Int. J. Morphol.,
41(6):1687-1697, 2023.

Development, Histological, and Ultrastructural Evaluation of Sheep Hyperimmune Serum Therapy for COVID-19

Desarrollo, Evaluación Histológica y Ultraestructural de la Terapia con Suero Hiperinmune de Oveja para COVID-19

Elif Kervancioglu Demirci[1]; Erva Sevic Yilmaz[1] & Engin Alp Onen[2]

KERVANCIOGLU DEMIRCI, E.; YILMAZ, E. S. & ONEN, E. A. Development, histological, and ultrastructural evaluation of sheep hyperimmune serum therapy for COVID-19. *Int. J. Morphol., 41(6)*:1687-1697, 2023.

SUMMARY: In response to the threat posed by new variants of SARS-CoV-2 and the urgent need for effective treatments in the absence of vaccines, the aim of this study was to develop a rapid and cost-effective hyperimmune serum (HS) derived from sheep and assess its efficacy. The utilization of a halal-certified, easily maintained in certain geographic regions, easy-to-handle animal such as sheep could provide a viable alternative to the expensive option of horses. Sheep were immunized with a whole inactivated SARS-CoV-2 antigen to produce HS, which was evaluated for neutralizing potency using the PRNT50 assay. K18-hACE2 transgenic mice (n=35) were divided into three groups: control, SARS-CoV-2 exposure through inhalation, and SARS-CoV-2 exposed mice treated with HS. HS efficacy was assessed through serum proinflammatory cytokine levels, qRT-PCR analysis, histopathological examination of lungs and hearts, and transmission electron microscopy. Purified HS exhibited significant neutralizing activity (1/24,576). The SARS-CoV-2+HS group showed lower levels of TNF-α, IL-10, and IL-6 ($P<0.01$) and relatively lower levels of MCP-1 compared to the SARS-CoV-2 group. HS prevented death, reduced viral RNA levels in the lungs and hearts, protected against severe interstitial pneumonia, preserved lung tissue integrity, and prevented myocyte damage, while the SARS-CoV-2 group exhibited viral presence in the lungs. This study successfully developed a sheep-derived HS against the entire SARS-CoV-2 virus, resulting in a significant reduction in infection severity, inflammation, and systemic cytokine production. The findings hold promise for treating severe COVID-19 cases, including emerging viral variants, and immunocompromised patients.

KEY WORDS: Antisera; Horse; SARS-CoV-2; Ovine.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel virus responsible for severe viral pneumonia and systemic symptoms since 2019, has prompted the urgent need for effective COVID-19 treatments. While antiviral drugs, vaccines and monoclonal antibodies have been extensively studied, their production on a large scale remains challenging and expensive (Haghighi *et al*., 2022). An alternative approach utilizing hyperimmune serum derived from horses, rich in neutralizing antibodies, has shown promise as a potential therapeutic intervention (Cunha *et al*., 2021; Leon *et al*., 2021; Li *et al*., 2023). A hyperimmune serum formula can be 150 times more potent than convalescent plasma (da Costa *et al*., 2021). On the other hand, with the emergence of new variants posing a potential threat, it is crucial to develop easy and cost-effective treatments.

This study aims to develop and evaluate the histological and ultrastructural effects of a sheep-derived hyperimmune serum against SARS-CoV-2. The SARS-CoV-2 virus is characterized by a single-stranded RNA genome and a spike protein (S) essential for host cell entry via interaction with angiotensin-converting enzyme-2 (ACE2) receptors. Transgenic K18-hACE2 mice, which represent a suitable animal model for the study of SARS-CoV-2, were employed to assess the effects of SARS-CoV-2 and the hyperimmune serum (Oladunni *et al*., 2020).

Sheep hyperimmune serum offers advantages due to its readily and fast production in large quantities (Findlay-Wilson *et al*., 2022). Although previous studies have demonstrated the efficacy of horse-derived hyperimmune

[1] Histology and Embryology Department, Istanbul Faculty of Medicine, Istanbul University, Istanbul, Turkey.
[2] Vaccine and Biotechnology R&D, Kocak Pharmaceuticals, Tekirdag, Turkey.

Received: 2023-07-16 Accepted: 2023-08-12

serum formulations with high neutralizing antibody levels against SARS-CoV-2, the cost and maintenance of horses pose limitations (Ainsworth *et al*., 2020). Sheep, with their cost-effective and easier maintenance, offer a preferable alternative for various geographic and economic conditions (Dowall *et al*., 2016). However, the development and evaluation of sheep-derived hyperimmune serum, particularly in terms of its histological and ultrastructural effects, remain understudied (Jacobson *et al*., 2023).

This study aims to address this knowledge gap by developing and evaluating a sheep hyperimmune serum formula against SARS-CoV-2. Histopathological assessments will focus on examining morphological changes and viral titers in target organs, including the respiratory system and heart. The findings of this research can contribute to the development of innovative and cost-effective treatment strategies, particularly in situations where widespread availability of other therapeutics may be limited.

MATERIAL AND METHOD

Inactivated SARS-CoV-2 antigen preparation. Virus seed lots were derived from a SARS-CoV-2 strain obtained from a patient during the COVID-19 outbreaks (Vial no.31242/12.05.2020). The Turkish Ministry of Health provided these seed lots. The genome sequences of SARS-CoV-2 passages (ON653597, ON653599, and ON653598) were identified and published in the NCBI database. The virus was cultured in VERO CCL-81 cell line, inactivated with b-propiolactone, purified using multimodal chromatography, and adsorbed with ALUM adjuvant (Alp Onen *et al*., 2022). The hyperimmune serum antigen formulation was adjusted to 2 mg of inactivated SARS-CoV-2 virus per dose (Fig. 1).

Ethical statement. This study was conducted in strict accordance with the recommendations of the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. The research protocol with animal experimentation was approved by the KODEHAL Animal Care and Use Committee (Protocol Number: KOHADYEK 2021-6 for sheep, 2022-5 for mice). All surgery was performed under ketamine xylazine anesthesia and every effort was made to minimize suffering.

Animal immunization for sheep hyperimmune serum production. Animals underwent standard prophylactic vaccination and deworming programs. Additionally, they were screened for general health conditions (hematologic parameters and blood biochemistry), in compliance with Turkish Ministry of Agriculture regulations.

