REPUBLIC OF TURKEY
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF VETERINARY PATHOLOGY

ECAM AND TYROSINE KINASES EXPRESSION PROFILING OF CANINE & FELINE NATURALLY OCCURRING SOFT TISSUE SARCOMAS AND 3-MCA INDUCED SARCOMAS IN MICE TREATED WITH PHOSPHODIESTERASE-5 INHIBITOR

DOCTORAL THESIS

Ishtiaq AHMED

Samsun

February – 2018

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The thesis prepared by **Ishtiaq Ahmed** entitled as ***"ECAM And Tyrosine Kinases Expression Profiling of Canine & Feline Naturally Occurring Soft Tissue Sarcomas and 3-MCA Induced Sarcomas in Mice Treated with Phosphodiesterase-5 Inhibitor"*** has been supervised by **Prof. Dr. Mahmut SÖZMEN** and accepted by the panel as DOCTORAL thesis following the examination on the date of 09/02/2018.

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ÖZET

KEDİ VE KÖPEKLERDE DOĞAL YUMUŞAK DOKU SARKOMLARI VE 3-MCA İLE SARKOM OLUŞTURULARAK PHOSPHODIESTERASE-5 İNHİBİTÖRÜ UYGULANAN FARELERDE ECAM VE TİROZİN KİNAZLARIN EKSPRESYON PROFİLLERİ

Amaç: Bu çalışma, köpek ve kedi yumuşak doku sarkomlarında ve farelerde 3-MCA ile indüklenen rhabdomyosarkomlarda PDGFA, PDGFR-alfa, integrin alfa v ile E-selektin ekspresyonları ile tadalafilin kemoterapötik etkilerinin değerlendirilmesi amaçlandı.

Materyal ve Metot: Çalışmada, 25 (14 kedi ve 11 köpek) olgu incelendi. Ayrıca, 60 deney faresinde tümör oluşturuldu. Fareler 6 gruba ayrıldı. Üç grup kontrol grupları olarak kullanıldı, diğer üç grupta tümör oluşturuldu; bunlardan birisi, tadalafil ile intraperitoneal ve biri de intratümöral olarak 14 gün süreyle tedavi edildi. Köpek, kedi ve fare tümörlerinin klasifikasyonun için bir dizi antikor kullanıldı. Daha sonra tümörler, PDGFA, PDGFR-alfa, integrin alfa v ve E-selektin ile boyandı.

Bulgular: Kedilerde 13 tümör enjeksiyon bölgesi fibrosarkomu, bir tanesi mikzoid liposarkom, köpeklerde ise 10'u fibrosarkom, biri ise mikzosarkom olarak teşhis edildi. Kedi ve köpek tümörlerinde PDGFA, PDGFR-alfa, integrin alfa v ekspresyonlarının yüksek E-selectin salınımının ise düşük olduğu belirlendi. Farelerde, sırası ile PDGFA, PDGFR-alfa, integrin alpha v, ve E-selectin salınımlarının %88, %58, %96 ve %46 olduğu görüldü. IP grubundaki tümör hücrelerinde PCNA ekspresyonunun daha differensiye oldukları ve PCNA salınımının daha düşük olduğu görüldü.

Sonuç: Köpek ve kedi yumuşak doku sarkomları ile 3-MCA ile oluşturulan fare sarkomlarında tirozin kinazlar ile ECAM'ın arttığı görüldü. Tadalafilin ise 3-MCA ile oluşturulan RMS'larda önemli bir terapötik etkisinin olmadığı belirlendi

Anahtar Kelimeler: Yumuşak doku sarkomları, 3-MCA, tadalafil, PDGF, İntegrin, E-selektin

Ishtiaq AHMED, Doktora Tezi

Ondokuz Mayıs Üniversitesi-Samsun, Şubat-2018

ABSTRACT

ECAM AND TYROSINE KINASES EXPRESSION PROFILING OF CANINE & FELINE NATURALLY OCCURRING SOFT TISSUE SARCOMAS AND 3-MCA INDUCED SARCOMAS IN MICE TREATED WITH PHOSPHODIESTERASE-5 INHIBITOR

Aim: The objective of the current study was to determine the expression of PDGFA, PDGFR-alpha, integrin alpha v and E-selectin in the canine and feline soft tissue sarcomas and in 3-MCA induced rhabdomyosarcomas in mice, and evaluation of chemotherapeutic effects of tadalafil.

Materials and Methods: The study was conducted on the 25 (14 cats & 11 dogs) cases of soft tissue sarcomas. Furthermore, tumor induced in 60 mice. Three groups served as control while tumor was induced in other 3 groups; one was tumor control and others were treated with tadalafil intraperitoneally or intratumorally for 14 days. A panel of antibodies was used to classify the tumors of dog, cat and mice. Later tissue sections were stained with PDGFA, PDGFR-alpha, integrin alpha v and E-selectin.

