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IMMUNOHISTOCHEMICAL PROFILING OF MACROPHAGES AND T LYMPHOCYTES AS FORENSIC INDICATORS OF ASPHYXIATION

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Many studies have shown that experimental hypoxia may change the morphology, viability, phagocytosis, metabolic activity, and release of cytokines by macrophages. Results are contradicting in several studies: some found activation of macrophages especially in cases of protracted asphyxiation; others confuted these reactions. Our results show that intra-alveolar macrophages were found both in peripheral and central regions of the lungs in death due to asphyxia of a girl after prolonged agony, while in the case of short-term agony a significantly higher number of macrophages was found in the peripheral (subpleural) lung regions, which partly differs from the results that other authors have obtained. The larger number of activated T cells in the lungs was found in death due to asphyxia of a girl with prolonged agony. Increased number of macrophages, and T lymphocytes, may represent a contributing factor in forensic diagnostics of violent death due to asphyxia.

Keywords: Forensic medicine, Autopsy, Asphyxiation, Alveolar macrophages, T lymphocytes

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IMUNOHISTOHEMIJSKO PROFILISANJE MAKROFAGA I T-LIMFOCITA KAO FORENZIČKIH POKAZATELJA ASFIKSIJE

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Mnoge studije su pokazale da eksperimentalno izazvana hipoksija može promeniti morfologiju, vitalnost, fagocitozu, metaboličku aktivnost i oslobađanje citokina od strane makrofaga. Rezultati u pojedinim studijama se razlikuju: neke su utvrdile aktivaciju makrofaga, posebno u slučajevima produžene asfiksije, dok su druge osporile ove reakcije. Naši rezultati pokazuju da su intraalveolarni makrofagi prisutni i u perifernim i u centralnim delovima pluća kod smrti usled asfiksije kod devojčice sa produženim agonalnim periodom, dok je u slučaju kratkotrajne agonije uočen značajno veći broj makrofaga u perifernim (subpleuralnim) delovima pluća, što se delimično razlikuje od rezultata koje su dobili drugi autori. Veći broj aktiviranih T limfocita u plućima primećen je u slučaju smrti usled asfiksije kod devojčice sa produženim agonalnim periodom. Povećan broj makrofaga i T limfocita može predstavljati doprinoseći faktor u forenzičkoj dijagnostici nasilnih smrti izazvanih asfiksijom.

Ključne reči: sudska medicina, obdukcija, asfiksija, alveolarni makrofagi, T-limfociti

INTRODUCTION

Mechanical occlusions of the mouth and nose lead to suffocation. When there is a case of suspected smothering, one must seek for local signs to verify pressure on the face. (1). Fatalities brought on by obstrucion of the larynx, otherwise called bolus death, are connected with a reflex heart failure. The bolus material stuck in the throat is triggered by the impact on the larynx. Food may enter the larynx either by the act of swallowing, or it may be regurgitated from the stomach (2).

There is no anatomic or microscopic feature, which is pathognomonic for asphyxial deaths, as one of the leading causes of traumatic deaths in newborns, infants, and young children. Instead, pathologists rely on investigation information, including confessions and/or witness statements, and potential evidence at the scene (3). However, the most important results of some studies concern (4) the presence, localization, and distribution pattern of HIF1-α (HIF-1a is a transcription factor expressed in response to hypoxia) in lung vessels after hypoxic stimuli. HIF1-α was expressed in small-, medium-, and large-caliber lung vessels of the vast majority of mechanical asphyxia deaths and CO intoxications, with the number and intensity of positive-stained vessels increasing with the duration of the hypoxia. A notable number of activated macrophages, especially in the interstitial level, have been evidenced, and such phenomenon supports the hypothesis of a possible association between CO intoxication and pulmonary macrophages activity. The highlighted association could be mediated by changes of the surfactant, by impairing of mitochondrial respiration and by the release of pro-inflammatory cytokines (5). Some studies confirm that massive and intense granular SP-A (surfactant protein A) patterns in alveolar space could be associated with drowning, while it is not clear if its expression could help in other fatal asphyxiations (6). Many studies have shown that experimental hypoxia may change the morphology, viability, phagocytosis, metabolic activity, and release of cytokines by macrophages (7). Results are contradicting in several studies: some found activation of macrophages especially in cases of protracted asphyxiation; others confuted these reactions (8).