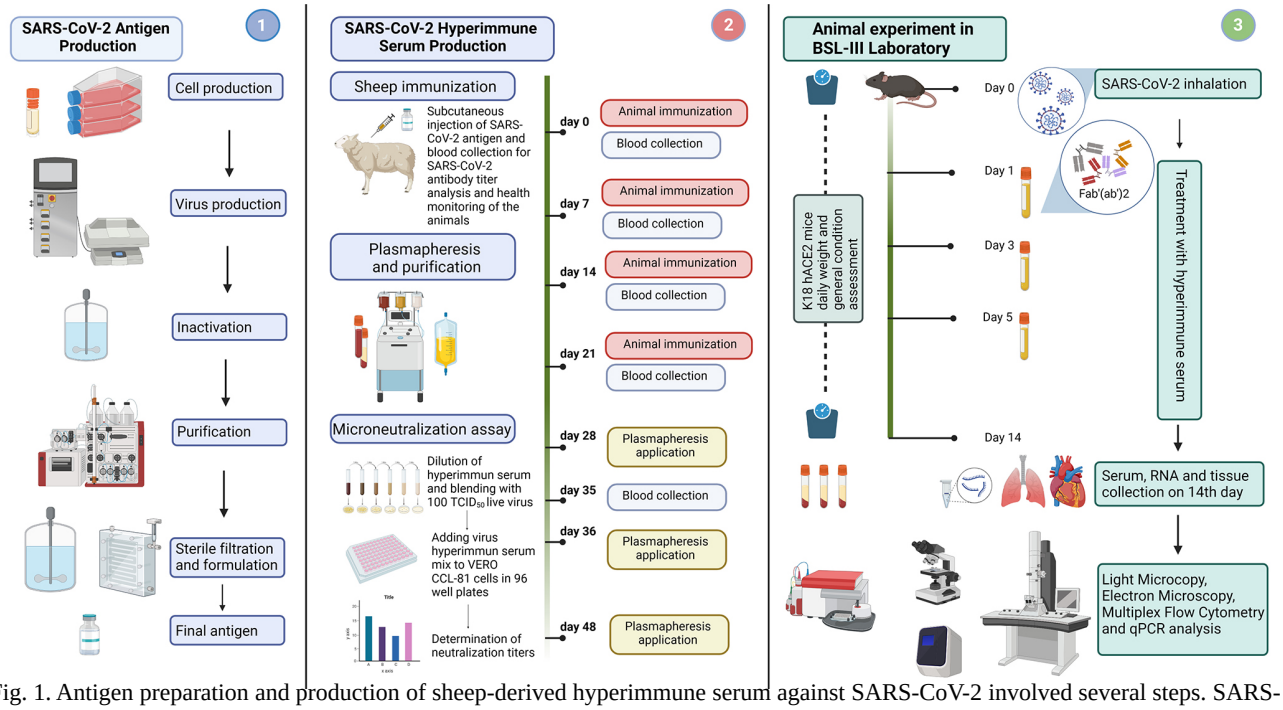

Fig. 1. Antigen preparation and production of sheep-derived hyperimmune serum against SARS-CoV-2 involved several steps. SARS-CoV-2 obtained from a patient was used for virus production, which was followed by inactivation, purification, sterile filtration, and formulation of the final antigen. This antigen was then used to immunize sheep through repetitive injections. Serum from the immunized sheep underwent plasmapheresis, followed by final filtration and assessment of microneutralization and titers. Standardized sheep hyperimmune serum was administered to SARS-CoV-2 infected mice at two-day intervals. After two weeks, tissue samples from these mice were collected and subjected to evaluation using light and electron microscopy, as well as qPCR analysis.

Two healthy sheep (2 males), aged 3-5 years and weighing approximately 40- 50 kg each, were selected to produce polyvalent hyperimmune serum. Prior to immunization, blood samples were collected from the jugular vein and stored at -20°C as negative controls for binding and neutralizing antibody assessments. Subcutaneous immunization was administered four times at seven-day intervals in different dorsal positions, with each immunization consisting of 2 mg of inactivated SARS-CoV-2 virus mixed with Alhydrogel adjuvant.

Daily monitoring included food and water intake, clinical evaluation, and behavioral assessment. Suitable sheep had blood samples collected for laboratory tests, including complete blood cell count, chemistry panel, and coagulation tests (prothrombin time and activated thromboplastin time). Samples were obtained from the jugular vein and sent to the Turkish Central Veterinary Research and Control Institute for analysis. Blood collection occurred every 7 days before subsequent inoculations and 14 days after the final inoculation (up to 48 days from the initial inoculation). Plasma was stored at -20°C for measuring binding and neutralizing antibody titers.

Sheep Plasma Collection. Serum samples obtained on days 0, 28, 36, and 48 after the initial antigen dose were used to assess serum neutralizing antibody levels. Plasmapheresis was performed on day 48 using the Haemonetics PCS2® system for therapeutic apheresis. Plasma separation occurred through continuous flow centrifugation based on cell-specific gravity, and aseptic transfer was made to a custom bag.

Production and Purification of Sheep F(ab')2 Fraction. Plasma from all animals was pooled and mixed with water, phenol, and enzymes. pH adjustment and enzymatic digestion removed the Fc portion. F(ab')2 fragments were separated, precipitated, and filtered. Ammonium sulfate precipitation and diafiltration were performed. Buffer exchange and addition of phenol solution were carried out. The final F(ab')2 product was sterile-filtered and stored at 4°C (Fig. 1).

Microneutralization Assay. To evaluate the neutralizing capability of sheep serum against SARS-CoV-2 in vitro, serum samples were collected on day 0 and weekly after three booster immunizations on days 28, 36, and 48 post-initial immunization. Heat-inactivated serum samples were diluted with Eagle's Modified Eagle Medium (EMEM). Each sample was then combined with diluted live virus and incubated at 37 °C. Vero CCL-81 cells in 96-well plates were inoculated with the serum-virus mixture and incubated at 37 °C. After washing and fixing, the cells were stained. The neutralization titers were determined by assessing the serum dilution that caused a 50 % reduction in infection. Reed & Muench method was used for titer calculation based on four replicate tests (Alp Onen *et al*., 2022).

Mice Experiments. Fourty-five 8-9-week-old adult K18-hACE2 male and female mice weighing approximately 25-30 g were divided into four groups based on their susceptibility to intratracheal instillation of SARS-CoV-2, as demonstrated in previous studies (Bi *et al*., 2021). The study comprised a control group of 10 mice, an infected group of 10 mice, a group treated with sheep hyperimmune serum only (10 mice), and a group infected with SARS-CoV-2 and treated with sheep hyperimmune serum (15 mice). On day 0, the infected groups were intranasally challenged with live viruses (5×10^4 live virus/50 µl), while the treatment groups received intraperitoneal injections of purified sheep hyperimmune serum (2500 PRNT50/0.2 ml). Additional doses of serum were administered at 3- and 5-days post-infection (dpi). Daily monitoring of weight changes and health status was conducted for 14 days. On day 14, necropsy was performed, and serum and tissue samples were collected for biochemical and histological analyses.

Multiplex Flow Cytometry. The Multiplex Inflammation Panel Flow Cytometry Assay (MMX774, Antigenix America) was employed to assess serum levels of IL-1B, IL-6, IL-10, TNF-α, and MCP-1 as indicators of inflammation. This assay employs beads of varying sizes and fluorescence intensities, facilitating the identification of distinct bead populations in flow cytometer data. It enables the simultaneous measurement of up to 24 analytes within a single reaction well. The Super-X Plex Cytokine Assays can be utilized with any flow cytometer (Accuri C3, BD) featuring a 488 nm laser to determine analyte concentrations based on R-Phycoerythrin emission, using a standard curve for comparison.

