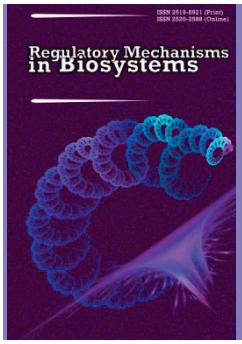

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Histological and immunohistochemical changes in equine sarcoids

M. Portenko, O. Shchebentovska, H. Blishch

**Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Lviv, Ukraine*

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Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies, Pekarska st., 50, Lviv, 79010, Ukraine. Tel.: +38-067-701-81-46. E-mail: schebentovskaolga@gmail.com

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The article presents the results of histopathological and immunohistochemical studies of three types of equine skin neoplasms, classified as type 2 fibroblastic sarcoid based on their morphological features. The tumours were localized in the abdominal area, macroscopically presented as dense, fleshy formations without a stalk, yet with small ulcers on the surface. Infection of horses could have occurred through direct or indirect contact with other infected horses and cattle, as well as through insects. Histological examination of the sarcoid established that the epidermis was in a state of hyperkeratosis and acanthosis, with the formation of numerous projections that penetrated deeply into the dermis. The boundaries between the papillary and reticular layers of the dermis were not visualized; the defining feature was the chaotic spindle-shaped fibroblasts that formed a significant number of dense whirl-like structures, or moire patterns, which encircled vessels of a varying caliber. The cell nuclei were predominantly round-shaped and hyperchromatic. Present were both typical and atypical mitotic figures. Replacement of loose connective tissue with collagen fibers was observed intradermally. Upon studying the morphological structure of the sarcoid on semi-thin sections, significant vascularization was observed. Spindle-shaped fibrocytes with branched processes and elongated nuclei were located around vessels of varying sizes, exhibiting well-differentiated single or double nuclei. Fibroblasts of various shapes and sizes with round nuclei were randomly distributed throughout the entire area of the neoplasm. The bulk of the sarcoid consisted of collagen fibers, which turned pink when exposed to methylene blue and further exposure to acid fuchsin. Immunohistochemical studies revealed intense vimentin-positive expression in the sarcoid cells, confirming the hypothesis of their mesodermal origin. Upon detecting Ki-67 antibodies in the sarcoids from three clinical cases, a significant number of cells in the G1 and S phases of mitosis were discovered; the cell nuclei and karyoplasm were stained brown. These actively proliferating cells of the neoplasm constitute the "growth fraction" of the tumour and point to a high risk of recurrence and malignancy.

Keywords: skin; fibroblasts; fibrocytes; proliferation; immunohistochemistry; Ki-67 marker; vimentin.

Introduction

Skin neoplasms in ungulates are a widely recognized global issue; however, the etiology of certain types is yet to be fully elucidated. Iran-based monitoring studies show that fibromas account for 38.4% of skin neoplasms in ungulates, followed by sarcoids (34.8%), squamous cell carcinomas (11.5%), melanomas (11.5%), and papillomas (3.8%). At the same time, 57.7% of the lesions were observed in donkeys, whereas horses accounted for 34.6% of the neoplasms, with sarcoids constituting 33.4% (Kaleibar et al., 2015). Other researchers claim that sarcoids are reported to account for 12% to 67% of equine skin neoplasms and 70% of all equine skin tumours (Lepage et al., 1998; Teifke & Weiss, 1991), while squamous cell carcinoma constitutes about 37% (Scott & Miller, 2003).

Jangir et al. (2013) described 16 small-sized skin lesions in horses, localized on the eyelids and various parts of the body. The histopathological research classified these cases as neoplastic (81.3%) and other (18.8%) conditions. Among them, benign tumours accounted for 62.5%, including myoma (37.5%), occult sarcoid (12.5%), fibromatosis (6.3%), and fibropapilloma (6.3%), while all malignant tumours (18.8%) were verified as squamous cell carcinoma. All skin biopsies were examined using the PCR method to identify the presence of BPV-1 and -2 DNA, resulting in 31.3% positive outcomes. Therefore, it is reasonable to agree with the viewpoint of scientists that sarcoid is a common equine skin tumour induced by papillomavirus (Knottenbelt & Matthews, 2001; Bogaert et al., 2010; Haralambus et al., 2010; Abel-Reichwald et al., 2016; Portenko & Shchebentovska, 2023). As of today, the majority of researchers have acknowledged that bovine papillomavirus (BPV) types 1 and 2 are indeed associated with the pathogenesis of sarcoid development (Nasir & Reid, 1999; Chambers et al., 2003; Nasir & Campo, 2008; Christen et al., 2014; Semik-Gurgul, 2021). Using a variety of molecular research methods, including Southern hybridization (Southern blot) and polymerase chain reaction (PCR), BPV DNA has been detected in 86% to 100% equine sarcoid cases (Trenfield et al., 1985; Angelos et al., 1991; Teifke & Weiss, 1991; Otten et al., 1993; Martens et al., 2001). Nasir & Reid (1999) demonstrated that in 95% of sarcoid cases where BPV DNA was detected using PCR, viral gene expression was also present. Carr et al. (2001) showed the presence of the BPV E5 protein in equine sarcoids they examined. Recently, a genetically related BPV13 has been found in equine sarcoids in Brazil (Jindra et al., 2021).