MATERIALS AND METHODS

Histology

Lung tissue samples were stored in a neutral, buffered 4% formaline solution, for 18-24 hours, dehydrated in ethanol of progressive concentration and embedded in paraffin. From the paraffin blocks, the tissue was cut into 4-5 μm thin samples, stained with the standard hematoxylin-eosine (H&E) method and microscopically analysed using Leica DM1000 (Wetzlar, Germany), with digital microscopic camera Leica EC3 (Wetzlar, Germany) with Leica LAS EZ imaging software V 1.8.0.

Immunohistochemistry

The immunohiostochemical stainings were performed using commercially available monoclonal anti-human antibodies CD68, LCA and CD45RO. All the reactants were produced by the DAKO Company (Glostrup, Denmark).

For immunohistochemical analysis all tissue sections were deparaffined in xylene and rehydrated with graded alcohols and finaly in aqua destillata. The samples were microwave pretreated with citrat buffer pH 6,0 (20 minutes monoclonal CD45R0, clone UCHL1 to detect subpopulation T cells within both CD4 and CD8 subsets, and mature, activated T cells and 30 minutes to detect leukocyte common antigen (LCA) positive leukocytes, monoclonal LCA, clone PD7/26 and 2B11, and also CD68, monoclonal antibody PG-M1 labels human monocytes and macrophages). After cooling at room temperature for about 20 minutes, the slides were washed in distilled water for 5 minutes and treated with 3 % H_2O_2 to block endogeneous peroxidase. After washing, the slides were transferred into freshly prepared phosphate buffered saline (PBS) which was gently removed after 5 minutes and primary antibodies were applied on sections for 30 minutes in moist chambre at room temperature. The second and the third links were applied (each for 20 minutes and washings with PBS). After that, the samples were treated with diaminobenzidine (DAB) as a chromogen and stained in Hematoxylin for 1 minute, rehydrated and mounted in DPX. Dark brown granules of DAB on the cells were regarded as positive staining. Human tonsilar tissue was used as a positive control. In all tests, a negative control was performed without monoclonal antibody.

Microscopic evaluation

The macrophages, giant cells and lymphoid cells were counted on H&E sections and immunohistochemical preparations. Only alveolar macrophages were evaluated. Interstitial macrophages in the lung were not evaluated. The intra-alveolar and interstitial lymphoid cells were evaluated. The semiquantitative evaluation is calculated as the score of the positive cells in central and peripheral (subpleural) areas at x 200 magnification: determine as (1+) = low; (2+) = moderate; (3+) = high.

RESULTS

In this study two case were analyzed: one with short protracted asphyxiation and the other with long protracted asphyxiation.

Case 1: A two-year old girl was found dead in the apartment, where she was with her ten-year-old brother, who claimed that he had hit her and that she fell and remained dead. The total of five stamp-like hematomas, in the shape and size of fingertips, were found on the face of a little girl, around the mouth and the nose. The inner side of the labial mucosa was bloodshot and individual small mucosal abrasions, which in surface and location corresponded to the nearby primary teeth, were found in that area. The autopsy showed acute pulmonary emphysema, petechiae in the conjunctiva and serous membranes of internal organs, blood stasis in the internal organs, liquid blood. Pathological examination confirmed the enhanced emphysema of the lungs, fresh bleedings in lung tissue and disseminated intravascular coagulation in the lungs. Chemical and toxicological findings were negative, and no violations of hyoid bone and thyroid cartilage were found. Violent mechanical asphyxia was established due to the blockage of the nose and mouth with hand. According to information on the circumstances of the case and the autopsy findings, the death of this girl occurred quickly, with estimated time period of suffocation 5-10 minutes, which is why this was the case of short protracted asphyxiation (7).

Results in the first case (Case 1 - short protracted asphyxiation) revealed the following findings:

- Light microscopy. Lung tissue showed moderate vascular congestion and striking intra-alveolar macrophages and giant cells (Fig. 1) with very scarce fields of fresh hemorrhage.

- CD68. A high score of macrophages (3+) was observed in the peripheral areas of the lungs (subpleural) (Fig. 3a), while a moderate presence was detected in the central parts of the lungs.

- CD45RO. A moderate number of T lymphocytes (2+) was found in intra-alveolar and in the interstitial tissue, both in the central and peripheral areas of the lungs (Fig. 4a).

- LCA. Lymphoid cells immunoreactive to LCA were moderately present (2+) intra-alveolarly and within the interstitium, in both the central and peripheral areas of the lungs (Fig. 5a).

