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Electron microscopic changes in fibroblastic sarcoid in horses

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The article presents the results of ultrastructural research on fibroblastic sarcoid, which is one of the most aggressive types of sarcoid in horses. A retrospective study on the prevalence of sarcoids in horses in Ukraine's western regions was conducted in 2019–2023. It was found that during the period of 2022–2023, the number of horses with sarcoids increased sharply by 15.6% compared to the period 2020–2021. This was associated with the mass movement of animals from eastern regions due to the onset of the war in Ukraine. The fibroblastic type of sarcoid was predominantly localized in the abdominal wall and groin area. To study the ultrastructure of sarcoids, they were surgically removed by excising undamaged skin portions under general anesthesia, adhering to all requirements of the European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes. Electronograms revealed that the tumour formation mainly consisted of fibroblasts of varying differentiation degrees: significant number of vesicles, markedly expanded endoplasmic reticulum cisterns, and irregularly shaped nuclei with numerous invaginations. Open nuclear pores were observed in most nuclei. Active formation of a large number of capillaries was noted between the collagen matrix, fibroblasts, and fibrocytes, indicated by the increased number of endotheliocytes with pseudopodia on the cytoplasm's marginal part and the basal surface. This suggests their embryonic type. Endotheliocytes contained large round nuclei and a significant number of mitochondria in the cytoplasm. There were both bright and dark endotheliocytes in the blood capillaries' venous section. The cytoplasm of bright endotheliocytes contained myofibrils, individual mitochondria, free ribosomes, peroxisomes, and vesicles of various sizes. The plasmalemma formed small pseudopodia. The capillary lumen showed moderate electron density. Additionally, high platelet activity was observed, manifested by cell adhesion to the marginal part of the endotheliocyte cytoplasm. It should be noted that most capillaries were of the venous type, as indicated by the endotheliocytes' height, the presence of an increased number of mitochondria and vesicles. The entire cellular pool was localized among the massive framework consisting of collagen fibers. Elastic fibers could not be detected in the fibrillar component.

Keywords: horses; ultrastructure; fibroblasts; fibrocytes; collagen fibers; capillaries; endotheliocytes; myofibrils.

Introduction

Numerous studies on the prevalence of equine sarcoids worldwide demonstrate significant interest in this disease. Information about sarcoid as a tumour in horses was first published in 1936 (Jackson, 1936; Ragland et al., 1970). It was initially considered a benign fibroblastic skin neoplasm characterized by rare regression, invasiveness, and a high recurrence rate after surgery. Additionally, sarcoids were regarded as one of the major complications in the wound healing process for horses (Pascoe & Summers, 1981; Ragland et al., 1970), resulting from fibroblast proliferation and changes in the dynamics of the extracellular matrix and its main components (Martano et al., 2018).

Nowadays, sarcoids are classified based on anatomical-topographical location and clinical manifestations, including occult, nodular, verrucous, fibroblastic, mixed, and malignant types (Martens et al., 2000). Verrucous and fibroblastic sarcoids are among the most aggressive types. Malignant sarcoids are rare, and there is a belief they may metastasize, which is atypical for other sarcoid types, leading some researchers to doubt whether it is genuinely a sarcoid. Macroscopically, sarcoids are characterized by focal skin thickening, depigmentation in affected areas, and excessive densification. Morphological diagnosis of sarcoid remains challenging as repeated biopsy manipulations may induce inflammatory reactions masking the classic presentation of sarcoid (Schaffer et al., 2013).

It is important to consider other tumours in horses that morphologically resemble sarcoids, such as fibrosarcomas, fibromas, and excessive granulation tissue formation. Some authors suggest that molecular-biological

studies detecting bovine papillomavirus (BPV) in areas affected by sarcoids theoretically could simplify diagnosis. However, numerous studies have shown the presence of BPV DNA is not prognostic in sarcoid diagnosis, as even outwardly normal skin in horses sometimes contains BPV DNA (Müller, 1991; Nasir & Reid, 1999; Chambers et al., 2003; Knottenbelt, 2005). Sarcoids are known for their variability, leading clinicians to advise against biopsy in cases of suspected sarcoid due to its tendency to transform into a clinically more aggressive type.

