

PATHOLOGICAL AND IMMUNOHISTOCHEMICAL CHARACTERISTICS
OF BILATERAL SMOOTH MUSCLE HAMARTOMAS IN BROAD LIGAMENT
OF A SLAUGHTERED RIVER BUFFALO (*BUBALUS BUBALIS*)
SHORT COMMUNICATION

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Abstract. This report is related to bilateral and symmetrical smooth muscle hamartomas (SMH) in broad ligament of a 5-year-old slaughtered river buffalo. The reddish pink masses with triangle-shaped and fleshy consistence had occupied mesovarium of broad ligament. Microscopic findings in histopathologic sections revealed smooth muscle bundles elongated randomly which appeared haphazardly and there was fibroadipose tissue between them in variable amounts. The nuclei of smooth muscle cells were cigar shaped, vesicular and had rounded blunt endings. Neither mitotic figure nor pleomorphism was seen in the nuclei of smooth muscle cells. Also, prominent vascular proliferation was present in some areas of stroma. No degenerative changes, necrosis neither inflammation was noted. Immunohistochemical examinations showed strong positive reaction for smooth muscle actin, desmin and faint positive reaction for S100. Meanwhile the special staining for estrogen, progesterone, vimentin, cytokeratin, von Willebrand factor and CD34 were negative. According to the macroscopic, microscopic and immunohistochemistry findings, the diagnosis of SMH was made. As best of our knowledge, there are no reports on SMH in broad ligament of animals. The present report for SMH of broad ligament in river buffalo seems to be the first one.

Keywords: broad ligament, river buffalo, smooth muscle hamartoma, pathology, immunohistochemistry.

AZIJOS BUIVOLĖS (*BUBALUS BUBALIS*) PLAČIAJAME GIMDOS RAIŠTYJE APTIKTOS
BILATERALINĖS LYGIOJO RAUMENS HAMARTOMOS
PATOLOGINĖ IR IMUNOHISTOLOGINĖ CHARAKTERISTIKA
TRUMPAS PRANEŠIMAS

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Santrauka. Šiame straipsnyje aprašoma 5 metų Azijos buivolės plačiajame gimdos raištyje aptikta bilateralinė simetriška lygiojo raumens hamartoma. Rausvas trikampio formos mėsingas darinys buvo susiformavęs plačiojo gimdos raiščio kiaušidžių pasaite. Mikroskopinės histopatologinių pavyzdžių analizės metu nustatytos lygiajame raumenyje netvarkingai išsidėsčiusios įvairaus dydžio pailgos pūslės ir tarp jų esantis fibroadiposinis audinys. Lygiojo raumens ląstelių branduoliai buvo pailgos vezikulinės formos su apvaliu buku galu. Branduoliuose nebuvo matyti nei mitozinių figūrų, nei pleomorfizmo. Kai kuriose stromos vietose buvo ryškiai matoma vaskuliarinė proliferacija. Kokie nors degeneraciniai pokyčiai, nekrozė ar uždegimas nepastebėti. Imunohistocheminės analizės metu nustatyta stipri teigiama reakcija lygiojo raumens aktino ir desmino atžvilgiu ir silpna teigiama reakcija S100 atžvilgiu. Tuo tarpu rezultatai nustatant (dažant) estrogeną, progesteroną, vimentiną, citokeratiną, von Willebrando faktorių ir CD34 buvo neigiami. Remiantis makroskopinės, mikroskopinės ir imunohistocheminės analizės rezultatais, diagnozuota lygiojo raumens hamartoma. Kiek mums žinoma, iki šiol gyvūnų lygiojo raumens hamartoma nebuvo aprašyta. Manome, kad šis straipsnis yra pirmas, kuriame aprašoma Azijos buivolės plačiajame gimdos raištyje aptikta lygiojo raumens hamartoma.

Raktažodžiai: platusis gimdos raištis, Azijos buivolė, lygiojo raumens hamartoma, patologija, imunohistocheminė analizė.