Case 2: 19-year-old girl, a resident of the Home for Mentally Challenged Persons, died when during food consumption started vomiting and aspirated a greater amount of gastric semi-mushy and semi-friable content, with time period > 20 minutes. The autopsy found a greater amount of whitish-yellowish, semi-mushy and partly softer semi-granular content in the area of guttural cover and laryngeal chamber and in the initial part of the esophagus. Mucous membranes were more vascularized and discreetly bloodshot in that area. Acute pulmonary emphysema, petechiae in the serous membranes of internal organs, liquid blood, and blood stasis in the internal organs were determined, which was also histopathologically confirmed. Chemical and toxicological findings were negative. Violent mechanical asphyxia was established due to food blockage of the pharynx and larynx by the food taken into the mouth and due to stomach vomit aspirated. According to information on the circumstances of the case and autopsy findings established, the death of the girl most likely occurred with time period > 20 minutes, which is why this was the case of long protracted asphyxiation (7).

Results in the second case (Case 2 - long protracted asphyxiation) revealed the following findings:

- Light microscopy. Lung tissue showed present marked vascular congestion and edema, with less conspicuous intra-alveolar macrophages (Fig. 2a), and in places marked areas of fresh hemorrhage (Fig. 2b).

- CD68. A moderate number of macrophages (2+) was present within the alveoli in both the central and peripheral areas of the lungs (Fig. 3b).

CD45RO. The cell populations of T lymphocytes present in the indicated number (3+) intra-alveolarly in the interstitial tissue, in the central and peripheral areas of the lungs (Fig. 4b).

LCA. Lymphoid cells immunoreactive for LCA were moderately present (2+) intra-alveolarly and within the interstitium, in both the central and peripheral areas of the lung (Fig. 5b).

DISCUSSION

Many of the increased numbers of macrophages present in diseased tissues are seen to amass in or next to poorly vascularized, hypoxic sites where considerable tissue damage may have occured (8).

Other authors claim that suffocation is not proven by the occurrence of essentially expanded numbers of macrophages and giant cells since they also occur in other causes of death and are not dependent on the cause of death. However, a useful criterion to assure the diagnosis of asphyxiation is high number of such cells, e.g. in the case of a victim with morphological findings such as emphysema, petechiae, heavy congestion, fluid blood, etc. which leads to the suspicion of a suffocation death but shows a lack of conclusive tools (e.g. in soft tissue covering) (7).

It was Grellner and Madea (9) who found that pulmonary giant cells and numerous alveolar macrophages were not restricted to asphyxia. Noteworthy contrasts in the substance of these cells between asphyxiated persons (causes of death: strangulation, drowning, hypoxia, thoracic compression) and controls incorporating cases with very short survival periods could not be detected.

Jansen (10) described a mobilization and proliferation of alveolar cells with the formation of multinuclear giant cells in 4 deaths with protracted oxygen deficiency (throtting, thoracic compression, smothering). On the other hand, Betz et al. (11) recently showed that these cell populations appeared in both fatal asphyxia/suffocation (strangulation, drowning, thoracic compression, hypoxia) and control cases in almost the same frequency.

Sacco and Aquila (12) through their analysis demonstrated that a significantly higher number of giant cells, together with early-stage macrophages, is present in cases of prolonged asphyxia. This indicates that the duration of the agony period in asphyxias is associated with the production and proliferation of alveolar macrophages and giant cells at the pulmonary level, as well as with the time required for the development of the inflammatory reaction.

In protracted asphyxiation, the number of intra-alveolar macrophages was definitely elevated. A significant increase of giant cells was observed in the cases of long protracted asphyxiation. CD 68 showed clearly elevated numbers in both asphyxiation groups.

Our results show that moderate number intra-alveolar macrophages were found both in peripheral and central lung parts in death due do asphyxia of a girl with prolonged agony, while in the case of short-term agony a significantly higher number of macrophages was found in the peripheral lung parts (subpleural), which partly differs from the results other authors have obtained.

The larger number of activated T cells in the lungs was found in death due do asphyxia of a girl with prolonged agony.

There are no significant differences in the number and distribution of LCA-positive leukocytes in the lungs in death due do asphyxia in relation to the duration of the agony.

CONCLUSION

Alveolar macrophages and T lymphocytes show different distribution patterns depending on the duration of hypoxia in mechanical asphyxia, therefore increased number of macrophages, and T lymphocytes, may represent a contributing factor in forensic diagnostics of violent deaths due do asphyxia.

LEGEND FOR FIGURES

Figure 1. Pulmonary congestion and intraalveolar macrophages in cases short protracted asphyxiation. (H&E, x200).

Figure 2. Long protracted asphyxiation. a) pulmonary congestion and edema, and intraalveolar macrophages, (H&E, x200); b) fields of fresh intraalveolar bleeding, (H&E, x200).