In cases of detecting a large-sized sarcoid, veterinary doctors perform surgical intervention involving the complete neoplasm removal. This allows for macroscopic evaluation of the neoplasm structure, examining its appearance on the cross-section, and further studying the morphological structure. The histological diagnosis of sarcoid is based on the identification of fibroblastic proliferation and replacement of the loose connective tissue of the dermis papillary layer with thick collagen fibers. These fibers typically form formations from fibroblasts perpendicular to the epidermis basal membrane in the shape of a "picket fence" (Wobeser et al., 2010; Portenko & Shchebentovska, 2022; Portenko et al., 2023).

Even nowadays, many aspects of sarcoid biology in horses remain unclear. In Ukraine, there is a lack of information about sarcoids, their prevalence, and treatment methods, despite the rapid increase in the number of horses. Until there is a precise diagnosis for these neoplasms, the issue will remain relevant.

The aim of our research was to study the ultrastructural organization of fibroblastic sarcoids in horses.

Materials and methods

During the research, ethical requirements for the use of animals in experimental studies were strictly adhered to (Strasbourg, 1986; Kyiv, 2002). During 2022–2023, there was a sharp increase in the number of horses with sarcoids in Ukraine's western regions, namely by 15.6% compared to the years 2020–2021. This phenomenon was associated with the mass movement of animals from the eastern regions due to the onset of war in Ukraine. Various clinical types of sarcoids were anatomically localized predominantly in the abdominal wall, chest, and groin. Some of the neoplasms increased in size over several months. Surgically, tumours that significantly increased in size were removed by excising undamaged skin portions. All manipulations with the horses were performed under general anesthesia, adhering to the requirements of the European Convention for the Protection of Animals used for Experimental and Other Scientific Purposes. For ultrastructural research, fragments of sarcoids were collected from five horses. They were fixed in a 2% solution of osmium tetroxide in 0.1 M Millonig's phosphate buffer at pH 7.36 for 2 hours in a thermos at the temperature of melting ice. After fixation, they were rinsed in chilled Millonig's phosphate buffer, dehydrated in ethanol with increasing concentrations at 10% intervals, starting from a 70% ethanol solution in distilled water for 10 minutes each.

The dehydrated fragments were kept in three portions of absolute ethanol for 10 minutes each, then transferred to two portions of propylene oxide for 5 minutes, and then soaked for 24 hours in a mixture of araldite with the following composition: araldite M, hardener HY964 1:1, thoroughly mixed. To 20 mL of this solution, 0.4 mL of catalyst DY064 and 0.6 mL of dibutyl phthalate were added. The soaked fragments were transferred to polypropylene molds with a fresh araldite mixture for 24 hours at 60 °C for polymerization (Uikli, 1975). The formed blocks were trimmed into trapezoidal shapes and secured in a block holder using a glass knife. Semi-thin sections with a thickness of 1 µm and ultra-thin sections with a thickness of 90 µm were obtained using an ultramicrotome LKB-2188 (Sweden).

The sections were mounted on glass slides, heated on an LKB-2208 Multiplate device (Sweden), stained with methylene blue, counterstained with acid fuchsin, and then covered with a cover glass after adding a drop of synthetic balsam solution. Ultra-thin sections, retrieved from distilled water, were mounted on support grids, then dried in a thermostat at 60 °C during 24 h, followed by contrasting with lead citrate according to Reynolds and uranyl acetate (Koster & Klumperman, 2003). Samples were examined and photographed using a Tesla BS-500 transmission electron microscope at an accelerating voltage of 60 kV. Photo fixation was performed using FT-41P photographic film. Negative images were digitized using an Epson Perfection V500 photo scanner and associated software package.

Results

The ultrastructural investigation revealed that fibroblasts, fibrocytes, cross-sectional and longitudinal collagen fibers, and a significant number of capillaries constituted the main mass of the tumour formation. In malignantly transformed fibroblasts, a branched network of smooth endoplasmic reticulum, wide cisterns with pronounced vacuolization, and granular material were evident (Fig. 1a). The cytoplasmic matrix contained a substantial amount of ribosomes, occasional peroxisomes, and mitochondria. The outer and inner nuclear membranes were characterized by high electron optical density, with a clear space between the membranes. Heterochromatin with high electron density and well-defined fibrillar centers were prominent in the nuclei (Fig. 1a). Thin filaments were present in the cytoplasmic matrix. Collagen fibers were represented by numerous collagen fibrils with periodic alternation of denser and less dense matrix, resembling cross-striations (Fig. 1b).