Introduction. Smooth muscle hamartoma (SMH) is a tissue abnormality which is known to be originating from smooth muscles fibres. According to previous clinical reports, this impairment (SMH) in man is usually manifested in the form of congenital skin lesions as indurated macule or plaque. Typically, SMH is located on the trunk as thick bundles of smooth muscle fibres scattered throughout the dermis and sometimes extending into the subcutaneous tissue (Yamaguchi et al., 2004). According to our knowledge, no evidence of malignant transformation has been reported for SMH, whether congenital or acquired, show no evidence of malignant transformation and do not appear to be associated with other congenital abnormalities (Eda et al., 2007). Most of the hamartomas are detectible in very early ages and in neonates. Moreover, its development in most of the species correlates with those of the environmental tissues (Sugiyama et al., 2007). Due to the importance of SMH and its low distribution in domestic animals, the current histo-pathological examinations were considered as complementary studies for clinical observations which were demonstrated in a 5 years old non pregnant multiparous slaughtered river buffalo. Since smooth muscle hamartoma is a tumour-like lesion and it is difficult to differentiate it from other lesions and tumours of the muscles such as leiomyoma, we combined immunohistochemistry and histopathology for the confirmation of smooth muscle hamartoma in broad ligament of river buffalo (*Bubalus bubalis*).

Case description

During a daily clinical examination of the animals which were considered for slaughtering, a 5 years old river buffalo was marked to impair from SMH. The clinical appearance of the buffalo was examined for fever, vaginal secretions, heart monitoring, rumen activities, rectal touch and food consumption. No fever was detected and the animals' feeding was evaluated normal. No problem was manifested in heart, rumen activities. No vaginal secretions and changes were observed. Through a diagnostic rectal touch, symmetrical bilateral masses were detected on broad ligament.

After slaughtering, the uterus and ovaries were dissected out and the surrounding tissues were cleaned carefully. Pair of masses bilaterally demonstrated on the ligaments. The reddish pink masses which occupied a triangle-shaped region measuring $11 \times 9 \times 3$ cm in mesovarium of broad ligament had a "fleshy" consistency in palpation. The thickness of the affected areas was 3 cm which was 3-4 times larger than its normal form in healthy animals (Fig. 1). The ovaries were in dioestrus stage of reproductive cycle and corpus luteum 2–2.2 cm in size in the left ovary was observed. The ovaries were normal and implantation sites and cystic structures were not detected. No remarkable uterus abnormalities were manifested in macroscopic examinations.

Materials and methods. The tissue samples (sexual organs) were dissected out. The masses were dissected and fixed in 10% neutral buffered formalin fixative through histopathological investigations. The histological sections were prepared in 5µm thickness by rotary

microtome. For general observations the Haematoxylin and Eosin (HE) and Masson's trichrome staining techniques were used.