Figure 3. Intraalveolar macrophages, CD 68-positive: a) short protracted asphyxiation, (IHH, x100); b) long protracted asphyxiation, (IHH, x200).

Figure 4. Intraalveolar and interstitial lymphoid cells, CD 45RO-positive: a) short protracted asphyxiation, (IHH, x200); b) long protracted asphyxiation, (IHH, x200).

Figure 5. Intraalveolar and interstitial lymphoid cells, LCA-positive: a) short protracted asphyxiation, (IHH, x200). b) long protracted asphyxiation, (IHH, x200).

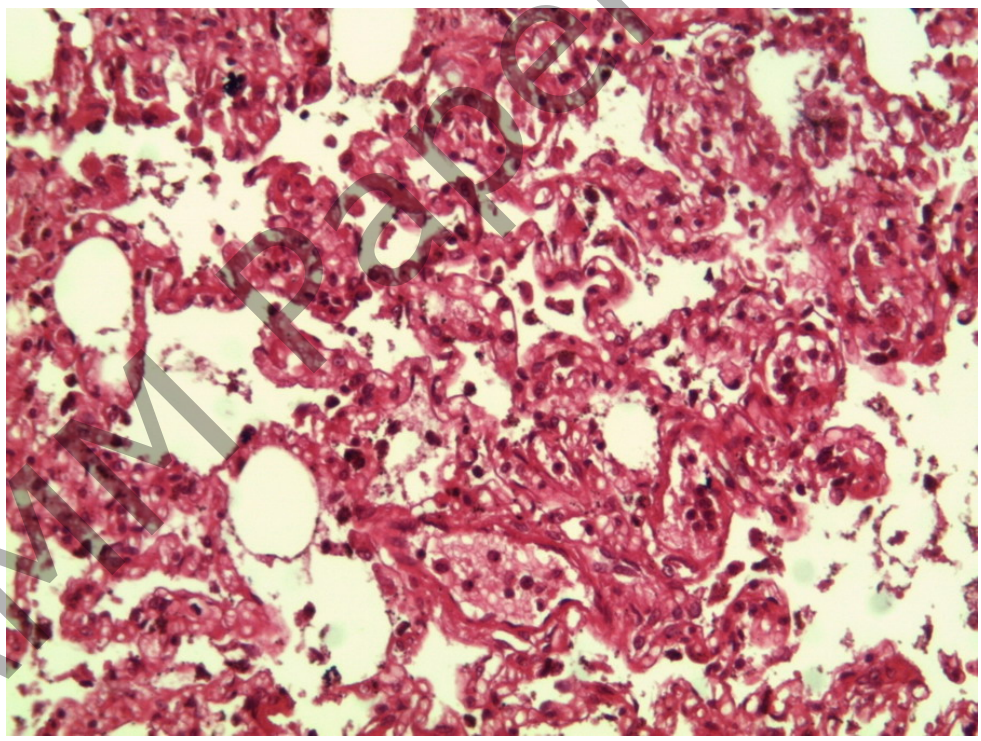

Figure 1.