The relatively high frequency of BPV infection in apparently healthy horses living in close proximity to the papilloma-infected cattle points to the cross-species transmission of BPV from cattle to ungulates. 50% of horses that had direct contact with sarcoid-affected horses showed latent BPV infection (Bogaert et al., 2008). BPV-1 and -2 DNA have been detected in sarcoids globally, either separately or as a mixed infection of both types within a single horse (Teifke & Weiss, 1991, Nasir & Reid, 1999; Bogaert et al., 2007; Kidney & Berrocal, 2008; Yamashita-Kawanishi et al., 2021). In Europe, the majority of detected DNA is attributed to BPV-1, with only a small number of sarcoids containing BPV-2 (Otten et al., 1993). US-based research has shown that BPV-1 and -2 are distributed in approximately equal proportions in the eastern part of the United States (Teifke, 1994), while BPV-2 dominates the western part of the United States, accounting for 63% of amplified DNA (Carr et al., 2001). In western Canada, equine sarcoids are most commonly associated with BPV-2 (Carr et al., 2001; Wobeser et al., 2010). Since horses and cattle are often kept in shared facilities or graze on shared pastures, the potential for

cross-species transmission is always present. Unlike BPV infection in cattle, where complete viral particles are formed, horses experience non-productive infection. Hypotheses about equine infection include direct or indirect contact with other infected horses and cattle, as well as transmission through insects (Reid et al., 1994; Chambers et al., 2003; Gaynor et al., 2016). According to the researchers (Lazary et al., 1985; Meredith et al., 1986; Broström et al., 1988; Chambers et al., 2003), direct contact with the virus is insufficient for tumour proliferation. Skin trauma, compromised immune status, and genetic predisposition of certain horse breeds play significant roles in the proliferation of neoplasms (Lazary et al., 1985; Meredith et al., 1986; Broström et al., 1988; Chambers et al., 2003). According to one theory, sarcoids may develop in areas of chronic local skin damage due to physical trauma, which could activate latent BPV infection, lead to pathological wound healing, and scar formation, which can transform scar tissue into a sarcoid (Haddow, 1972; Chambers et al., 2003; Wobeser et al., 2012). Interestingly, the presence of BPV DNA has also been detected in normal horse skin, and it has been found that the virus is transcriptionally active in some cases of inflammatory skin conditions (Bogaert et al., 2005; Yuan et al., 2007). Most studies confirm the ability of sarcoids to transform from a mild condition to a more severe one or transition from one type to another while significantly affecting certain skin areas. In addition, veterinarians assert that 30% of neoplasms recur after prevention measures or treatment (Knottenbelt & Matthews, 2001; Knottenbelt, 2005). Due to the diversity of equine sarcoid tumours, effective and universal treatment methods do not currently exist. Most treatment methods are effective only for a specific type of tumour. Considering the aforementioned data, it can be concluded that a sarcoid is a tumour that typically has low metastatic potential but a high likelihood of recurrence after treatment. Some BPV genes influence host cell cytoskeletal proteins, which could also contribute to the migration of sarcoid cells into the surrounding layers of affected tissue (Rector & Van Ranst, 2013).

Clinically and morphologically, equine sarcoid has six different types, but some of them need to be differentiated from fibroma and fibrosarcoma (Knottenbelt, 2005). Fibrosarcoma typically grows rapidly and its consistency is firm upon palpation. As the neoplasm grows, it may become red in colour with an ulcerated surface, which closely resembles a sarcoid (Olson & Cook, 1951). Histologically, this tumour exhibits pronounced cellular structure: the majority of cells are pleomorphic, with high cytological atypia, and there are numerous mitotic figures, both typical and atypical. Furthermore, fibrosarcoma exhibits a marked disorganization in the arrangement of nuclei. Fibroblastic sarcoid, which is one of the most aggressive types, is characterized by intense dermal proliferation, especially in cases of large ulceration. It is composed of granulation tissue with a significant number of fibrous elements located at right angles to newly formed capillaries, which resemble palisade structures with noticeable inflammatory swelling and pronounced vascularity (Martens et al., 2000; Yuan et al., 2008; Epperson & Castleman, 2017; Portenko & Shchebentovska, 2022). As of today, systematic studies of equine skin lesions, including sarcoids, have not been conducted in Ukraine. In order to develop an effective treatment strategy for skin lesions in valuable sport horse breeds, it is necessary to perform biopsies of the affected skin tissues to classify the type of neoplasm.

The purpose of our study was to investigate the histopathological changes in sarcoids in three horses, apply the immunohistochemical analysis to detect the presence of the intermediate filament protein of connective tissue – vimentin – within dermal cells and assess the proliferative activity of fibroblasts using the Ki-67 marker.

Materials and methods

During the research, full compliance with ethical requirements regarding the use of animals in experimental studies was strictly observed (Strasbourg, 1986; Kyiv, 2002). Throughout the year 2022, small neoplasms were identified in three horses housed in a stable in Lviv region. The neoplasms were located in the lower third of the abdomen and gradually grew in size over several months. Upon gathering the anamnestic data from the animal owners, it was established that the ages of the horses ranged from 4 to 10 years. Initially, the neoplasms were of small size (3–4 cm), but over time, they grew to 6–8 cm with subsequent local ulceration due to constant trauma. The decision was made to surgically remove the neoplasms by excising the undamaged portion of the skin. All manipulations with the horses were performed under general anesthesia using analgesic drugs and adhering to all rules of asepsis and antiseptics, in accordance with the requirements of the European Convention for the Protection of Pet Animals. Upon removal, fragments of the neoplasms were immersed in a 10% neutral formalin solution, followed by embedding in paraffin using standard histological techniques. Seven-micrometer-wide histological sections were prepared from the paraffin blocks. These sections were then deparaffinized and stained with hematoxylin and eosin as per Ehrlich's hematoxylin and eosin staining method (Merkulov, 1969). In addition, immunohistochemical analysis was performed on the paraffin sections of the sarcoid to investigate the expression of Ki-67 and the vimentin activity.