The electronogram depicts a fibrocyte with numerous nuclear invaginations (Fig. 2a), and a sizable nucleus with a pronounced fibrillar center, indicating its high functional activity. In the fibroblast's karyoplasm, a marked expansion of the Golgi complex cisterns with the accumulation of osmiophilic material was visualized (Fig. 2a). In the cytoplasmic matrix, there is a significant amount of collagen fibers, round and oval mitochondria with fragmented cristae. Collagen fibers are available in close proximity to fibroblasts and fibrocytes (Fig. 2a).

Capillaries with electron-dense endothelial cells are positioned alongside fibroblasts and fibrocytes (Fig. 2b). Some nuclei exhibit numerous invaginations and contain electron-dense heterochromatin, with less prominent nucleoli. The endothelial cells' perikaryon contains a substantial number of mitochondria, well-developed Golgi complex, granular and agranular endoplasmic reticulum, polysomes, ribosomes, and myofilaments. The marginal portion of cells with uneven edges and well-defined pseudopodia is filled with vesicles. The lumen of the capillaries shows moderate electron density.

Light and dark endotheliocytes were present in the venous section of the blood capillary (Fig. 3a). The cytoplasm of light endotheliocytes contained myofilaments, occasional mitochondria, free ribosomes, peroxisomes, and vesicles of various sizes. The plasmalemma formed insignificant pseudopodia. The capillary lumen had a moderately electron-dense appearance (Fig. 3a). Among the cellular pool, segmented neutrophilic granulocytes with typical segmented nuclei were also identified (Fig. 3b). Heterochromatin was located perinuclearly, while euchromatin, to a lesser extent, occupied the central position with clearly defined intracytoplasmic granules of various shapes. In venules, endotheliocytes of moderate electron density were observed, with nuclei showing clear surface invaginations. The perikaryon contained a significant number of mitochondria, polysomes, and ribosomes. Nuclear pores were open. The space between the perinuclear cistern was not visualized (Fig. 3c).

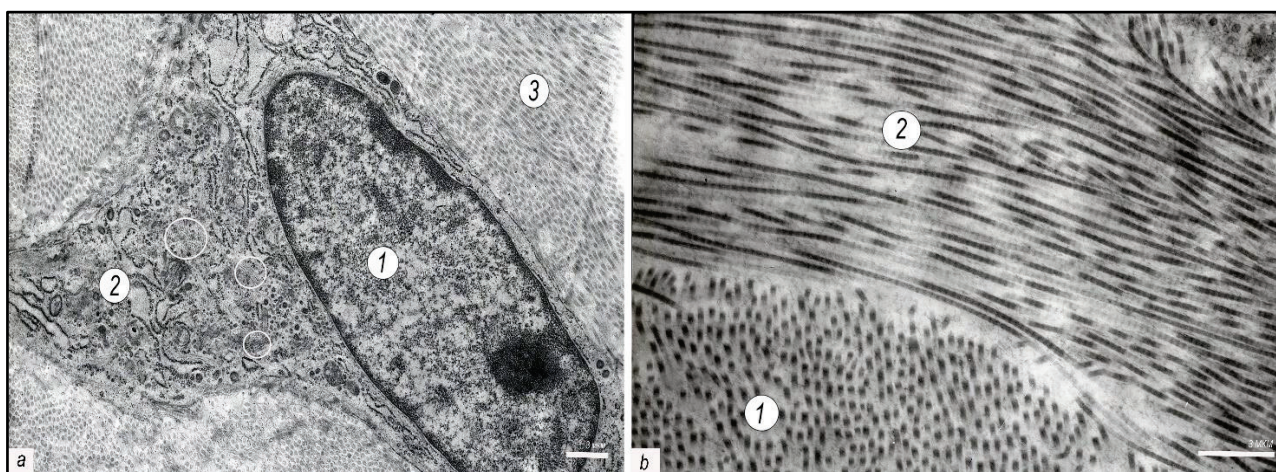


Fig. 1. Fibroblastic sarcoid: *a* – fibroblast: nucleus with a significant amount of euchromatin (1), endoplasmic reticulum with ribosomes (2), longitudinal collagen fibers (3), thin filaments in the cytoplasmic matrix (encircled), x8000; *b* – collagen fibers: cross-section (1), longitudinal arrangement (2), x14000