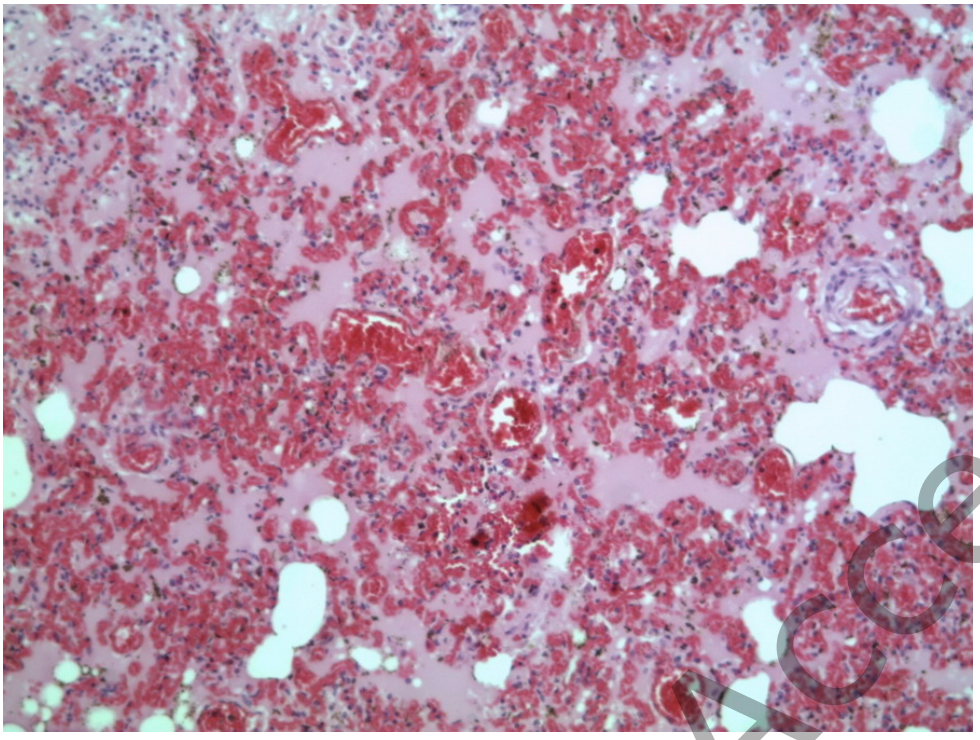

Figure 2a.

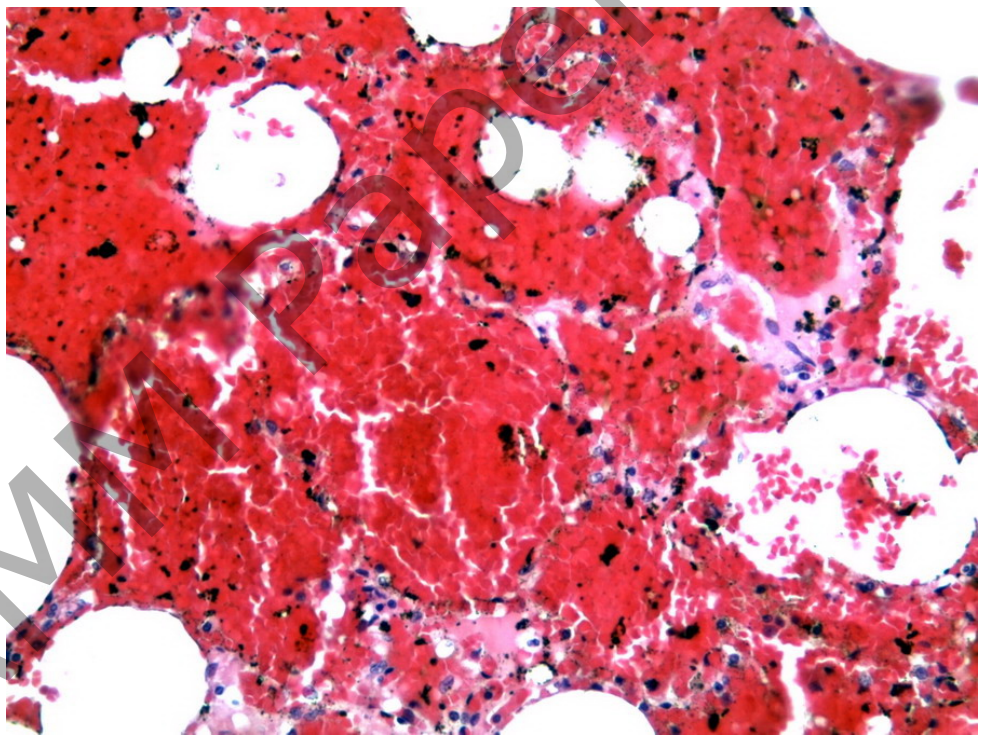

Figure 2b.