For the preparation of semi-thin sections, the tumour fragment was fixed in a 2% osmium tetroxide solution 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, the samples were washed in chilled Millonig's phosphate buffer (Uikli, 1975), and dehydrated in increasing concentrations of ethanol for 10 minutes each, starting with a 70% ethanol solution in distilled water and increasing the concentration by 10%. The samples were subjected to three changes of absolute ethanol for 10 minutes each, followed by two changes of propylene oxide for 5 minutes each. Then, the samples were infiltrated with a mixture of Araldite resin composed of Araldite M and HY964 hardener in a 1:1 ratio, thoroughly mixed, and allowed to impregnate for 24 hours. Afterward, 0.4 mL of DY064 catalyst and 0.6 mL of dibutyl phthalate were added to 20 mL of this solution. The resin-impregnated fragments were then transferred into polypropylene moulds containing a fresh mixture of Araldite resin and allowed to polymerize at 60 °C for 24 hours while ensuring proper orientation (Uikli, 1975). The formed blocks were trimmed into a trapezoidal shape and securely attached to block holders using a glass knife. Semi-thin sections with a thickness of 1 micron were obtained using a LKB-2188 ultramicrotome (Sweden). These sections were mounted on glass slides and heated on an LKB-2208 Multiplate instrument (Sweden). The attached sections were stained with methylene blue with subsequent counterstaining with acid fuchsin (Hunter, 1993). Then, 1 drop of synthetic balsam solution was added and the sections were covered with a cover glass. The obtained specimens were examined using a Leica DM-2500 light microscope (Switzerland). Image capture was performed using a Leica DFC450C digital camera and Leica Application Suite Version 4.4 software.

Results

The clinical picture of three horses classified the neoplasms as localized type 2 fibroblastic sarcoid with a broad base and fleshy appearance, ranging from 6 cm to 8 cm in width (Fig. 1a, 1b), and with a firm consistency. During its growth, the sarcoid acquired a brown colour and lost its hair covering, resulting in subsequent soft tissue trauma and the formation of small local ulcers.

Histologically, the boundary between the epidermis and dermis was uneven, with numerous finger-like projections of the epidermis penetrating deep into the dermis and forming significant acanthosis. The stratum corneum was thickened, and the basal cells of the basal layer were cylindrical in shape with elongated nuclei, oriented vertically to the line separating the epidermis and dermis. Hyperkeratosis with elongation and widening of the epidermal papillae was observed (Fig. 2a). No granular layer in areas of cornification was detected. One of the characteristic features of fibroblastic sarcoid is the replacement of the loose connective tissue of the papillary layer of the dermis with thick collagen fibers, which typically form perpendicular formations to the basal membrane of the epidermis, creating a "palisade" appearance from fibroblasts. Anisocariosis and anisocytosis of fibroblasts were moderately pronounced. The boundaries between the papillary and reticular layers of the dermis were not visualized, and there were chaotic spindle-shaped fibroblasts present, forming a significant number of dense curls, or moire structures, which were located around vessels of varying calibers (Fig. 2c). The cell nuclei were mostly round and hyperchromatic (Fig. 2d). Both typical and atypical mitotic figures were present.