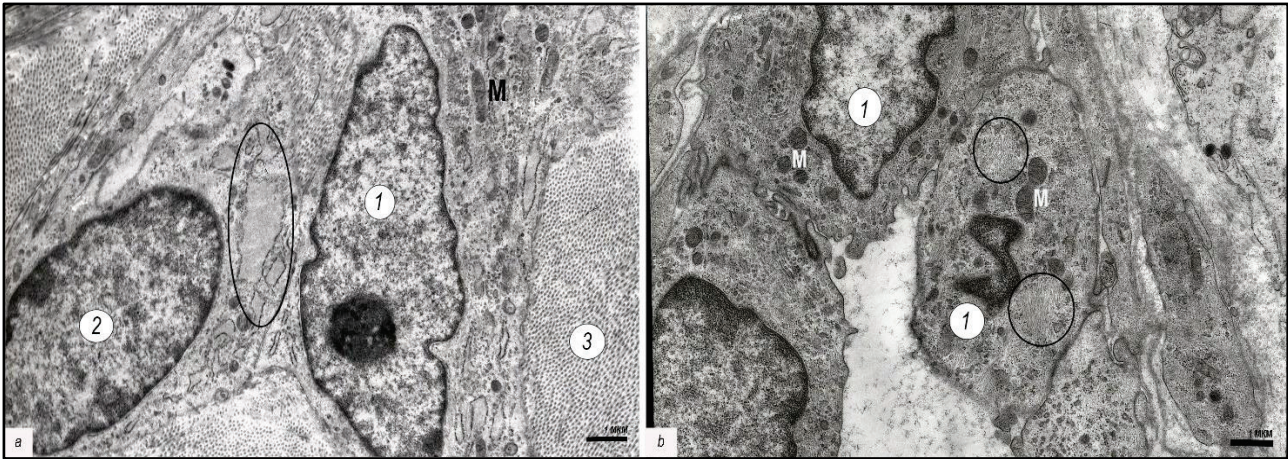


Fig. 2. Fibroblastic sarcoid: *a* – fibrocyte (1), fibroblast (2), cross-section of collagen fibers, marked expansion of the Golgi complex cisterns (in circle), mitochondria (M), x6000; *b* – capillary: endothelial cells (1), mitochondria (M), myofilaments (in circle), x8000

In most capillaries (Fig. 3d), both light and dark endotheliocytes with a clear perinuclear cistern were visible. The cytoplasm contained a substantial amount of polysomes, ribosomes, and lysosomes. Euchromatin predominated in the nucleus. Fibroblasts with spindle-shaped nuclei, open nuclear pores, pronounced perinuclear cistern, and a Golgi apparatus with expanded cisternae were located near the capillaries (Fig. 3d).

The fibroblastic type of sarcoid in all examined cases was characterized by the main pool of neoplastic tissue formed by fibrocytes and fibroblasts, between which a network of capillaries and venules developed. In some capillaries, endotheliocytes were of a round shape with oval nuclei; chromatin was compactly arranged, and some cells were without nuclei (Fig. 4a). Numerous mitochondria, ribosomes, and peroxisomes were visualized in the cytoplasm (Fig. 4a). The capillary space was filled

with masses of moderate electron density. Capillaries in which endotheliocytes lacked nuclei contained a significant number of small mitochondria, vesicles, and myofilaments. Granules were located in the cytosol. Intercellular spaces were narrow, and the cells themselves took on elongated forms (Fig. 4b). Intercellular contacts overlapped in the form of complex short junctions. The plasmalemma contained insignificant pseudopodia (Fig. 4b).

Fibrocytes with a well-defined nucleus and fibrillar centers were located in the neoplasm collagen matrix. Nuclear pores were open, and the perinuclear cistern was pronounced. A significant number of expanded cisterns of the Golgi complex and occasional mitochondria were visualized in the cytoplasm (Fig. 4c). An agranular reticulum was visible in the perikaryon.