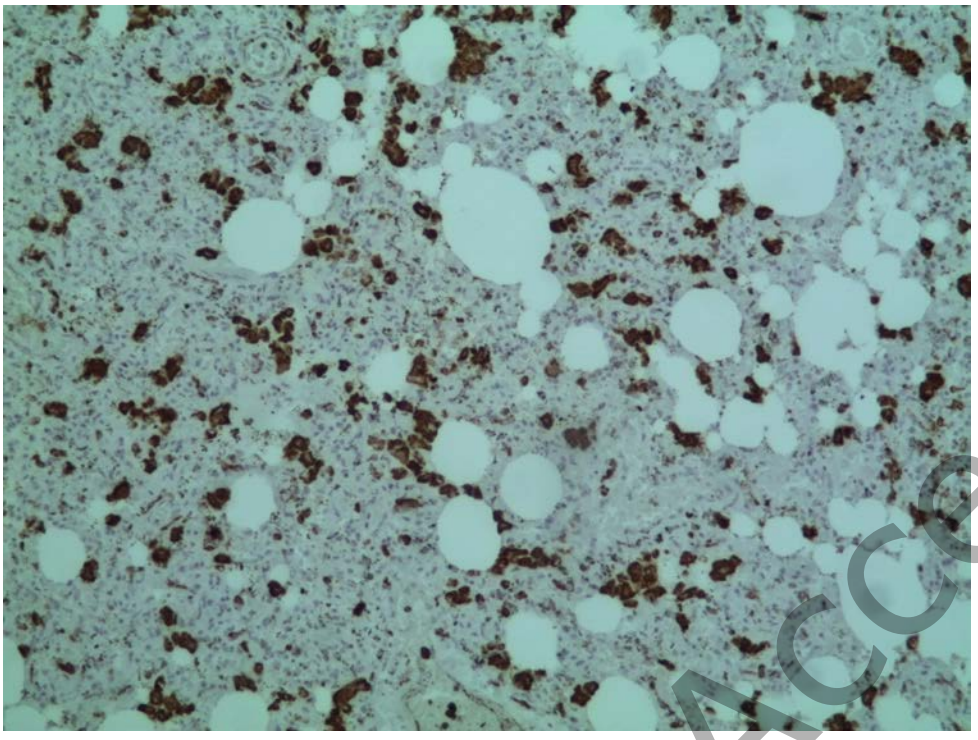

Figure 3a.

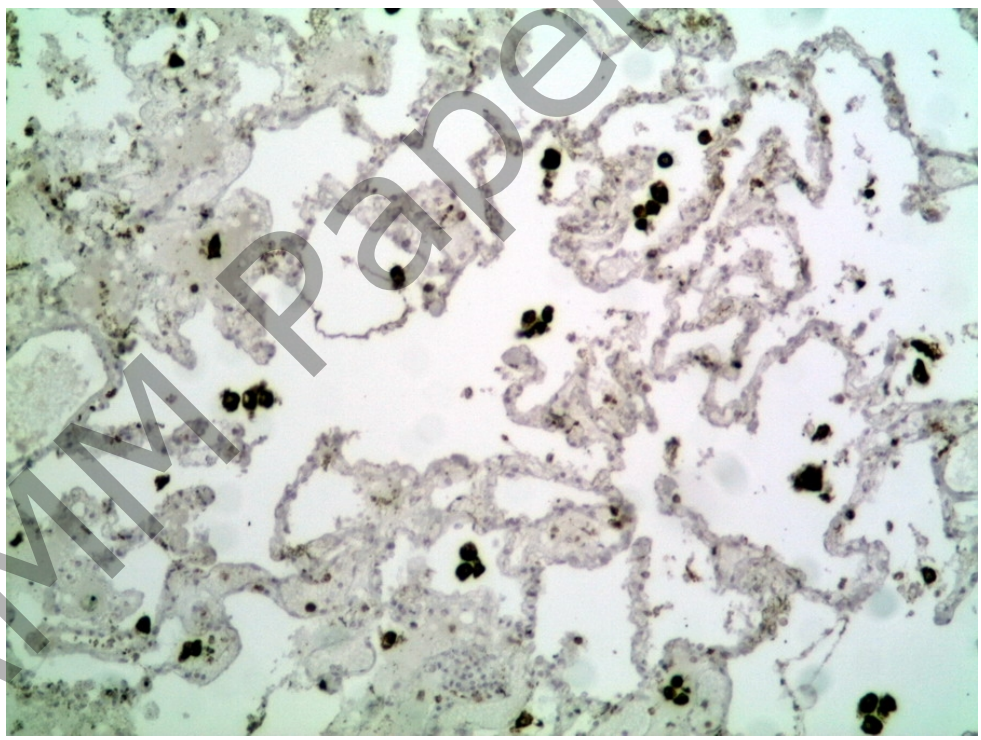

Figure 3b.