Results: Thirteen tumors in cats were diagnosed as injection site fibrosarcoma and 1 as myxoid liposarcoma while 10 tumors in dogs were fibrosarcoma, 1 was myxosarcoma. PDGFA, PDGFR-alpha, integrin alpha v was upregulated in cat and dog tumors while E-selectin expression was low. In mice, PDGFA, PDGFR-alpha, integrin alpha v and E-selectin expression was observed in 88% and 58%, 96% and 46% tumors, respectively. Tumor cells in IP group was more differentiated and showed less PCNA expression.

Conclusion: Tyrosine kinases and ECAM are upregulated in canine & feline soft tissue sarcomas and 3-MCA induced RMS and tadalafil does not have significant chemotherapeutic effects against 3-MCA induced RMS at the dose rate used in the current study.

Keywords: Soft tissue sarcomas, 3-MCA, tadalafil, PDGFs, Integrin, E-selectin

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ABBREVIATIONS

CAF	**:**	Cancer associated fibroblasts
cAMP	**:**	Cyclic adenosine monophosphate
cGMP	**:**	Cyclic guanosine monophosphate
CNP	**:**	C- type natriuretic peptide
ECAM	**:**	Endothelial cell adhesion molecules
FAK	**:**	Focal adhesion kinases
FNCLCC	**:**	Federation Nationale des Centers de Lutte Contre le Cancer
GFAP	**:**	Glial fibrillary acidic protein
HE	**:**	Hematoxylin and eosin
H-score	**:**	Histo score
IHC	**:**	Immunohistochemistry
IL-1	**:**	Interleukin 1
MCA	**:**	Methylcholanthrene
MMP	**:**	Matrix metalloproteinase
MyoD	**:**	Myogenic differentiation
PBS	**:**	Phosphate Buffered saline
PCNA	**:**	Proliferating Cell Nuclear Antigen
PDE-5	**:**	Phosphodiesterase-5
PDGF	**:**	Platelet-derived growth factor
PDGFR	**:**	Platelet-derived growth factor
PKA	**:**	cAMP dependent protein kinase
PKG	**:**	cGMP dependent protein kinase
PNST	**:**	Peripheral nerve sheath tumor
pRB	**:**	Retinoblastoma protein
RMS	**:**	Rhabdomyosarcoma
SFKs	**:**	Src- family tyrosine kinases
SMA	**:**	Smooth muscle actin
Sr. Actinin	**:**	Sarcomeric Actinin
STSs	**:**	Soft tissue sarcomas

TNF- α	**:**	Tumor necrosis factor α

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1. INTRODUCTION

Cancer occupies an important place in human and animal diseases and is associated with significant morbidity and mortality in both these species (Withrow et al., 2013). It can be classified either on the basis of histology/tissue of origin or anatomical location in the body. Tumors in animals and humans share similarities and thus research on animal oncology can be beneficial not only for animal health but also for humans (Vail and Macewen, 2000; Di Cerbo et al., 2014). Therefore, the current project was designed to study soft tissue sarcomas including rhabdomyosarcoma (RMS) in the companion animals and mice model.

1.1. Soft Tissue Sarcomas in Cats and Dogs

Soft tissue sarcomas (STSs) include a group of heterogeneous tumors having similar histological features and biological behavior. STSs originates in connective tissue including muscles, neuro-vascular, adipose, fascial and fibrous tissue giving rise to benign or malignant growths and accounts for approximately 15% and 7% of all the skin and subcutaneous tumor cases in dogs and cats, respectively. This group of tumors normally occurs in middle to old aged animals with no breed or sex predisposition, generally (Liptak and Forrest, 2013). This group includes peripheral nerve sheath tumor (PNST, non-brachial plexus), myxosarcoma, liposarcoma, perivascular wall tumor, pleomorphic sarcoma (anaplastic sarcoma with a giant cell or malignant fibrous histiocytoma), malignant mesenchymoma, undifferentiated sarcoma, and fibrosarcoma. (Dennis et al., 2011). Some authors do include RMS in this category (Liptak and Forrest, 2013).

Generally, these mesenchymal tumors have infiltrative or expansive nature but metastasis is infrequent. Mitotic count, histological grade and completeness of excision margins are considered determinants of the recurrence (Hendrick, 2016). Where complete surgical margins envisage reduced recurrence, histologically grade III tumors have a higher likeliness of recurrence and metastasis while high mitosis index is associated with reduced survival time (Dennis et al., 2011). Only 5% of the recurrence is reported in patients with tumor cell-free surgical margins, while the ratio is 25% for the cases with incomplete resected margins (Hendrick, 2016).