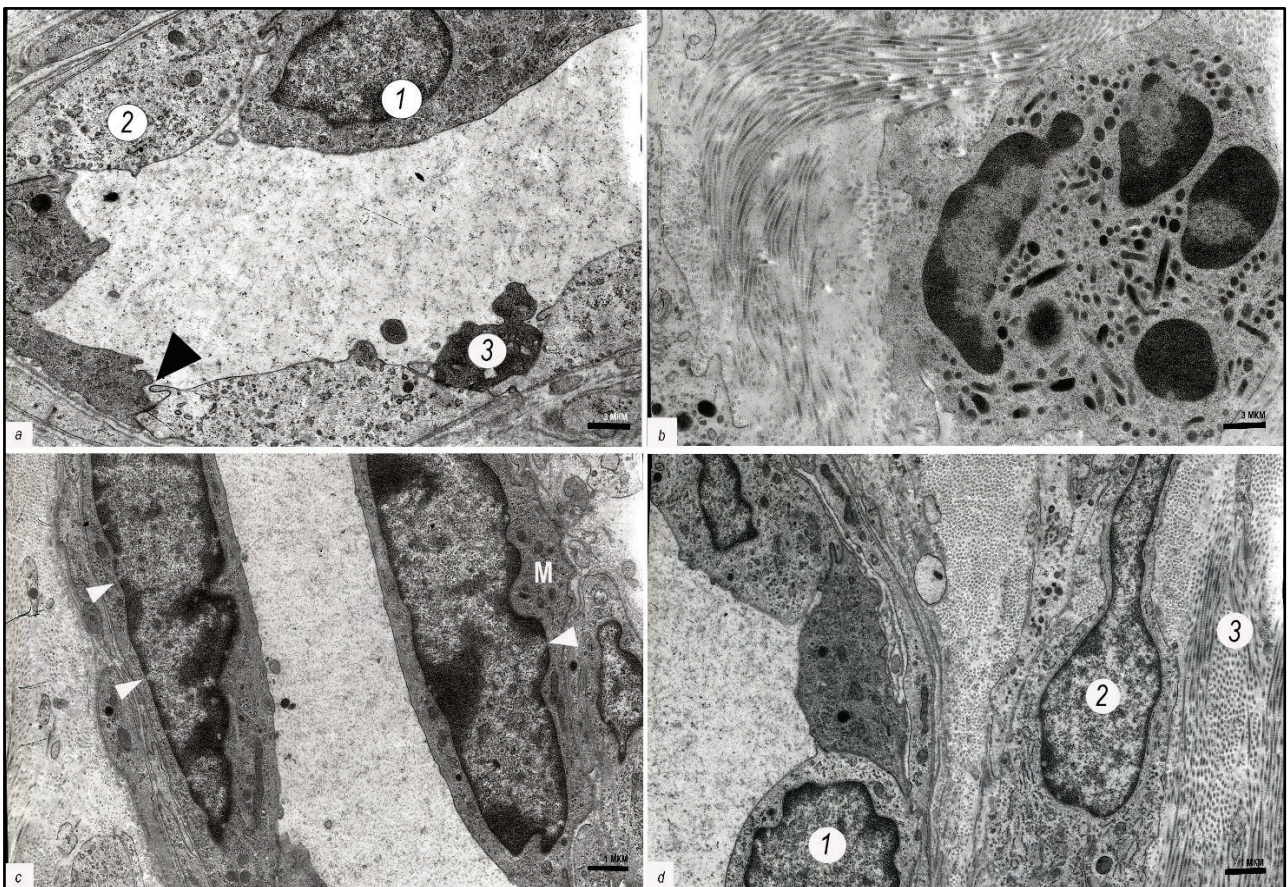


Fig. 3. Fibroblastic sarcoid: *a* – capillary: dark endotheliocyte (1), light endotheliocyte (2), platelet (3), pseudopodia (arrow), x8000; *b* – segmented neutrophilic granulocyte, x8000; *c* – venule: endotheliocyte nuclei with pronounced invaginations and open nuclear pores (arrows), mitochondria (M), x6000; *d* – capillary: endotheliocyte (1), fibroblast with spindle-shaped nucleus (2), collagen fibers (3), x6000

The fibroblasts' nuclei were somewhat rounded and also contained nucleoli with high electron density and pronounced fibrillar centers; heterochromatin was not pronounced. Euchromatin occupied the larger portion of the nucleus (Fig. 4d). The perinuclear cistern was pronounced, nuclear pores were open, and the nuclear membrane had slight invaginations. Expanded cisterns of the Golgi complex, ribosomes, occasional mitochondria, peroxisomes, and numerous vesicles were visible in the cytoplasm (Fig. 4d).