Q-PCR. Quantitative Real-time RT-PCR was conducted to measure viral load in lung and heart tissues. Lung and heart samples were collected from each mouse group and homogenized using TissueLyser II equipment (Qiagen, Germany). After centrifugation, viral nucleic acids were extracted using the Viral Nucleic Acid Kit isolation kit (ROCHE, Germany). A commercial one-step RT-PCR Master Mix (Primer design) was used for the polymerase chain reaction. Amplification was performed in 96-well plates on a LightCycler 480 Real-Time PCR instrument (ROCHE, Germany). The real-time PCR conditions included reverse transcription at 55 °C, Taq polymerase activation at 95 °C, and 40 cycles of amplification. Viral load was determined using a standard curve (Dhama *et al*., 2020).

Light microscopy. After serum collection, the thorax was opened through the sternum, and the lungs and hearts were

extracted. Each tissue sample was fixed in neutral buffered formalin (pH: 7) for 24 hours.

The tissues were subjected to routine paraffin tissue processing, involving sequential treatment with ethanol (70 %, 90 %, 96 %, 100 %), toluene, and paraffin. Sections measuring 4 μm in thickness were prepared using a Leica RM22555 Rotary Microtome.

For histochemical staining and evaluation, the deparaffinized sections underwent a series of alcohol rinses and were stained with hematoxylin-eosin for lung and heart histopathological assessment. Additionally, Masson-Trichrome staining was performed to detect connective tissue changes in the lung.

Using a Leica DMLB light microscope, two histologists unaware of the study groups examined staining patterns, cell differentiation, morphological structure, inflammation, and damage.

Transmission Electron Microscopy. The tissue samples were collected from the experimental subjects and immediately immersed in a fixative solution of 2.5 % glutaraldehyde for 4 hours at 4 °C to preserve the tissue structure. After fixation, the tissues were rinsed in a phosphate buffered saline solution to remove excess fixative. A secondary fixation step was carried out using a solution containing 1 % osmium tetroxide. To prepare the tissues for electron microscopy analysis, they were dehydrated by transferring them through a series of ethanol solutions. The dehydration process removed water from the tissues, and they were subsequently embedded in Epon 816 (Sigma) for sectioning. Ultrathin sections of 90 nm were obtained and contrasted with uranyl acetate and lead citrate. The specimens were then evaluated using a JEOL transmission electron microscope (Tokyo, Japan) by two histologists who were blinded to the study groups.

Statistical Analysis. The statistical analysis was performed using GraphPad Prism version 9.4.0 software (San Diego, CA, USA). The normality of the data was assessed using the Shapiro-Wilk test. The data followed a normal distribution and are represented as means ± SEM in a column bar graph. One-way ANOVA followed by Tukey's multiple comparison tests were conducted. A significance level of $p<0.05$ was used to determine statistical significance.

RESULTS

Sheep Hyperimmune Serum. We initially assessed the in vitro efficacy of sheep hyperimmune serum against SARS-CoV-2 by measuring its neutralizing activity. Samples were collected at day 0 and subsequently after three booster immunizations (at days 28, 36, and 48). The PRNT50 titers of the sheep serum consistently increased over time, reaching an average titer of 1:24,576 on day 48. Following purification and sterile filtration, the hyperimmune serum was formulated to contain 2500 PRNT50 units per 0.2 ml for subsequent in vivo experiments in transgenic mice to evaluate its effectiveness (Fig. 1).

Mice Experiments. The general condition of the mice was recorded and monitored through weight measurements. When comparing the two infected groups, it was found that there were no fatalities in the group receiving sheep hyperimmune serum, whereas half of the mice in the SARS-CoV-2 infected group succumbed to the infection. No deaths were observed in the control groups. Significant statistically differences in weight were observed among the groups ($p<0.0001$). The SARS-CoV-2-infected mice lost weight, whereas the control and SARS-CoV-2+HS groups did not (Weight percent of change of SARS-CoV-2 vs. control groups $p <0.0001$and SARS-CoV-2 vs. SARS-CoV-2 + HS groups $p<0.0001$) (Fig. 2).

Proinflammatory Cytokines. When examining the levels of proinflammatory cytokines among the control, virus-infected, and treatment groups, statistically significant differences were observed in the levels of IL-10, MCP-1, TNF-α, and IL-6 ($p=0.0002$, $p=0.0147$, $p=0.0003$, $p<0.0001$, respectively), while no significant difference was found in the levels of IL-1β ($p=0.0725$). In pairwise comparisons, the levels of these proinflammatory cytokines were found to be significantly higher in the virus-infected group compared to the control group. Moreover, the administration of sheep hyperimmune serum effectively reduced the levels of IL-10, TNF-α, and IL-6 in comparison to the virus-infected group ($p=0.0018$, $p=0.05$, $p=0.0023$, $p=0.0076$, respectively). Except for IL-6, the levels of cytokines were similar between the control and treatment groups (Fig. 2).

Viral RNA Analyses. Animals infected with the virus exhibited a high viral load in their lungs, as measured by quantitative RT-PCR (Fig. 2). Although the viral load in the heart was lower but positive, it was statistically significant compared to the negative control group (Hearts of SARS-CoV-2 vs. hearts of control groups p= 0.,0118). Treatment with sheep HS significantly reduced the viral RNA load in the lungs and heart compared to the group infected with the virus alone (Lungs of SARS-CoV-2 vs. lungs of sheep HS groups $p <0.0001$ and hearts of SARS-CoV-2 vs. hearts of sheep HS groups $p=0.0122$).

Morphological Evaluation. In the SARS-CoV-2 group, there was significant interstitial pneumonia with lymphocyte-dominant mononuclear leukocyte infiltration, alveolar collapse, and thickening of the alveolar septum. Bronchioles were affected, and peribronchiolar vessels showed congestion. Edema and erythrocytes near the infected areas were occasionally observed. In contrast, the SARS-CoV-2 + HS group exhibited mild infection, with small vessel edema, congestion, and leukocyte infiltration of neutrophils, lymphocytes, and macrophages near these areas. Patchy infiltration was rare and limited in the SARS-CoV-2 + HS group. Overall, the alveolar morphology was well-preserved (Fig. 3).

The SARS-CoV-2+HS group showed milder cardiac abnormalities with reduced inflammation and preserved tissue compared to the significant small vessel endotheliosis, inflammation in the outer layer of small vessels, scattered myofiber degeneration, myocyte necrosis, and signs of myocardial ischemia in the SARS-CoV-2 infected group (Fig. 4).

The control group had normal heart and lung morphology.

TEM. In the SARS-CoV-2 group, there were constricted airways due to the recruitment of inflammatory cells, increased early fibrosis, and thickened basal membrane, resulting in damage to the blood-air barrier. Type II pneumocytes exhibited disrupted surfactant structure and frequently showed apoptotic changes. Some type II pneumocytes contained free virus within vesicles and apoptotic cells. The bronchiolar epithelium demonstrated an increase in Club cell activity (Fig. 5).

