytosis and CD8+ T-cell bone marrow response were observed in critical cases of COVID-19. SARS-CoV-2 infection can also lead to cardiovascular manifestations in COVID-19 patients, mainly due to the interaction between the viral spike protein and ACE2, which triggers virus entry into host cells. Many patients infected with COVID-19 had increased fibrinogen, fibrin degradation products, D-dimer, and von Willebrand factor, and these elevations appear to correlate with disease severity and thrombotic risk. Previous reports show a substantial burden of myocardial injury in patients who were critically ill or who died from COVID-19. This pattern of cardiac injury could be due to endothelial dysfunction and coronary microvascular thrombosis in these patients, rather than to macrovascular thrombosis. In an analysis of hearts from COVID-19 patients who died early in the pandemic, myocyte necrosis was observed in approximately 35% of patients. Most patients with myocardial injury had these small areas of infarction and microthrombi in small vessels. Our investigation found no evidence of large infarcts. We detected SARS-CoV-2 in cortical neurons and observed pathological features associated with infection with minimal immune cell infiltrates. These results provide evidence of the neuroinvasive capacity and unexpected consequence of the direct infection of neurons by SARS-CoV-2, and may explain the mental fog developed by patients who survive COVID-19 infection. Liver damage in patients with COVID-19 infections could be directly caused by viral infection of liver cells. Significantly elevated bilirubin levels- three times the upper limit- have been observed in patients with COVID-19. Other liver function markers are known to be elevated in COVID-19 patients, including Aspartate Transaminase (AST) and Alanine Aminotransferase (ALT), with markers such as albumin decreased. The presence of ACE2 receptors in the liver together with the local effects of systemic inflammation and possible iatrogenic toxicity seem to be the main mechanisms involved in the appearance of liver damage in patients with COVID-19. In particular, during the progression of COVID-19, the liver could be involved either as a direct target of SARS-CoV-2 (for example, hepatocyte apoptosis or caspase- dependent pathways) or affected by complex pathways of systemic alterations promoted by viral infection, mainly including inflammation and release of cytokines (including IL- 1, IL-6, IL-10), immune response, impaired coagulation, hepatic ischemia and hypoxia, and sepsis-related abnormalities. Placentas from infected patients showed prominent syncytiotrophoblast positivity by SARS-CoV-2 IHC, meeting published criteria for transplacental viral transmission as confirmed in fetal cells by identification of viral antigens by IHC. The concurrence of chronic histiocytic intervilli and trophoblastic necrosis appears to be a risk factor for SARS-CoV- 2 placental infection, as well as maternal-fetal viral transmission and suggests a potential mechanism by which the coronavirus may disrupt the maternal-fetal interface [1-16].

Conclusions

Histologically, COVID-19 showed diffuse organ damage, according to the phase of the disease (acute to fibrotic), divided into 3 main injury patterns: epithelial, vascular, and fibrotic. According to estimates, cytokine storms occur in up to 5% of severe cases of COVID-19, with high levels of various inflammatory cytokines, including IL-1β, IL-6, and TNFα. Detection of multiple cytokines can provide clinicians and researchers with a detailed picture of SARS-CoV-2 pathogenesis, facilitating the development of a personalized approach to treatment. Autopsies provide critical information to correlate with clinical, pathophysiological, and epidemiological data; In this sense, it is the way to achieve a true diagnosis and effective treatment.

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