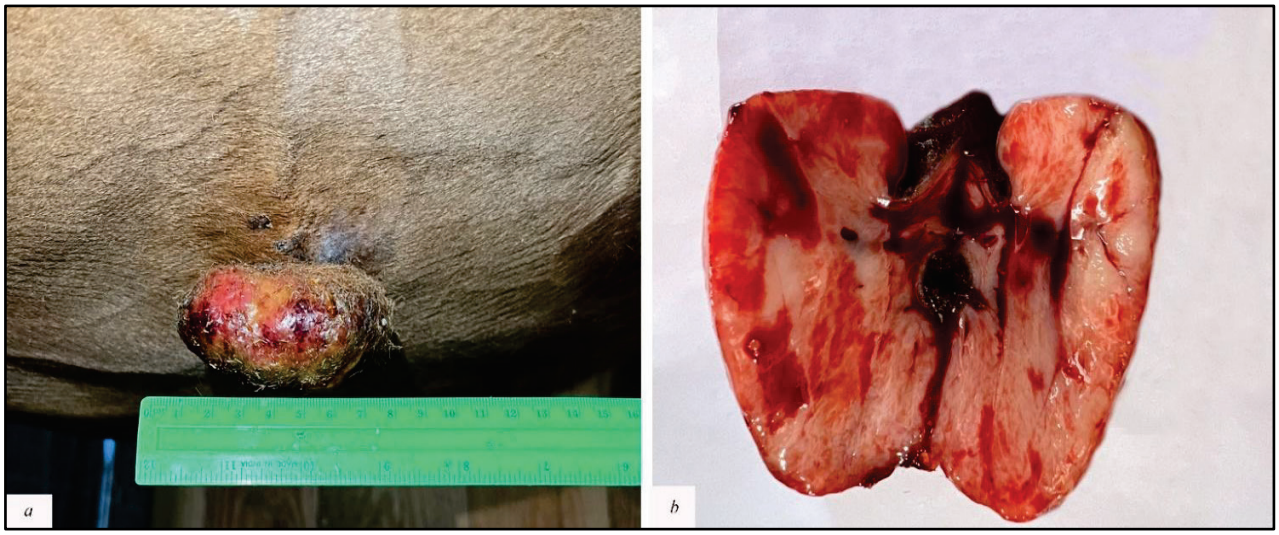

Fig. 1. Fibroblastic sarcoid type 2: large-sized tumour, localized in the lower third of the abdomen with a wide base, without a hair covering (*a*); cross-sectional view of the sarcoid – a dense fleshy formation of grey-white colour (*b*)

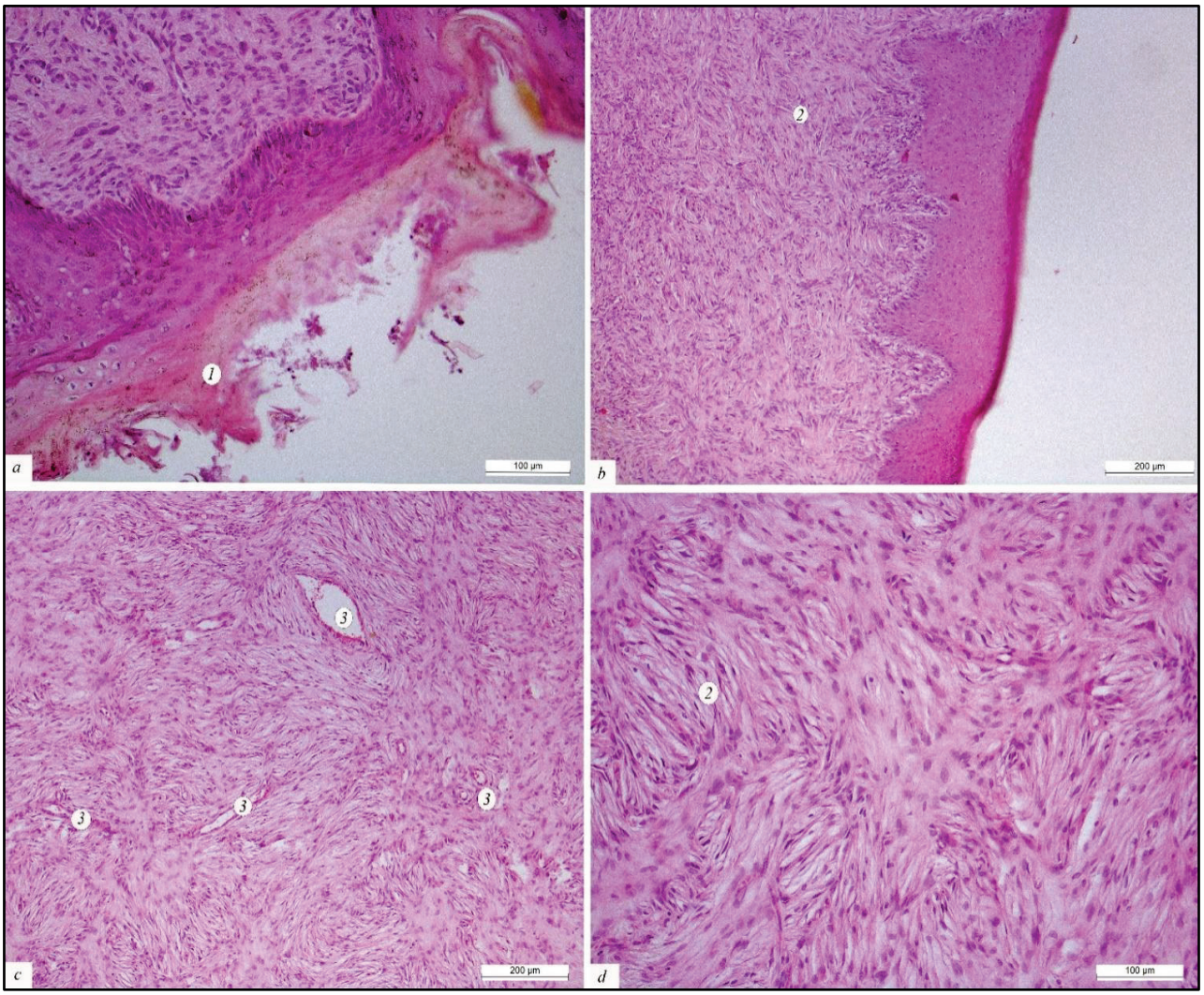

Fig. 2. Fibroblastic sarcoid: *a* – hyperkeratosis (*1*), *b*, *d* – formation of dense bundles of fibroblasts in the dermis (*2*), *c* – vessels of various calibers; hematoxylin and eosin staining

In semi-thin sections stained with methylene blue, elongated spindle-shaped fibrocytes with branched processes of various sizes are clearly visualized, contacting both with each other and with round nuclei of fibroblasts. The cellular membrane of fibroblasts and fibrocytes is well delineated, and nuclei have clearly defined one or two nucleoli (Fig. 3a, 3b). Fibrocytes contained elongated nuclei with minor invaginations. Upon additional staining of the sections of the sarcoid with acid fuchsin, it became clearly visible that the majority of the neoplasm is composed of collagen fibers, which are stained pink (Fig. 3c, 3d). Furthermore, it was established that the fibroblastic type of sarcoid exhibits well-defined vascularity, characterized by arteries, venules, and capillaries of varying sizes (Fig. 3d).