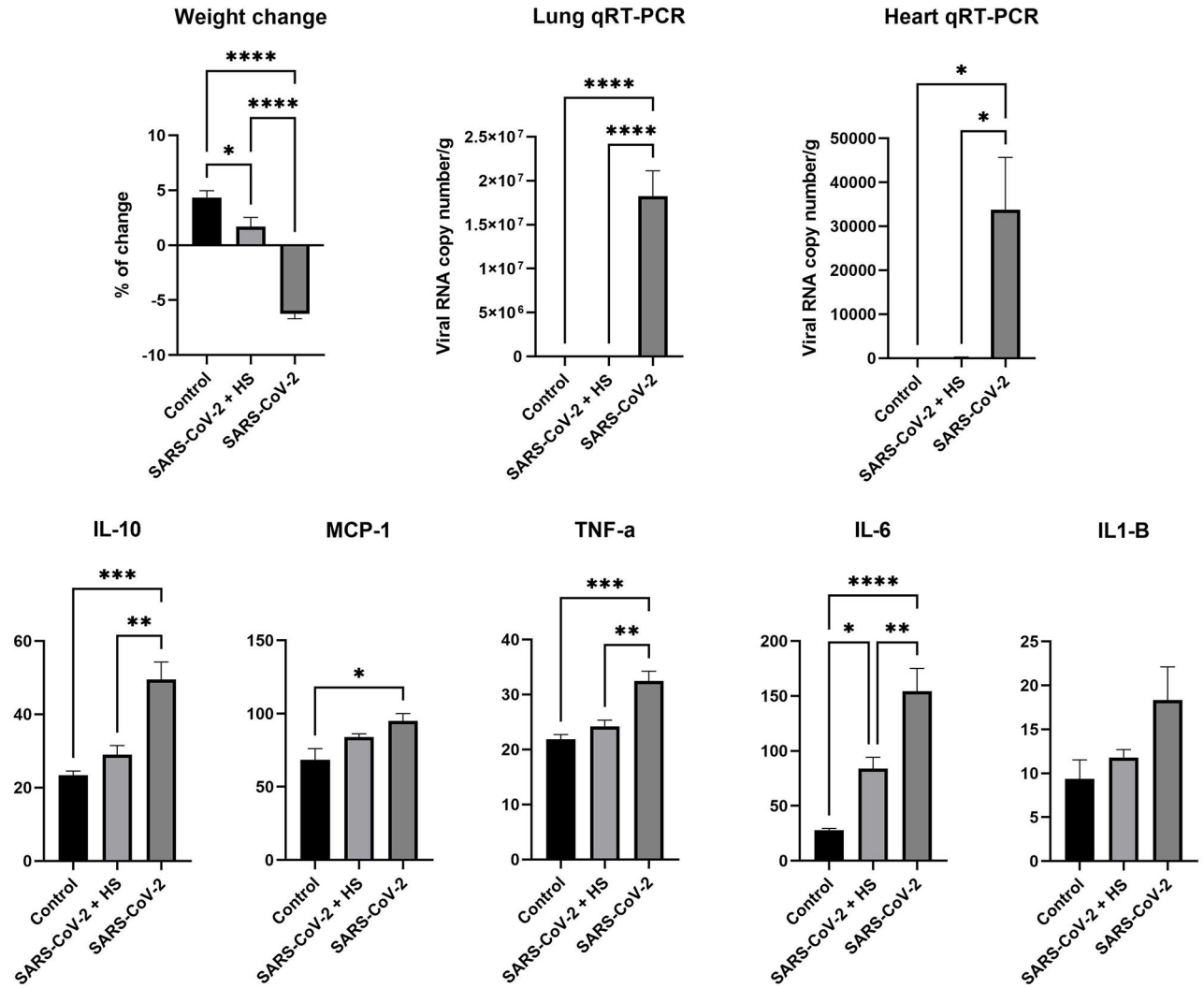

Fig. 2. Weight percent change, lung and heart SARS-CoV-2 RNA levels, and serum proinflammatory cytokine levels in pg/ml in the study groups. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