On electronograms, some nuclei of fibrocytes revealed electron-dense round formations (Fig. 5a). The perinuclear cistern was not pronounced. Myofilaments, open nuclear pores, and collagen fibers were visualized in the cytoplasm. In the perikaryon, expanded cisterns of the Golgi complex were noted, in which electron-dense inclusions were also found (Fig. 5b, c).

Therefore, the electron microscopic examination of the sarcoid revealed that the main neoplasm cellular pool is represented by fibroblasts of varying degrees of differentiation, predominantly immature with high activity, as indicated by the significant number of the Golgi complex

expanded cisterns and nuclei with numerous invaginations, open pores, and myofilaments in the perinuclear and intracytoplasmic spaces. The nuclei of fibroblasts, mostly of irregularly rounded shape, occasionally exhibited intranuclear spherical electron-dense formations. Nucleoli with pronounced fibrillar centers were electron-dense. Some nuclei had multiple nucleoli. Active formation of numerous capillaries was observed between the collagen matrix, fibroblasts, and fibrocytes, as indicated by the large number of endotheliocytes with pseudopodia on the marginal part and the basal surface of the cytoplasm. This suggests an embryonic type. Endotheliocytes had large round nuclei, and the cytoplasm contained a significant number of mitochondria.

Additionally, a high platelet activity was observed, manifested by cell adhesion to the marginal part of the endotheliocytes' cytoplasm. It is noteworthy that most capillaries belonged to the venous type, as evidenced by the height of the endotheliocytes, the presence of a significant number of mitochondria, and a large quantity of vesicles. Filaments were also detected in the cytoplasm.