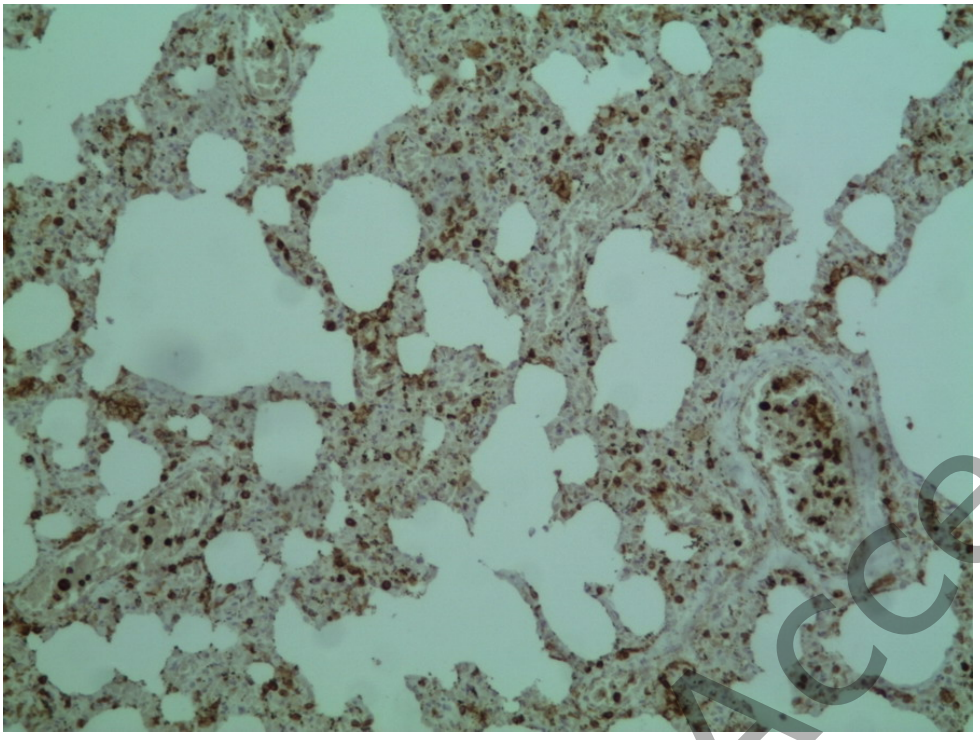

Figure 4a.

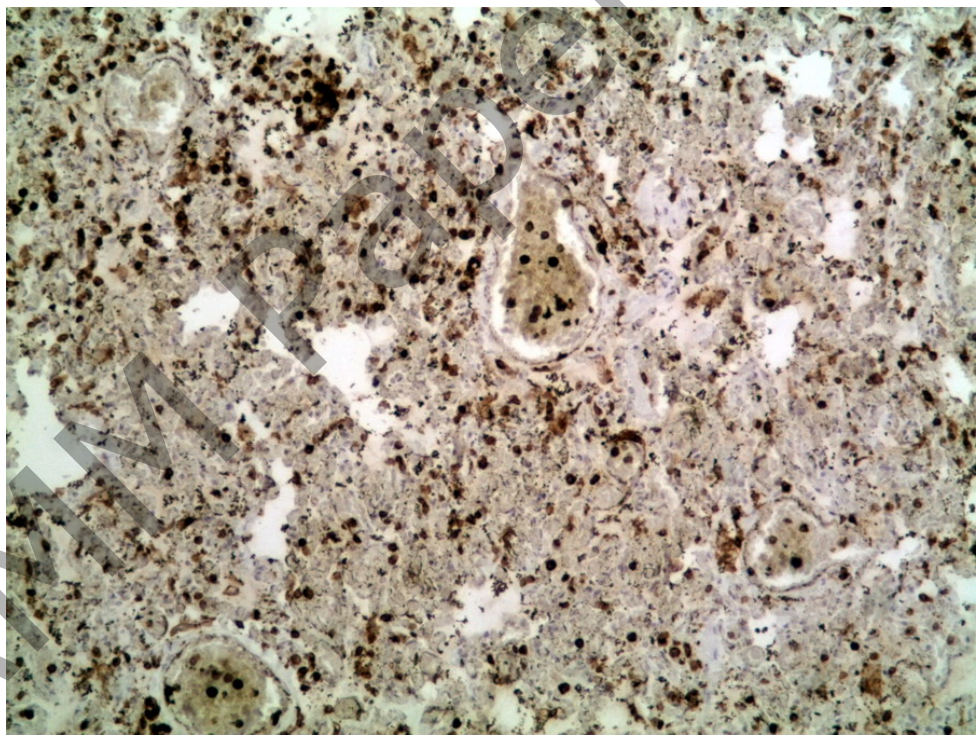

Figure 4b.