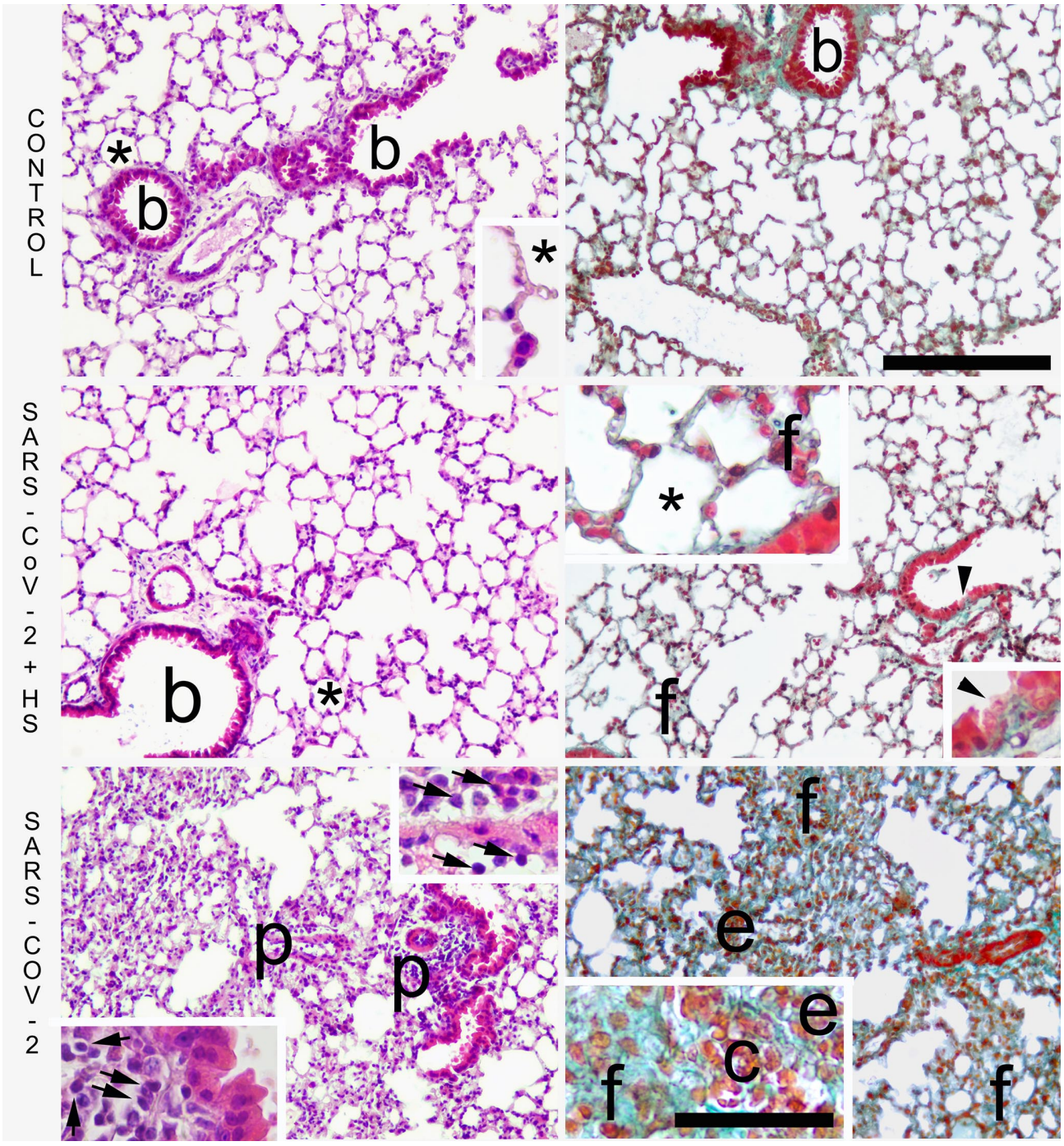

Fig. 3. Light microscopical analyses of lungs. The control and SARS-CoV-2 + HS groups exhibit open bronchi (b), open air spaces (asterisk) and thin alveolar septae (insets). In the SARS-CoV-2 + HS group, occasional small areas of fibrosis (f) and rarely bronchiolar epithelial damage (arrowhead) can be observed. The SARS-CoV-2 group displays interstitial pneumonia (p). Lymphocytes and other mononuclear leukocytes (arrow) are located in the interstitial area, surrounding the blood vessels, and around the bronchioles. Some blood vessels show congestion (c), extravasation of erythrocytes (e), and early signs of fibrosis (f). Left panel: Hematoxylin and eosin stain; right panel: Masson's trichrome stain. All images were captured at 10X magnification, with insets at 40X objective, with the scale bar indicating 200µm and 50 µm, respectively.

In the SARS-CoV-2+HS group, the alveolar air spaces were open, with thin type I pneumocytes and non-reactive type II pneumocytes compared to the SARS-CoV-2 group. Type I pneumocytes occasionally showed a

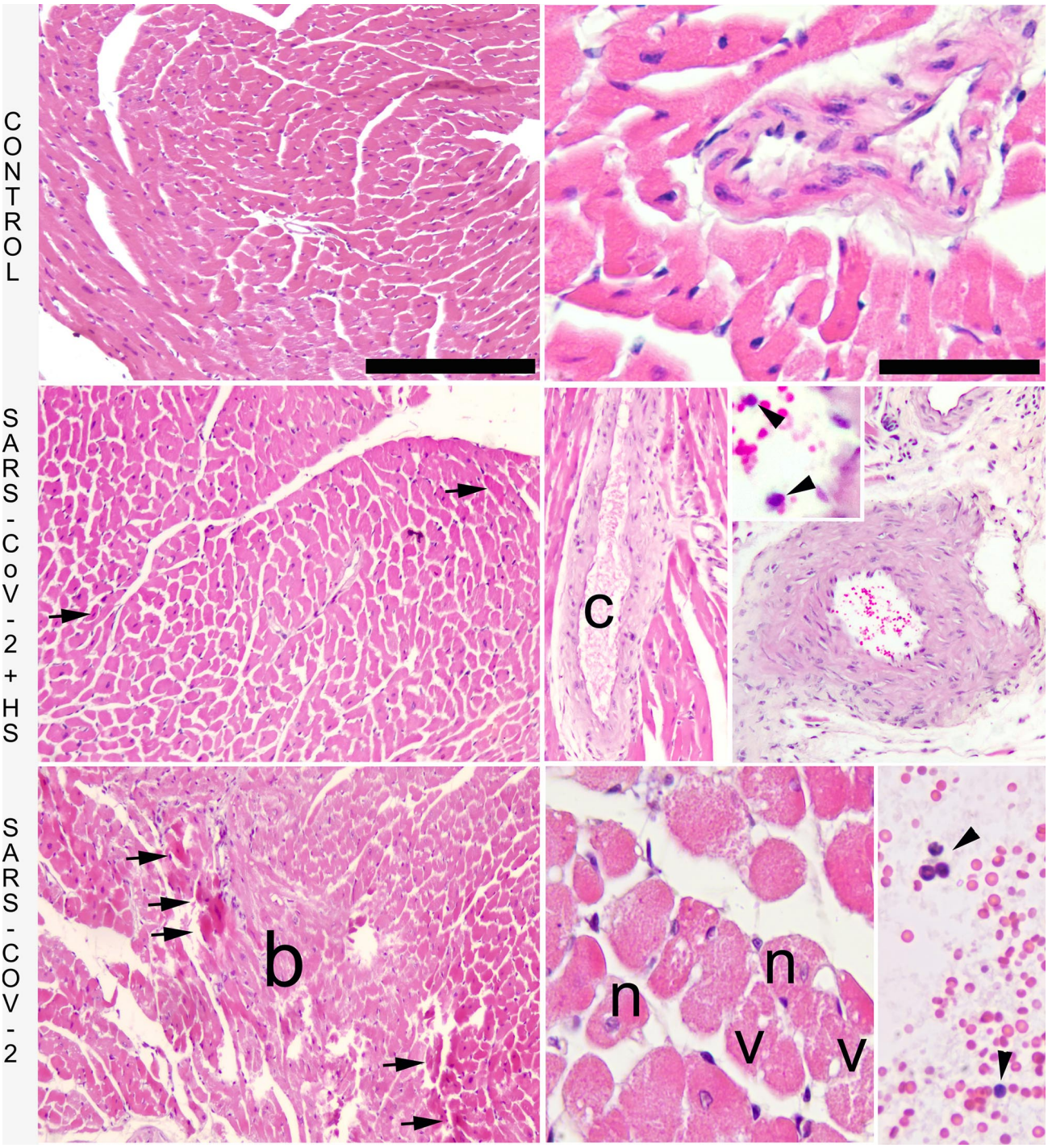

Fig. 4. Light microscopical analyses of hearts. The control group displayed normal heart muscle and vessels. In the SARS-CoV-2 + HS group, the heart muscle was mostly unaffected, except for few eosinophilic-stained cells (arrow). Some of the cardiac vessels were congested (c) without surrounding inflammation. However, inflammatory cells (arrow head) were rarely seen in the coronary artery. The SARS-CoV-2 group exhibited blurred cardiac muscle areas (asterisk) and eosinophilic-stained cells. At higher magnification, vacuoles (v) and nuclear blurring (n) indicated hypoxia. Monocytes and lymphocytes were commonly observed in the ventricular blood. Hematoxylin and eosin stain. Left images of all groups, SARS-CoV-2 (middle), and SARS-CoV-2 (right) were captured at 10X magnification, with the scale bar indicating 200 µm. The Control (right), SARS-CoV-2 + HS (inset), and SARS-CoV-2 (middle and right) are at 40X objective, with the scale bar indicating 50 µm.

thickness less than three times the normal level. The presence of fibrotic foci was significantly reduced and very small. The number of encountered inflammatory cells was greatly diminished.