The procedures of immunohistochemical staining were performed on the basis of standard protocol of manufacturer. Method of staining was the Envision plus dual link system. Stages of immunostaining which were performed included slicing of the tissue into 3 µm thick sections. The slices then were put on the silanized slides S3003 (Dako, USA) for drying and attaching them together for 1 hour. Next the slides were put in xylene, absolute and 96% ethanol and washed. The tissue sections were placed in Tris - EDTA Buffer (TBS, pH 9.0) and immersed by 3% H₂O₂ in methanol for 10 minutes for quenching endogenous peroxidase. Then the slides were transferred into microwave for antigen retrieval. First the machine was set to maximum power to take the buffer to spot welding. Then microwave power was reduced to 40% and the sections were incubated in this situation for 15 minutes. Since discharging the sections with buffer, they were incubated at room temperature to cool them down. For blocking of the sections, they were incubated in TBS owning 5% bovine serum albumin (BSA) for an hour (Alkafafy et al., 2010). After washing by water and TBS, environments of tissues were defined by Dako pen. They were incubated in a dark humid room and surface of tissues coated by primary antibody perfectly. All of the primary antibodies which were used, were ready-to-use (RTU) formation including: monoclonal mouse anti-human estrogen receptor α (ER α) (clone: 1D5, Dako, USA), monoclonal mouse anti-human progesterone receptor (PR) (clone: 1A6, Dako, USA), monoclonal mouse anti-human α -smooth muscle actin (clone: 1A4, Dako, USA), polyclonal anti-human von willebrand factor (vWF) (clone: F8/86, Dako, USA), monoclonal mouse anti-human CD34 (clone: BL-3C5, Dako, USA), monoclonal mouse anti-cow vimentin (clone: vim 3B4, Dako, USA), monoclonal mouse anti-human cytokeratin (clones: AE1/AE3, Dako, USA), monoclonal mouse anti-human desmin (clone: D33, Dako, USA), monoclonal mouse anti-human p53 protein (clone: D33, Dako, USA), polyclonal rabbit anti-cow S100 protein (Dako, USA). After washing by TBS for 5 minutes and incubating in humid room, the sections were coated by labeled polymer with peroxidase (secondary antibody). The temperature of humid room was 37 °C. Then the sections were washed by TBS for 5 minutes 2 times. For staining tissue surfaces were coated by chromogen plus 3,3-diamino benzidine (DAB) substrate solution (1 drop chromogen in 1 ml substrate) and incubated in humid room in which the temperature was 37 °C for 10 minutes. Other procedures were performed routinely and the sections counterstained by Harris haematoxylin for 1 minute and the slides mounted by mounting media.

We used the human tissues immunoreaction positivity of which had been confirmed previously (Ruiz et al., 2005). These tissues consisted of: normal breast (Estrogen, Progesterone); skeletal muscle (Vimentin, Desmin); skin epidermis (Cytokeratin); blood vessel (CD34, vWF); peripheral nerve (S100); smooth muscle

(SMA) and squamous cell carcinoma (P53). Negative controls were performed by omitting the primary antibody (Alkafafy et al., 2010). For internal positive control, normal blood vessels in broad ligament tissue were spotted for detecting of smooth muscle markers (Ramos-Vara et al., 2008).

Results. Haematoxylin-Eosin staining showed that the smooth muscle bundles elongated randomly which appeared haphazardly and there was fibroadipose tissue between them in variable amounts. The nuclei of smooth muscle cells were identified as cigarette-shaped and with vesicular and rounded blunt endings. There was no mitotic figure and pleomorphism in the nuclei of smooth muscle cells. Also, there were vessels of variable sizes which were numerous in various regions of tissue stroma. No degenerative changes, necrosis or inflammation were identified under light microscopic examinations. In Masson's trichrome stained-sections the fibrous tissue was manifested surrounding the smooth muscle cells and as well on the septa between smooth muscle masses (Figure 2). Remarkably powerful reactions of smooth muscles actin and desmin with significantly faint reactivated sites for S100 were demonstrated in immunohistochemistry examinations (Figure 3). Further investigations showed negative reactivated sites for estrogen, progesterone, vimentin, cytokeratin, von Willebrand factor and CD34 as well.