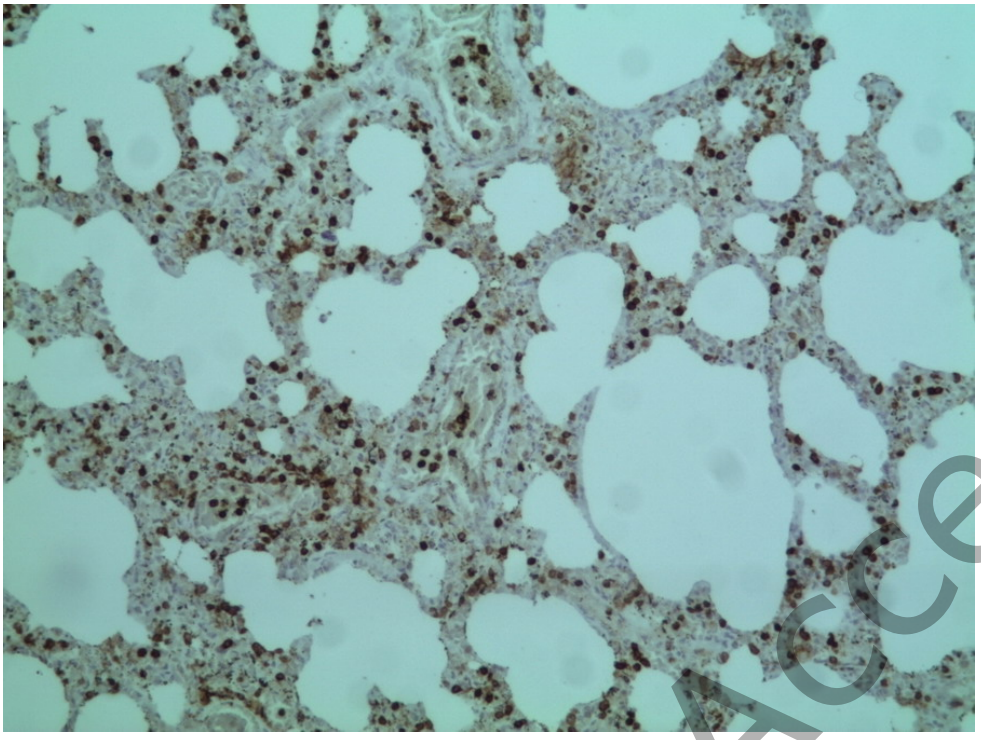

Figure 5a.

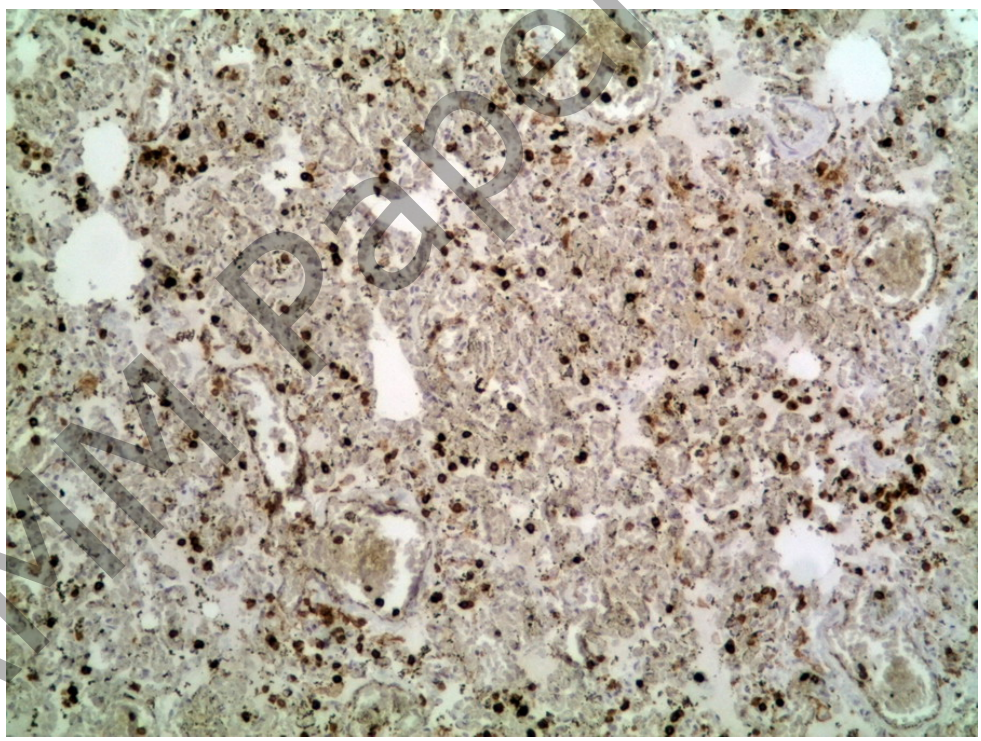

Figure 5b.

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