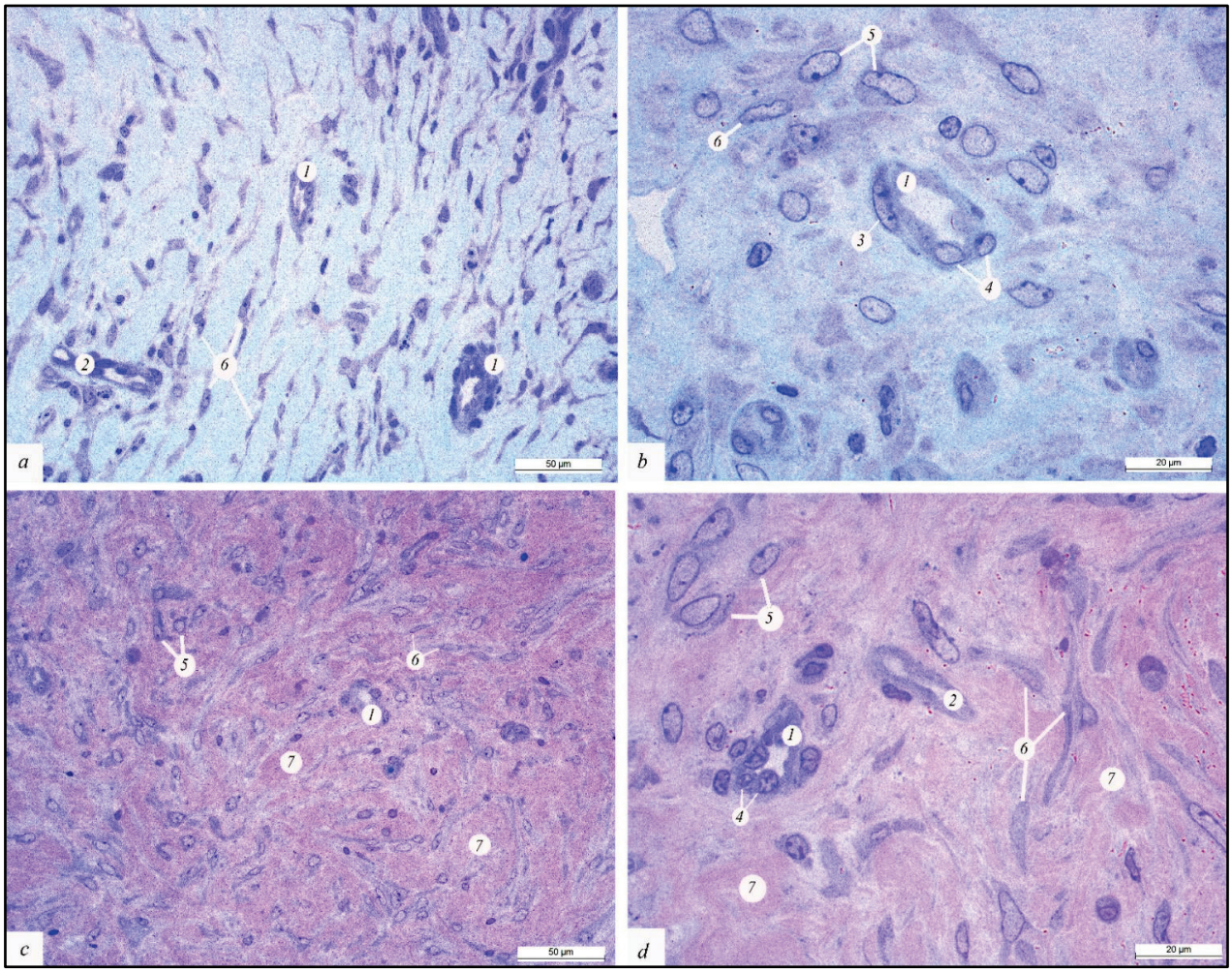

Fig. 3. A sarcoid (semi-thin section): fine morphological organization of fibroblastic type of equine sarcoid:
a, *b* – stained with methylene blue; *c*, *d* – methylene blue with counterstaining by acid fuchsin; significant tissue vascularity of the tumour formation (*a*, *d*);
1 – arterioles, *2* – venules, *3* – pericytes, *4* – endotheliocyte, *5* – fibroblasts (round nuclei), *6* – fibrocytes, *7* – collagen fibers (pink colour)