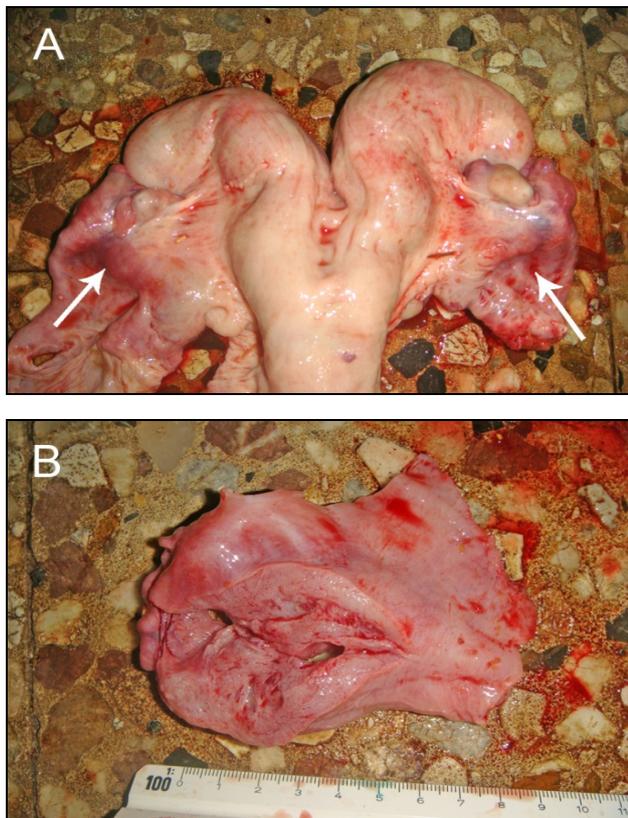


Fig. 1 A – Macroscopic appearance of bilateral masses in mesovarium of broad ligament (arrows). B – Reddish pink fleshy consistent masses with triangle-shaped region and 3–4 times larger than its natural forms in thickness

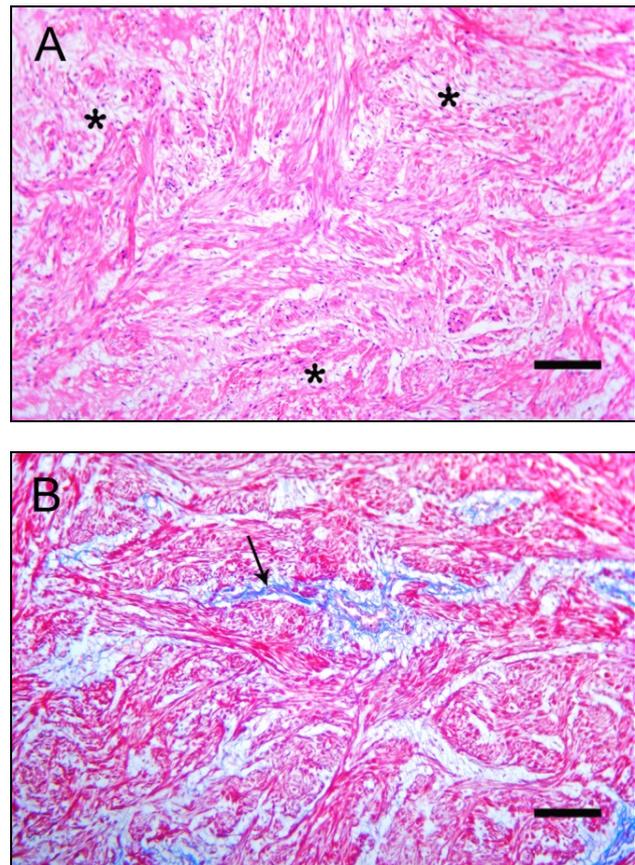


Fig. 2. A – Smooth muscle hamartoma with haphazardly arranged smooth muscle bundles and fibro-adipose tissue (asterisks) and variable size of vessels between them. H&E; B – Blue – stained fibrous tissue (arrow) surrounding the smooth muscle cells and in the septa between the smooth muscle cells. Masson's trichrome. Scale bars = 200 μ m

Discussion. According to previous reports, the genital neoplasm such as leiomyoma, fibroma and squamous cell carcinoma can impair artificial insemination attempts and also is capable to cause dystocia (Sendag et al., 2007; Meyers and Read 1990). Meanwhile the impacts of tumor-like lesions (hamartomas), especially SMH, on animal reproduction and genital tract's physiological functions have not been defined for certain until now.

Anatomically the broad ligaments consist of double serosal folds which are containing muscles, connective tissue, nerves and blood vessels between the folds. The musculature structures are well developed in adult non-pregnant buffaloes broad ligaments. In normal condition no difference can be identified between the amount of musculature in the right and left side ligaments. In contrast, during pregnancy on the pregnant side the broad ligament consists of relatively larger muscle fascicles with frequent syncytial appearances of muscle fibers. According to the fundamental structure of broad ligaments in buffaloes, they appear to be the cause of uterine torsion. (Brar et al., 2008a). Brar et al. (2008b) has reported that in uterine torsions (mostly in buffaloes with uterine torsions) the muscular development of broad

ligament is significantly lower than those of dysocia cases. In general, it could be concluded that the greater part of the buffalo population have weak broad ligament (with thinner musculature between folds). Thus these animals are highly susceptible for uterine torsions.