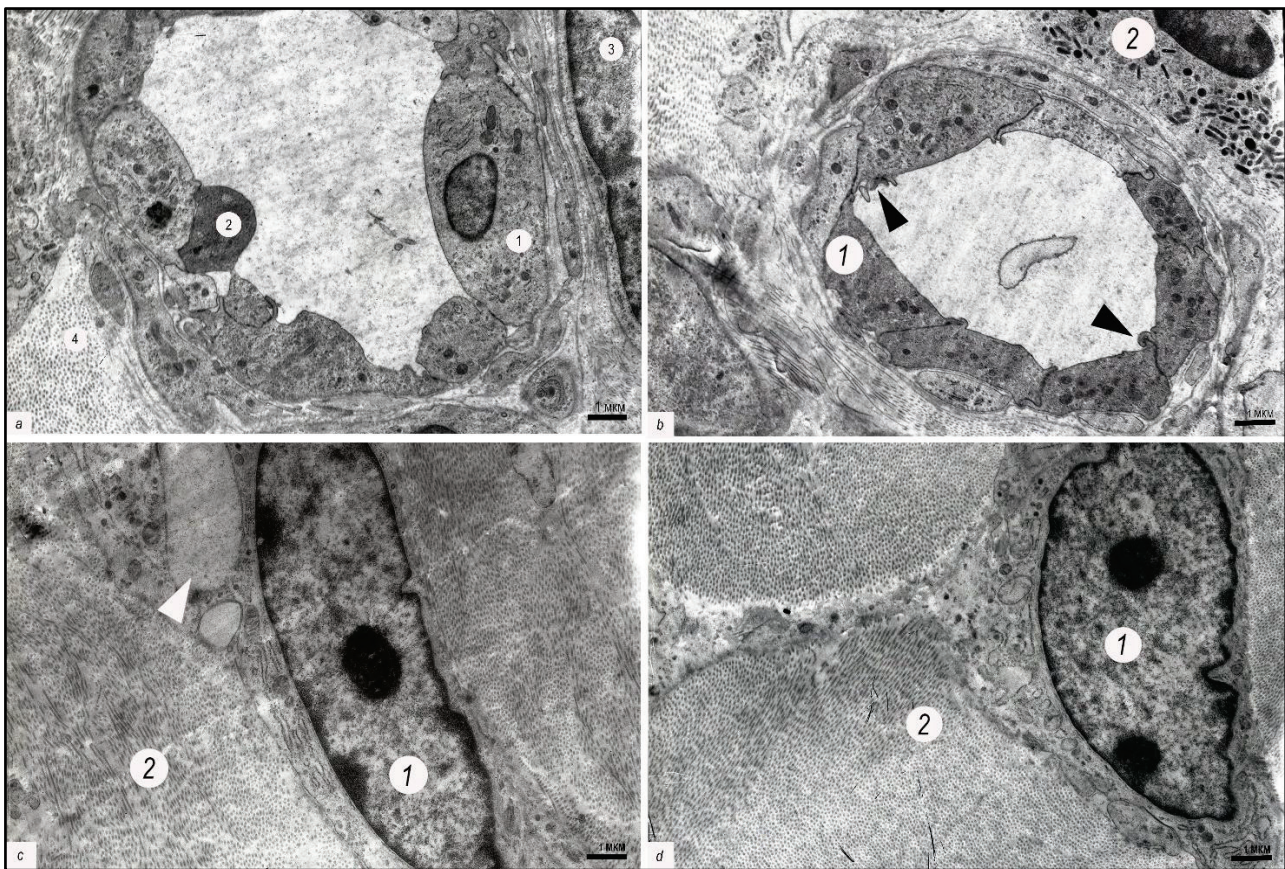


Fig. 4. Fibroblastic sarcoid: *a* – capillary: endotheliocyte (1), platelet (2), pericyte fragment (3), cross-section of collagen fibers (4), x6000; *b* – capillary: endotheliocyte with a significant number of mitochondria in the cytoplasm (1), segmented neutrophilic granulocyte (2), pseudopodia (arrows), x6000; *c* – fibrocyte: nucleus with a nucleolus with pronounced fibrillar centers, collagen fibers (2), expanded cisterns of the Golgi complex (arrow), x6000; *d* – fibroblast: nucleus with an electron-dense nucleolus (1), collagen fibers (2), x6000

Discussion

Nowadays, it is already known that virtually all tumours have their own blood supply. Previously, it was believed the vascular system of tumours is more developed than in normal tissues. This misconception arose because the vessels of neoplasms were predominantly large and, therefore, more visible than the smaller but functionally more efficient blood vessels of normal tissues. In the early 1970s, it became clear that the overall blood flow in tumours was significantly lower than in normal tissues, and tumours needed to induce vascular supply to grow from small to large sizes. It was also suspected that tumours induced their newly formed vascular system by secreting angiogenic factors, but the identity of these factors was just beginning to be investigated. In the following years, discussions arose about the molecular basis of angiogenesis, particularly the role of the angiogenic factor, i.e. the vascular endothelial growth factor. In the re-

search (Martano et al., 2016), a hypothesis was proposed that the vascular endothelial growth factor could play a key role in the development of sarcoids by altering the extracellular matrix homeostasis through the fibroblasts' population, leading to disruption of degradation and their excessive accumulation in the extracellular matrix (Martano et al., 2018). The excessive and progressive deposition of connective tissue (collagen) in sarcoids was not only a result of increased synthesis by fibroblasts but also contributed to matrix degradation through altered expression of matrix metalloproteinase and tissue inhibitor of metalloproteinase (Bao et al., 2009; Potocki et al., 2012; Potocki et al., 2014).

As a result of our studies on the fibroblastic sarcoid, we observed the endothelium functional heterogeneity within a single microvessel. In capillaries, three types of endothelial cells were identified: cells with brighter cytoplasm were young, cells with grey cytoplasm – mature, and cells with darker cytoplasm – old. Most endotheliocytes in capillaries were flat, com-

pletely lacking muscle elements. The basement membrane was intermittently interrupted. Perforations, i.e. intercellular gaps, were somewhere visualized. A significantly pronounced fibrillar component in the form of thin filaments was present in the endotheliocytes' cytoplasm. The majority of capillaries had wide lumens, and a significant number of vesicles were present in the cytoplasm, most of which were concentrated along the luminal edge. All these facts indicate the evidently venous type of capilla-

ries; therefore, most of the identified capillaries belong to the diffusion section of the microcirculation system. Accordingly, hematotissue exchange in this section is the most pronounced and adapted. It is also noteworthy there is a significant number of bright cells and platelets adhered to the luminal surface of endotheliocytes, indicating intensive angiogenesis in the tumour tissue. Substantial cellular infiltration by segmented neutrophilic leukocytes may also indicate immune reactivity.