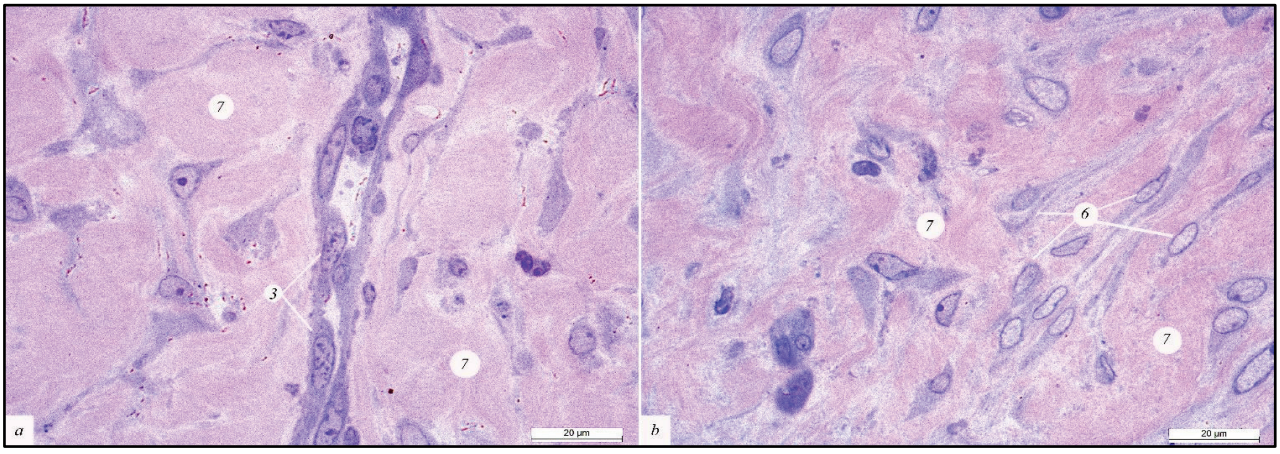

Fig. 4. A sarcoid (semi-thin section): *a*, *b* – the bulk of the neoplasm is represented by collagen fibers (*7*) and fibrocytes of varying shapes and sizes with distinctly round nuclei and cytoplasm (*6*); *3* – endotheliocyte; stained with methylene blue and counterstained with acid fuchsin

As is already established, each cell has a specific spatial internal and external shape, which can be quite dynamic depending on their functional load. For example, epithelial cells primarily utilize the protein cytokeratin in their cytoskeleton, while mesenchymal cells use vimentin. To determine the predominant cytoskeletal protein types in sarcoma, immunohistochemical typing was performed, which established that in all three cases of sarcoma, intense diffuse cytoplasmic expression of vimentin was observed. As shown in Figure 5, fibroblasts in the sarcoma exhibit intense vimentin expression, confirming its mesodermal origin. During the epithelial-mesenchymal transition, cytokeratin-positive epithelial cells start expressing vimentin, inducing stem cells and promoting tumour recurrence. The universal marker for assessing proliferative activity is the Ki-67 protein, which is a short-lived protein with a rapid turnover cycle. That is why antibodies to Ki-67 only detect dividing cells. Typically, the protein is not detected in either the G_0 phase of the cell cycle or at the beginning of the G_1 phase. Its level gradually increases during the S phase and reaches its maximum during mitosis. Microscopic examination of the sarcoid in all three cases revealed a sufficiently high level of Ki-67 expression, indi-

cating a high risk of recurrence and malignancy of the neoplasm. In Figure 6, it is evident that cells in an active phase of the cell cycle are distinctly stained in brown colour, both in their nuclei and cytoplasm. These actively proliferating cells of the neoplasm represent the "growth fraction" of the tumour.

Discussion

Numerous studies conducted in recent years indicate that sarcoid is a benign skin tumour that nonetheless affects the well-being of animals and their value. Since it does not spread to internal organs, affected horses can live perfectly normal lives, and with successful treatment, they can continue to work or be trained. The formation of sarcoid is also recognized as one of the major long-term complications during wound healing in horses (Hanson, 2008; Christen et al., 2014; Semik-Gurgul, 2021; Jindra et al., 2023). The authors speculate that on one hand in genetically predisposed healthy horses, BPV-1/BPV2 may be responsible for abnormal fibroblast proliferation, and on the other hand, it might lead to changes in the dynamics of the extracellular matrix and its key components (such as collagen). These changes could potentially alter the wound healing process and thus may play a significant role in the pathogenesis of the equine sarcoid (Martens et al., 2001; Jangir et al., 2013). The hypothesis that cancer may be a "wound that does not heal" has been supported by numerous studies (Dvorak, 1986; Schäfer & Werner, 2008; Haralambus et al., 2010), indicating that wound healing and tumorigenesis share histological similarities. Most researchers who have studied equine sarcoid suggest that a biopsy of the tumour leads to a marked deterioration and stimulates more aggressive fibroblast growth. The histological changes noted by the authors (Kraft & Austel, 1994; Portenko & Shchebentovska, 2022) are similar to those we observed in the three studied sarcoids, i.e. a dense dermal and hypodermal proliferation of spindle-shaped fibroblasts, which formed bundles interwoven with each other in the form of moiré structures. Typically, immunohistochemical studies assist in diagnosing sarcoids, although protein expression patterns are not considered highly specific. Certain researchers have noted that fibroblasts in sarcoids express vimentin, an intermediate filament that identifies the mesodermal origin of the neoplasm. They have also reported cases of positive reactions to protein components of the basement membrane of epithelial cells, including laminin, smooth muscle actin, and type IV collagen (Bogaert et al., 2011; Martano et al., 2016; Epperson & Castleman, 2017). Our research has revealed that in all three cases of fibroblastic sarcoid, there was intense vimentin expression, which is associated with cytoskeletal formation, regulation of signal transduction, and cellular adhesion (Bogaert et al., 2010; Yuan et al., 2010). For instance, laboratory studies conducted on mice have confirmed that the absence of vimentin is associated with impaired cell migration, while increased regulation of vimentin is closely linked to epithelial-mesenchymal transition in epithelial cell cultures, which plays a crucial role in cancer metastasis (Hanahan & Weinberg, 2000; Dave & Bayless, 2014; Battaglia et al., 2018). The observed differences in vimentin expression patterns may suggest that sarcoid cells exhibit higher expression levels of the analyzed gene. These findings could lead to the conclusion that sarcoid cells have an enhanced ability to migrate compared to fibroblasts.