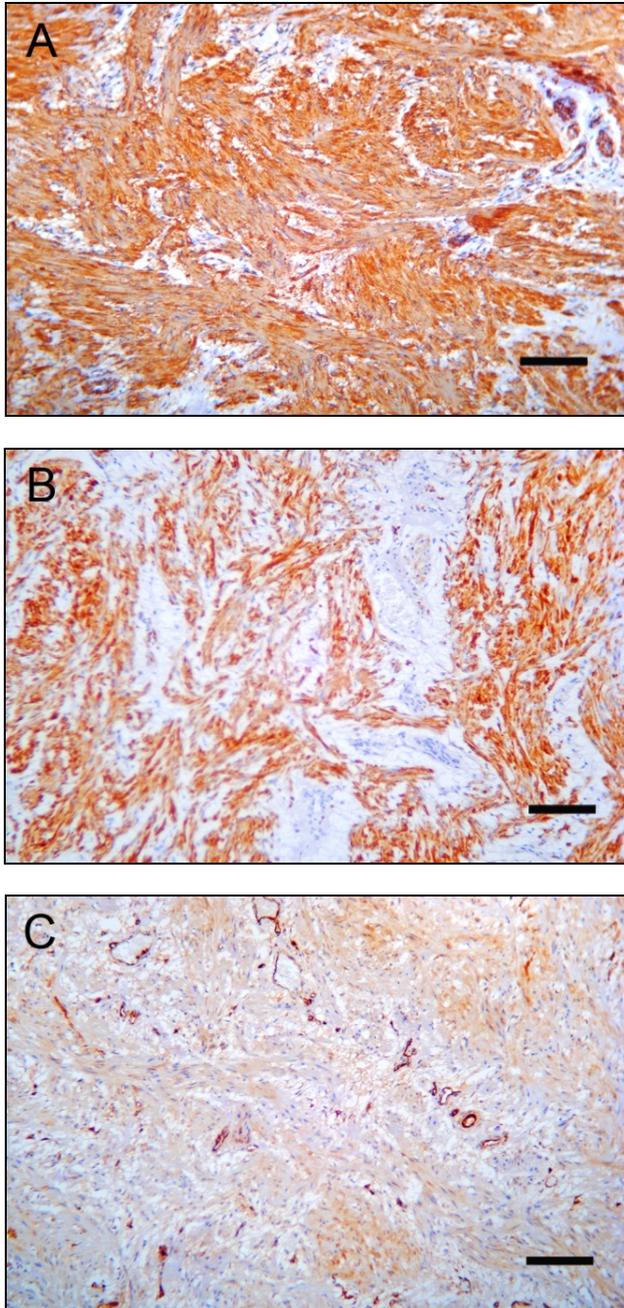


Fig. 3. Immunohistochemical labeling of smooth muscle hamartoma in the broad ligament. A – Strong positive reaction for smooth muscle actin. B – Cytoplasmic immunoreactivity for desmin. C – faint positive reaction for S100 in lymphatic vessels and scattered smooth muscle cells. Scale bars = 200 μ m

Previous reports show that the predisposing factors for both torsion of the uterus and prolapse of the vagina or uterus in buffaloes are anatomical in origin. Different

factors such as relatively long uterine ligaments and low distribution of smooth muscle cells in the broad ligament can be suggested (Noakes et al., 2001). On the basis of these hypotheses, we suggest that increase of smooth muscle cells in the broad ligament may cause dystocia and if smooth muscle hamartoma spreads to the uterus, it may impair artificial insemination.