Fibrosarcoma is a tumor having malignant spindle-shaped cells and mostly arise from the cutaneous, subcutaneous tissue, or oral cavity. Tumors can represent the variable degree of differentiation ranging from well-differentiated to poorly differentiated with a high number of pleomorphic cells and mitotic figures. The tumor is generally locally infiltrative with rare metastasis. Mostly old aged animals are at high risk of developing tumor with no breed or sex predilection; however, one publication reports higher predisposition of Golden retrievers and Doberman pinschers. Oral fibrosarcoma is biologically high-grade tumor with the capability to invade deeper tissues, growing large in size and up to 20% metastasis (Liptak and Forrest, 2007; Hendrick, 2016). A retrospective analysis of the cases in the Swiss canine cancer registry revealed that out of 121,963 patients, 63,214 (51.83%) were diagnosed as neoplastic and fibroma/fibrosarcoma was diagnosed in 3.40% dogs with soft tissue and skin being the major site of occurrence. The breed predisposition was noticed for Setter, Swiss mountain dog, Rottweiler, Labrador retriever, Dobermann, Boxer, and German shepherd (Gruntzig et al., 2015). Analysis of the 38 years' archive of skin tumors indicated that fibrosarcomas account for 1.73% of the cutaneous neoplasms in dogs in USA (Villamil et al., 2011) while in cats incidence was 15% (Miller et al., 1991). A study from Greece reports that 9.8% of skin mesenchymal tumors in cats fall into the category of fibrosarcoma (Kaldrymidou et al., 2002).

Histologically, well-differentiated fibrosarcomas manifest spindle-shaped cells having interwoven or herringbone pattern (Williamson and Middleton, 1998). Fibrosarcomas need to be differentiated from some other soft tissue sarcomas like haemangiopericytoma and schwannoma. Routinely histopathological tool is used for diagnosis and assessment of the degree of differentiation of various soft tissue sarcomas but immunohistochemistry and cDNA microarray is more useful to confirm the histogenic origin of neoplasm (Klopfleisch et al., 2013). In humans, fibrosarcoma with a typical herringbone pattern is a well-studied subject and termed as adult fibrosarcoma. The tumor has been classified into various clinically, morphologically and genetically distinct subtypes like low-grade fibromyxoid sarcoma, sclerosing epithelioid fibrosarcoma, dermatofibrosarcoma protuberans, and fibrosarcomatous dermatofibrosarcoma protuberans (Folpe, 2014). While in animals, to the best of our knowledge, there is no such classification exists.

A new entity of the fibrosarcoma known as vaccine-associated sarcoma was first described in 1991 by Hendrick and Goldschmidt. The development of the tumor was initially related to the administration of the vaccine (especially rabies) having aluminum-based adjuvants. However, recent advancements in the literature suggest that other foreign materials injected in the subcutaneous tissue or muscles can lead to a neoplastic growth following chronic inflammation. Hence vaccine-associated sarcomas are named as injection site sarcomas (Martano et al., 2011). Though most of the injection site sarcomas are fibrosarcomas other tumors like malignant fibrous histiocytoma, osteosarcoma, RMS, chondrosarcoma, and liposarcoma have also been observed by some authors (Hendrick and Brooks, 1994; Esplin et al., 1996; Chang et al., 2006). The predilection sites for feline injection site sarcoma are neck, thorax, lumbar, flank region, and limbs (Hendrick, 2016). The risk for the tumor development increases with the number of injections at the same site (Kass et al. 1993). The temperature of the vaccine (cold injection) prior to administration may also increase the risk of tumor development. Various drugs especially long-acting penicillin and methylprednisolone acetate (Kass et al., 2003), lufenuron an insecticide used for flea control in animals (Esplin et al., 1999) as well as suture material (Buracco et al., 2002) have also been mentioned as risk factors for sarcoma development in cats. Most of the tumors develop between 1-3 years post injection but may be detectable as early as 3 months and as late as 13-15 years (McEntee and Page, 2001; Wilcock et al., 2012).

Injection site sarcomas in the younger cats are more aggressive and had higher recurrence incidence as compared to non-injection site sarcomas (Hendrick et al., 1994). Injection site fibrosarcomas have also been reported in ferrets (Munday et al., 2003). A fibroblastic tumor with typical features of post-injection sarcoma has been observed in the cat (Daly et al., 2008) and dog (Vascellari et al., 2006) at the site of a microchip implant. Injection site sarcomas in both cats and dogs are usually located in the subcutis and have peripheral infiltration of lymphocytes. Most of these have myofibroblastic immunophenotype (Vascellari et al., 2003). Chronic inflammation is believed to play a significant role in the pathogenesis of these injection site sarcomas by DNA damage, cellular transformation, and clonal expansion. Expression of matrix metalloproteinases including membrane-type matrix metalloproteinases in the feline injection site sarcomas indicates the