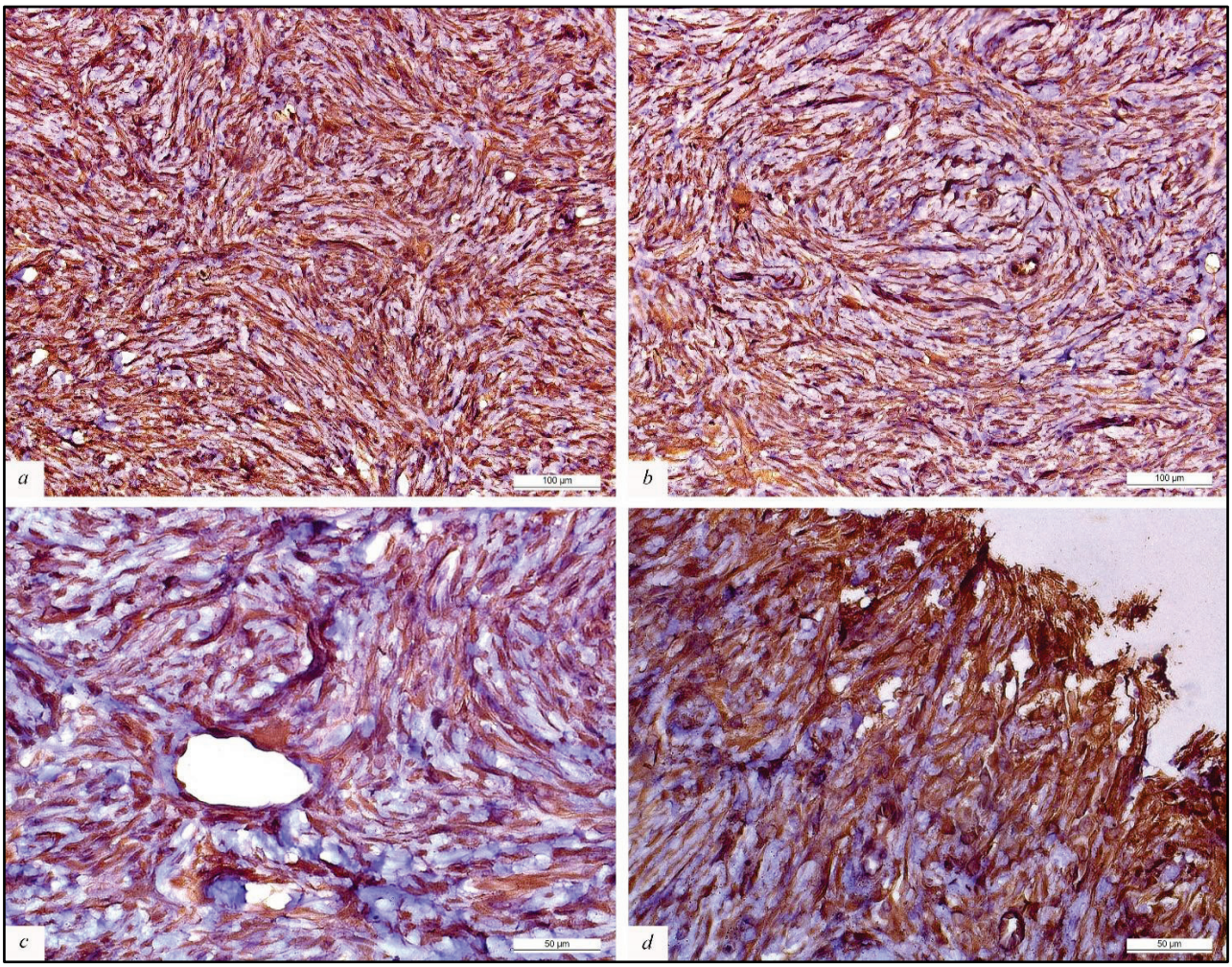

Fig. 5. Fibrous type of equine sarcoid: positive immunohistochemical reaction to vimentin; significant expression of vimentin by fibroblasts

Our histological and immunohistochemical studies of the three sarcoids revealed that fibroblasts constituted the main cellular population, with the remaining portion comprised of fibrocytes. In the semi-thin tissue sections of the sarcoid, there is an elevated content of collagen, which is chaotically and haphazardly distributed within the dermis. In their studies, Williams et al. (1982) noted that during normal healing of an aseptic skin wound, collagen type III is predominantly detected along with a significant number of myofibroblasts.