In the control group, the airways were open, and both type I and type II pneumocytes displayed normal surfactant content. The activity of bronchiolar epithelial cells was normal.

DISCUSSION

The objective of this study was to develop a rapid and cost-effective hyperimmune serum (HS) derived from sheep and assess its efficacy against SARS-CoV-2. This study showed a promising option for the rapid production of hyperimmune serum (HS). Considering selected geographies, using animals other than horses could be a

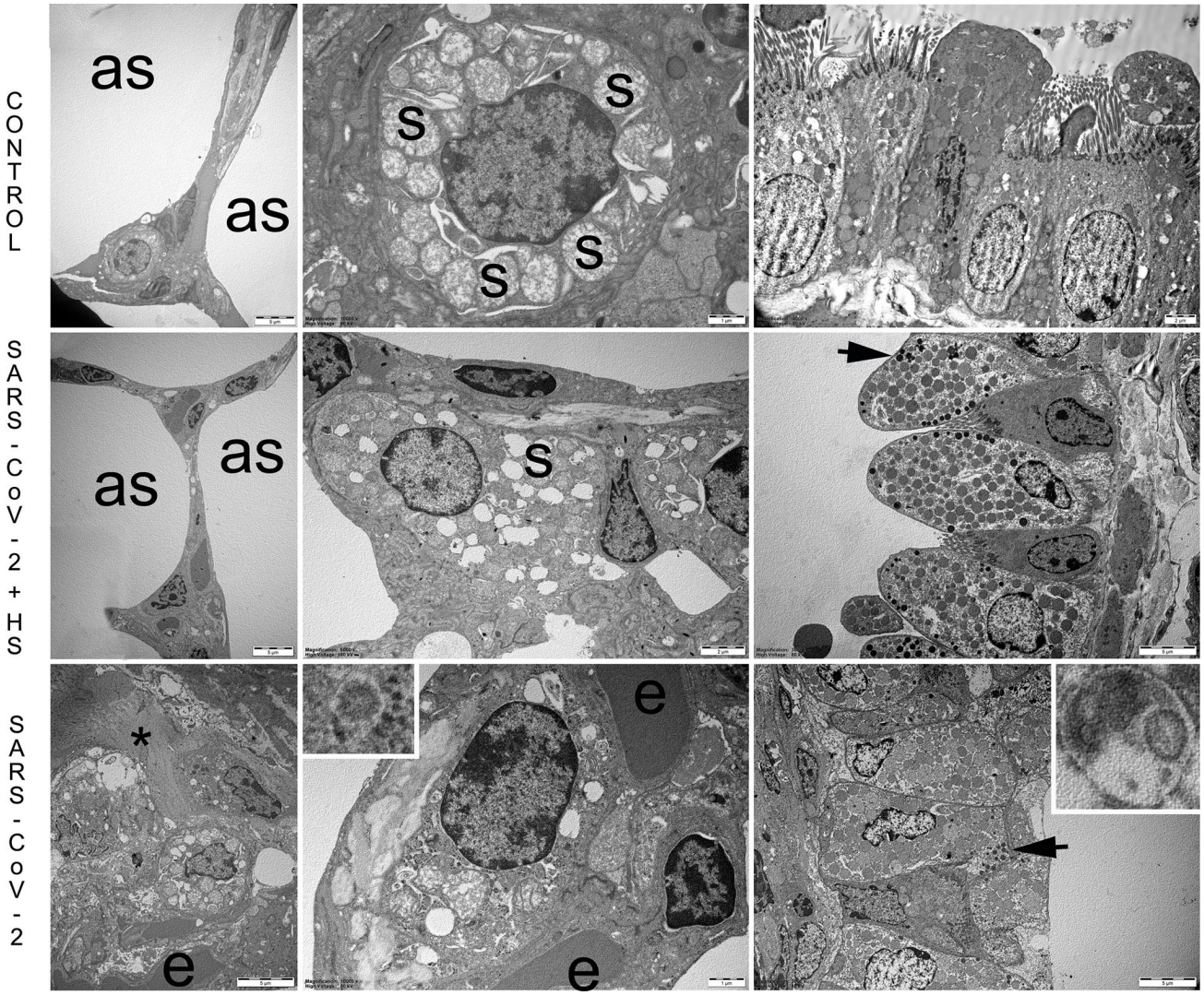

Fig. 5. Electron microscopical analyses of lungs. The control group had thin alveolar septae (left column), a regular basal membrane, type II pneumocytes with plenty of mature surfactant (S), and normal bronchiolar epithelium (right column). The SARS-CoV-2 + HS group exhibited slightly thicker corners, where type II pneumocytes reside. However, all of the air spaces were open (as). Type II pneumocytes were more active, and the surfactant appeared smaller. The SARS-CoV-2 group had large areas of closed airspaces due to inflammation. Damaged type II pneumocytes were accompanied by blood-air-barrier damage and a collection of connective tissue (*). Capillaries are filled with erythrocytes (e). Some type II pneumocytes and Club cells of bronchioles contained SARS-CoV-2 particles in vesicles measuring 90-120 μm. The bronchiolar epithelium displayed slightly increased secretory activity (arrow) in the SARS-CoV-2 and SARS-CoV-2 + HS groups. Magnifications of the ultramicrographs from left to right are 2000X, 10000X, 4000X for the control group; 2000X,6000X, 3000X for the SARS-CoV-2 + HS group; and 3000X, 10000X, 3000X for the SARS-CoV-2 group. Both insets are at 30000X and 2X optical magnification.

viable alternative (Dowall *et al*., 2016; Ainsworth *et al*., 2020). Sheep are easy to handle and breed (Tharmalingam *et al*., 2022). The most significant effect of the sheep-derived HS was the presence of neutralizing antibodies, which rapidly neutralize the virus and combat SARS-CoV-2 before any inflammatory response is initiated.

In previous studies, horses have been utilized to generate hyperimmune serum against various viral infections (Zylberman *et al*., 2020; Moreira-Soto *et al*., 2021; Botosso *et al*., 2022). However, horses are expensive to acquire and challenging to handle. Conversely, sheep can be infected with SARS-CoV-2 (Gaudreault *et al*., 2022). They are cost-effective to purchase and breed, easy to handle, commonly raised in herds in hot climates, and fully compliant with halal requirements. In the event of a need, sheep can be euthanized, and their serum can be fully utilized for hyperimmune serum production. In a recent study, sheep antisera were developed against the spike protein of SARS-CoV-2. However, a subunit protein derivative remains significantly limited (Findlay-Wilson *et al*., 2022). In our investigation, we generated an antiserum targeting the whole virus. Thus, it is superior due to its polyclonal cocktail nature, which encompasses both spike nucleoprotein and membrane protein. On the other hand, our study's findings demonstrate that the neutralizing activity of the sheep-derived HS increased progressively over time. The PRNT50 titers consistently elevated, reaching an impressive average titer of 1:24,576 on day 48. This notable enhancement in neutralizing potency highlights the potential of HS to effectively target and neutralize SARS-CoV-2.