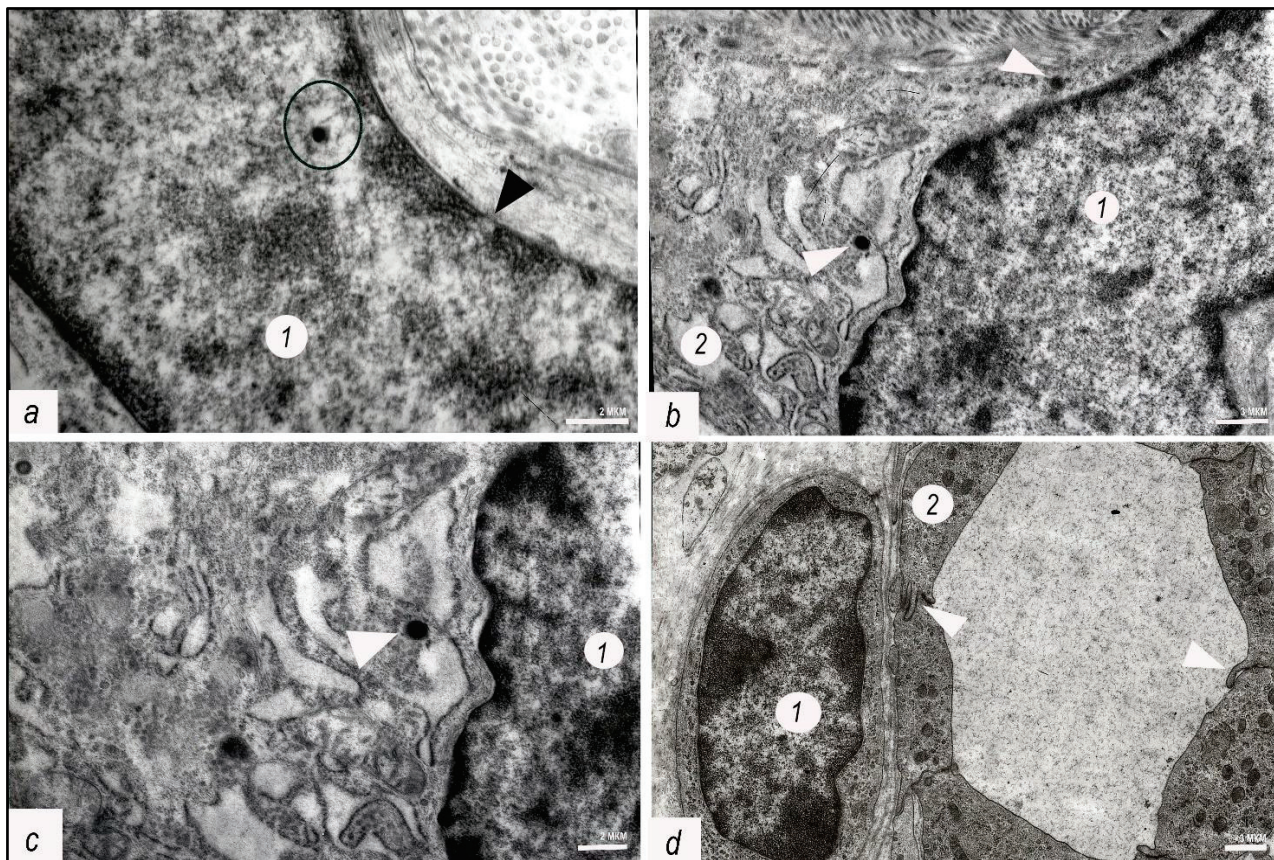


Fig. 5. Fibroblastic sarcoid: *a* – fibroblast nucleus (1), open nuclear pores (arrow), electron-dense round formation (encircled), x18000; *b* – fibroblast nucleus (1), expanded cisterns of the Golgi complex (2), electron-dense round formation (arrow), x10000; *c* – fibroblast nucleus (1), the Golgi complex with electron-dense round formation (arrow), x14000; *d* – capillary: pericyte (1), endothelial cells (2), pseudopodia (arrows), x8000

Conclusion

The fibroblastic type of sarcoid is characterized by a significant predominance of the fibroblastic cell population, the development of a branched system of venous-type hemocapillaries with pronounced angiogenesis. A substantial cytoskeleton of cells with numerous myofilaments was observed in endotheliocytes and fibroblasts. The entire cellular pool was localized amid a massive framework consisting of collagen fibers. Elastic fibers were not identified within the fibrillar component. The significant presence of blood platelets, i.e. thrombocytes adhering to the luminal surface of endotheliocytes, may indicate stimulation of proliferative activity in the endothelial component of the microcirculatory system.

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