Our immunohistochemical analyses showed faint reaction for smooth muscles actin (SMA) and as well for desmin. Thus we can conclude that the masses have originated from broad ligament's smooth muscles. On the other hand, the negative response for vimentin corroborated this theory very well and clarified that the bilateral masses are not tumorigenic. Further investigations such as vWF and CD34 confirmed all previous findings and revealed that the vascular structures in the masses are not originated from vascular neoplasm like haemangioma. Also the negative response to vWF and CD34 showing normal single layered endothelium with non dysplastic vessels, explained that the vessels of masses are not tumorigenic.

Negative or faint reaction for cytokeratin denotes another characteristic which suggested that the masses started from smooth muscles and the endothelial cells did not participate in lesions formation. Beside these findings, the absence of p53 immunoreaction, illustrated that no cell proliferation occurred. On the other hand, immunoreaction for estrogen and progesterone was negative. Also, the absence of follicular cysts on the ovaries shows independence of masses growth of steroidal hormones.

SMH must be differentiated from tumors such as leiomyoma and hyperplastic form of smooth muscle (physiologic). In this case, on the basis of macroscopical findings (unencapsulated and uncircumscribed), immunohistochemistry (negativity of ER, PR and vimentin markers) and histopathological appearance (presence of angio-lipo-fibromatous elements between smooth muscle bundles and disorientation of smooth muscle fibers) masses were diagnosed as SMH.

Kooten et al. (2004) assumed that various pathogeneses may attribute to hamartoma in human cases including: disorders of tissue embryogenesis and reaction to neoplastic cells. On the other hand, Lee and Maeda (2009) suggested that acquired proliferative reactions occur in patients with hematologic malignancy and as well in congenital malformations of spleen hamartoma. Also Stringer and Alizai (2005) reported further pathogenesis for mesenchymal hamartoma in liver which include: developmental, vascular, toxic insult and neoplasia. Most of the reports indicated that the pathogenesis of mesenchymal hamartoma is indistinct (Kafarnik et al., 2010). Referring to veterinary-reports, the pathogenesis of SMH has not been determined yet. Although cutaneous smooth muscle hamartoma in human is congenital and/or acquired, the researchers suggest CD34 positivity of the spindle cells (dendritic cells) which indicates its congenital nature (Saadat et al., 2007; Kooten et al., 2004). In contrast, in the veterinary medicine there is no classification for congenital or

acquired forms of hamartoma in animals (Eda et al., 2007). According to our knowledge, it has not been identified that SMH is congenital or acquired. However in authors' opinion if it is a primitive disorder according to the absence of clinical signs, developmental anomalies in genital tissues and animal age it seems that this lesion is acquired although its pathogenesis was not determined.

In a report about nasal vascular hamartoma in a domestic shorthair cat an inflammatory process was suggested as the probable factor that initiated its growth (Chambers et al., 2010). In our case, inflammatory reactions were observed in all genital organs. Although in this animal, SMH might have been caused by bleeding and bacterial infection and thickening of broad ligament. Yet we suggest that inflammation, especially its chronic type, in genital system may cause tumor like proliferation such as SMH.

There are few reports for SMH such as: smooth muscle hamartoma of the abomasums in a calf (Yamaguchi et al., 2004), smooth muscle hamartoma of the nipple in a dog (Eda et al., 2007) and gastric smooth muscle hamartoma in a cat (Smith et al., 2010). But no types of hamartoma in broad ligament of animals have been reported until now and this is the first report to authors' knowledge.

Conclusion. Smooth muscle hamartoma is a rare tumour-like lesion in humans and animals. There has been no report of SMH in broad ligament of animals yet. The causes of the lesion have not been defined for sure. According to special situation with the buffalo's broad ligament, it seems that smooth muscle hamartoma may cause disorders such as dystocia or artificial insemination impairs and even infertility compared with other animals. Contrarily, SMH may decrease the risk of torsion because of increasing smooth muscle fibres in broad ligament of buffaloes.

Acknowledgements

The authors are thankful to Dr. Mazdak Razi (Department of Comparative Histology & Embryology, Faculty of Veterinary medicine, Urmia University) for his assistance in editing the manuscript.

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Received 23 May 2012

Accepted 20 March 2013