The observed improvements in this study in the mice treated with HS support its therapeutic efficacy, as evidenced by the absence of deaths in the HS group compared to the high fatality rate in the SARS-CoV-2 infected group. On the other hand, the percentage change in weight provided further insight into the general condition of the living subjects, demonstrating the mortality-reducing effect of HS treatment. HS treatment also resulted in a significant reduction in proinflammatory cytokines, indicating its ability to attenuate and limit the inflammatory response induced by the virus. The neutralizing antibodies deactivate circulating viruses, rendering the inflammatory response unnecessary when subjects are treated with HS. Additionally, HS treatment effectively reduced the viral RNA load in the lungs and heart, demonstrating its antiviral effect on organs. The lung is the most affected organ, and the long-term and vaccine-related damages to the heart are also discussed in the literature (Puri *et al*., 2021; Davis *et al*., 2023). The viruses invading these organs are reduced by the other antibodies contained in HS, which contribute to the subject's immunity. Morphological evaluation revealed that HS preserved lung and cardiac tissue integrity and mitigated the severity of damage caused by SARS-CoV-2 infection. Electron microscopy analysis further confirmed these findings by showing the preservation of the blood-air barrier and reduced lung damage in the HS-treated group. The controlled inflammation in this group contributed to the protected structural and ultrastructural morphology of these organs.

This study represents the first production study of hyperimmune serum against whole SARS-CoV-2 derived from sheep in the existing literature. A functional formulation was developed, and its efficacy against SARS-CoV-2 was evaluated. This formulation holds potential for utilization against new variants and could be explored for other emerging viral infections. The need for a rapidly produced, economically viable alternative, compliant with halal requirements and adaptable to hot climates, for the production of antiviral treatments has been lacking in this field.

The advantage of this study lies in providing a potential alternative to human plasma therapy, which relies on immunized humans and requires a significant amount of time (Li *et al*., 2023). In vivo experiments conducted in k18-hACE-2 mice, recognized as ideal study animals for SARS-CoV-2 research, allowed for a robust evaluation of the serum's efficacy in terms of neutralizing activity, cytokine impact, viral load, and organ pathology. Furthermore, the study employed various analytical techniques to comprehensively evaluate the systemic response, including serum cytokine levels and organ damage. However, a notable limitation of the study is the lack of human data on the effectiveness and safety of the serum, although previous studies have shown no adverse effects (Botosso *et al*., 2022; Li *et al*., 2023). There is a low risk of serum sickness in the first two weeks after intravenously administering any human or animal-derived immunoglobulins (Brennan *et al*., 2003; Ainsworth *et al*., 2020). Additionally, the long-term effects of the serum remain unknown, but existing literature on hyperimmune serums of other animals has not reported any adverse reactions (Moreira-Soto *et al*., 2021).

CONCLUSION

In conclusion, this study demonstrates the successful development of a sheep-derived HS against SARS-CoV-2 and its efficacy in mitigating infection severity and reducing multiorgan damage. The neutralizing activity of HS,

combined with its ability to improve the general condition of infected mice, modulate proinflammatory cytokine levels, reduce viral load, and preserve lung and heart integrity, highlights its potential as a therapeutic option for severe SARS-CoV-2 infections. Further research and clinical trials are necessary to validate these findings and explore the full potential of HS in treating SARS-CoV-2 infections, especially in immunocompromised individuals and against emerging viral variants.

KERVANCIOGLU DEMIRCI, E. K.; YILMAZ, E. S. & ONEN, E. A. Desarrollo, evaluación histológica y ultraestructural de la terapia con suero hiperinmune de oveja para COVID-19. *Int. J. Morphol.; 41(6)*:1687-1697, 2023.

RESUMEN: En respuesta a la amenaza que suponen las nuevas variantes del SARS-CoV-2 y la urgente necesidad de tratamientos eficaces en ausencia de vacunas, el objetivo de este estudio fue desarrollar un suero hiperinmune (HS) rápido y rentable derivado de ovejas. y evaluar su eficacia. La utilización de un animal con certificación halal, de fácil mantenimiento en determinadas regiones geográficas y de fácil manejo, como las ovejas, podría proporcionar una alternativa viable a la costosa opción de los caballos. Las ovejas fueron inmunizadas con un antígeno de SARS-CoV-2 completamente inactivado para producir HS, cuya potencia neutralizante se evaluó mediante el ensayo PRNT50. Los ratones transgénicos K18-hACE2 (n = 35) se dividieron en tres grupos: control, exposición al SARS-CoV-2 mediante inhalación y ratones expuestos al SARS-CoV-2 tratados con HS. La eficacia de HS se evaluó mediante niveles de citoquinas proinflamatorias en suero, análisis qRT-PCR, examen histopatológico de pulmones y corazones y microscopía electrónica de transmisión. El HS purificado exhibió una actividad neutralizante significativa (1/24,576). El grupo SARS-CoV-2+HS mostró niveles más bajos de TNF-α, IL-10 e IL-6 ($P<0,01$) y niveles relativamente más bajos de MCP-1 en comparación con el grupo SARS-CoV-2. HS evitó la muerte, redujo los niveles de ARN viral en los pulmones y el corazón, protegió contra la neumonía intersticial grave, preservó la integridad del tejido pulmonar y evitó el daño de los miocitos, mientras que el grupo SARS-CoV-2 exhibió presencia viral en los pulmones. Este estudio desarrolló con éxito un HS derivado de ovejas contra todo el virus SARS-CoV-2, lo que resultó en una reducción significativa de la gravedad de la infección, la inflamación y la producción sistémica de citocinas. Los hallazgos son prometedores para el tratamiento de casos graves de COVID-19, incluidas las variantes virales emergentes y los pacientes inmunocomprometidos.

PALABRAS CLAVE: Antisueros; Caballo; SARS-CoV-2; Ovino.

REFERENCES

Ainsworth, S.; Menzies, S. & Pleass, R. J. *Animal derived antibodies should be considered alongside convalescent human plasma to deliver treatments for COVID-19.* Wellcome Open Res.; 5:115, 2020. Available from: https://wellcomeopenresearch.org/articles/5-115

Alp Onen, E.; Sonmez, K.; Yildirim, F.; Demirci, E. K. & Gurel, A. Development, analysis, and preclinical evaluation of inactivated vaccine candidate for prevention of Covid-19 disease. *All Life, 15(1)*:771-93, 2022.

Bi, Z.; Hong, W.; Yang, J.; Lu, S. & Peng, X. Animal models for SARS-CoV-2 infection and pathology. *MedComm (2020), 2(4)*:548-68, 2021.