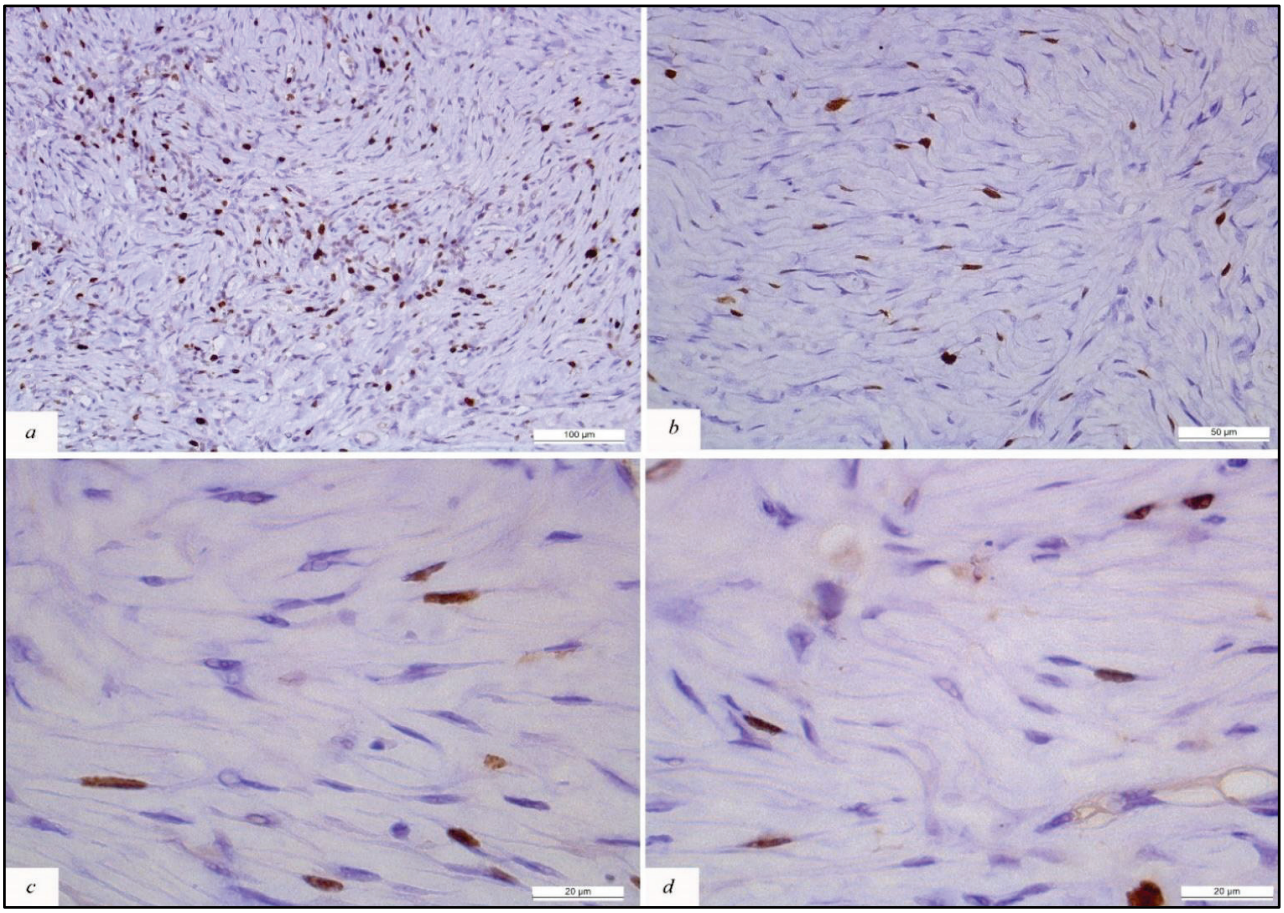

Fig. 6. Immunohistochemical typing of proliferative activity of connective tissue elements in fibrous equine sarcoid: *a*, *b*, *c*, *d* – intense mitotic activity of fibrocytes; Ki-67 marker

Based on our research findings as well as those of other scientists, it can be convincingly argued that sarcoid tumours indeed originate from mesenchymal cells, and the development of this neoplasm may result from stimulated proliferation of upper epithelial cells and fibroblasts in the dermis and hypodermis, leading to increased collagen production (Mohan et al., 2002; O'Toole et al., 2008).

Today, it is well known that Ki-67 is a non-histone nuclear protein that is expressed in proliferating cells during all active phases of the cell cycle. Increased expression of Ki-67 is consistently observed in inflammatory processes and malignant conditions. In the case of sarcoid, the risk of recurrence is directly linked to high expression of the proliferation factor.

Conclusions

As of today, there are no effective methods for treating equine fibroblastic sarcoids. Surgical excision of the neoplasm followed by histopathological and immunohistochemical verification and prognosis of possible metastatic risks remains the predominant approach. Based on the morphological and histological features observed in the three studied cases, the sarcoids were classified as fibroblastic type, characterized by a predominance of thick collagen fibers and the formation of diffuse moire patterns composed of fibroblasts and fibrocytes.

Immunohistochemical analysis revealed vimentin-positive reactions in fibroblast and fibrocyte cells within the dermis, as well as intense expression of the Ki-67 protein, indicating an active cell cycle phase encompassing G_1, S, and G_2 phases. These findings suggest rapid tumour growth and potential metastatic risks. Future investigations will focus on studying the ultrastructural characteristics of equine sarcoids.

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