Botosso, V. F.; Jorge, S. A. C.; Astray, R. M.; de Sa Guimaraes, A. M.; Mathor, M. B.; de Carneiro, P. D. S.; Durigon, E. L.; Covas, D.; de Oliveira, D. B. L.; das Neves Oliveira, R.; *et al*. Anti-SARS-CoV-2 equine F (Ab')2 immunoglobulin as a possible therapy for COVID-19. *Sci. Rep., 12(1)*:3890, 2022.

Brennan, V. M.; Salome-Bentley, N. J. & Chapel, H. M. Prospective audit of adverse reactions occurring in 459 primary antibody-deficient patients receiving intravenous immunoglobulin. *Clin. Exp. Immunol., 133(2)*:247-51, 2003.

Cunha, L. E. R.; Stolet, A. A.; Strauch, M. A.; Pereira, V. A. R.; Dumard, C. H.; Gomes, A. M. O.; Monteiro, F. L.; Higa, L. M.; Souza, P. N. C.; Fonseca, J. G.; *et al*. Polyclonal F(ab')2 fragments of equine antibodies raised against the spike protein neutralize SARS-CoV-2 variants with high potency. *iScience, 24(11)*:103315, 2021.

da Costa, C. B. P.; Martins, F. J.; da Cunha, L. E. R.; Ratcliffe, N. A.; Cisne de Paula, R. & Castro, H. C. COVID-19 and Hyperimmune sera: A feasible plan B to fight against coronavirus. *Int. Immunopharmacol., 90*:107220, 2021.

Davis, H. E.; McCorkell, L.; Vogel, J. M. & Topol, E. J. Long COVID: major findings, mechanisms and recommendations. *Nat. Rev. Microbiol., 21(3)*:133-46, 2023.

Dhama, K.; Khan, S.; Tiwari, R.; Sircar, S.; Bhat, S.; Malik, Y. S.; Singh, K. P.; Chaicumpa, W.; Bonilla-Aldana, D. K. & Rodriguez-Morales, A. J. Coronavirus Disease 2019-COVID-19. *Clin. Microbio.l Rev., 33(4)*:e00028-20, 2020.

Dowall, S. D.; Callan, J.; Zeltina, A.; Al-Abdulla, I.; Strecker, T.; Fehling, S. K.; Krahling, V.; Bosworth, A.; Rayner, E.; Taylor, I.; *et al*. Development of a cost-effective ovine polyclonal antibody-based product, EBOTAb, to treat Ebola virus infection. *J. Infect. Dis., 213(7)*:1124-33, 2016.

Findlay-Wilson, S.; Easterbrook, L.; Smith, S.; Pope, N.; Humphries, G.; Schuhmann, H.; Ngabo, D.; Rayner, E.; Otter, A. D.; Coleman, T.; *et al*. Development of a cost-effective ovine antibody-based therapy against SARS-CoV-2 infection and contribution of antibodies specific to the spike subunit proteins. *Antiviral Res., 203*:105332, 2022.

Gaudreault, N. N.; Cool, K.; Trujillo, J. D.; Morozov, I.; Meekins, D. A.; McDowell, C.; Bold, D.; Carossino, M.; Balaraman, V.; Mitzel, D.; *et al*. Susceptibility of sheep to experimental co-infection with the ancestral lineage of SARS-CoV-2 and its alpha variant. *Emerg. Microbes Infect., 11(1)*:662-75, 2022.

Haghighi, M.; Khorasani, A.; Karimi, P. & Mahdavi, M. Improvement of the inactivated SARS-CoV-2 vaccine potency through formulation in alum/naloxone adjuvant; Robust T cell and anti-RBD IgG responses. *Iran. J. Basic Med. Sci., 25(5)*:554-61, 2022.

Jacobson, G. M.; Kraakman, K.; Wallace, O.; Pan, J.; Hennebry, A.; Smolenski, G.; Cursons, R.; Hodgkinson, S.; Williamson, A. & Kelton, W. Immunogenic fusion proteins induce neutralizing SARS-CoV-2 antibodies in the serum and milk of sheep. *Biotechnol. Rep. (Amst.), 38*:e00791, 2023.

Leon, G.; Herrera, M.; Vargas, M.; Arguedas, M.; Sanchez, A.; Segura, A.; Gomez, A.; Solano, G.; Corrales-Aguilar, E.; Risner, K.; *et al*. Development and characterization of two equine formulations towards SARS-CoV-2 proteins for the potential treatment of COVID-19. *Sci. Rep., 11(1)*:9825, 2021.

Li, E.; Han, Q.; Bi, J.; Wei, S.; Wang, S.; Zhang, Y.; Liu, J.; Feng, N.; Wang, T.; Wu, J.; *et al*. Therapeutic equine hyperimmune antibodies with high and broad-spectrum neutralizing activity protect rodents against SARS-CoV-2 infection. *Front. Immunol., 14*:1066730, 2023.

Moreira-Soto, A.; Arguedas, M.; Brenes, H.; Bujan, W.; Corrales-Aguilar, E.; Diaz, C.; Echeverri, A.; Flores-Diaz, M.; Gomez, A.; Hernandez, A.; *et al*. High efficacy of therapeutic equine hyperimmune antibodies against SARS-CoV-2 variants of concern. *Front. Med. (Lausanne), 8*:735853, 2021.

Oladunni, F. S.; Park, J. G.; Pino, P. A.; Gonzalez, O.; Akhter, A.; Allue-Guardia, A.; Olmo-Fontanez, A.; Gautam, S.; Garcia-Vilanova, A.; Ye, C.; *et al*. Lethality of SARS-CoV-2 infection in K18 human angiotensin-converting enzyme 2 transgenic mice. *Nat. Commun., 11(1)*:6122, 2020.

Puri, G.; Singh, V. P. & Naura, A. S. COVID-19 Severity: Lung-Heart Interplay. *Curr. Cardiol. Rev., 17(4)*:e230421189016, 2021.

Tharmalingam, T.; Han, X.; Wozniak, A. & Saward, L. Polyclonal hyper immunoglobulin: A proven treatment and prophylaxis platform for passive immunization to address existing and emerging diseases. *Hum. Vaccin. Immunother., 18(2)*:1886560, 2022.

Zylberman, V.; Sanguineti, S.; Pontoriero, A. V.; Higa, S. V.; Cerutti, M. L.; Morrone Seijo, S. M.; Pardo, R.; Muñoz, L.; Acuña Intrieri, M. E.; Alzogaray, V. A.; *et al*. Development of a hyperimmune equine serum therapy for COVID-19 in Argentina. *Medicina (B. Aires), 80 Suppl. 3*, 1-6, 2020.

Corresponding Author
Dr. Elif Kervancioglu Demirci
Histology and Embryology Department
Istanbul Faculty of Medicine
Istanbul University
Fatih 34093
Istanbul
TURKEY

E-mail: elifkervanciogludemirci@istanbul.edu.tr