role of inflammation in the pathogenies of these tumors (Sorensen et al., 2004). Genetic studies focusing the role of p53 gene have indicated that an allele with a single thymidine nucleotide insertion in intron 7 (T3) bore a very strong association with injection site fibrosarcomas in cats (Banerji and Kanjilal, 2006; Banerji et al., 2007). However, another study from Germany on 150 cats (100 healthy and 50 feline injection site sarcomas) reports no significant difference in T insertion at SNP3 of p53 gene between healthy and cats with tumors (Mucha et al., 2014). DNA damage is a feature of feline injection site sarcomas as γH2AX (2.18-33.7%, median 16.2%) expression was observed in cats suffering from feline injection site sarcomas (Kang et al., 2017).

Liposarcoma is a malignant tumor of the adipose tissue with rare occurrence in animals. Though there is no internationally accepted nomenclature for the sub-classification of liposarcomas in animals, it can be divided into well differentiated, anaplastic (pleomorphic) and myxoid variants (Hendrick, 2016). Round cell variants of the myxoid liposarcoma have also been reported in a dog (Plumlee et al., 2016) and a Japanese Macaque (Kwon et al., 2007). Incidence is more in dogs as compared to other animal species. Liposarcoma may occur spontaneously (Kabak et al., 2011) or as a result of some foreign intervention like vaccine injection in cats (Esplin et al., 1996) or microchip implant and glass foreign body in dogs (McCarthy et al., 1996; Vascellari et al., 2004). Metastasis has been reported to lungs (Esplin et al., 1996) and kidney (Cramer et al., 2011) and many other organs (Diep and Fleis, 2012) in cats.

Myxosarcoma is a rare tumor of middle to old aged dogs and cats, characterized by myxoid matrix rich in mucopolysaccharides (Hendrick, 2016). Histologically, the tumor is composed of the spindle to stellate cells loosely arranged in a mucopolysaccharide rich matrix (Headley et al., 2011). Histological pattern of myxosarcoma has close similarity with myxoid variants of liposarcoma and PNST. Special stains like oil red o and S-100 may help in the differential diagnosis of these variants (Hendrick, 2016). Myxosarcoma is a locally infiltrative tumor with rare metastasis, a case of pulmonary metastasis was observed in a dog (Headley et al., 2011).

1.2. Rhabdomyosarcoma

Rhabdomyosarcoma which is excluded by some authors from the category of soft tissue sarcomas is an important malignancy in the human medicine. A neoplastic growth with the ability to differentiate into skeletal muscle lineage is termed RMS. The tumor may originate not only from resting myoblasts or satellite cells in the skeletal muscles but also from the primitive mesenchymal cell in the organs where the skeletal muscle is normally absent. The children and young adolescents are more affected as compare to adults. According to WHO, the tumor is classified into alveolar, embryonal, botryoid, pleomorphic and spindle-shaped or sclerosing variants (Fletcher et al., 2002; Jo and Fletcher, 2014). Histological classification is of prognostic importance in humans. The tumor is rather rare in animals. The classification scheme used in the human medicine is also acceptable in veterinary medicine. RMS has been reported in dogs (Yhee et al., 2008), cats (Miller et al., 2009), horses (Castleman et al., 2011), pigs (Vos et al., 1993), cattle (Kajiwara et al., 2009; Ulrich et al., 2014), steer (Taylor et al., 2002), rabbit (Park et al., 2016), birds (Gulbahar et al., 2005; Freundt Coello and Schaeffer, 2014), and rats (Chang et al., 2008). Gross pathology of embryonal RMS is described as a mass with pale, white to tan color, fleshy firm in consistency. Necrosis and hemorrhage may be evident (Cooper and Valentine, 2016).

Histologically, two patterns can be identified in embryonal RMS, either consisting of mainly large round to polygonal cells (rhabdomyoblasts) with deeply eosinophilic cytoplasm intermixed with small round cells with scant cytoplasm or those composed of primitive myotubes. However, there may be considerable pleomorphism and binucleated or multinucleated cells, and strap cells can be observed. Large rhabdomyoblasts have large, vesicular nuclei with one or more prominent nucleoli while small cells have a more basophilic nucleus (Cooper and Valentine, 2016). Botryoid is considered a subtype of embryonal RMS and reported to occur in the urinary bladder of the dogs (Gerbera and Rees, 2009). The tumor got its name because of its characteristic polypoid or grapes like projections in the lumen of bladder or other hollow organs. Histologically, the tumor contains undifferentiated myoblast cells and myotube cells within the myxomatous stroma. The tumor occurs in the submucosa and tumor cells are separated from the mucosa by a layer of connective tissue called as cambium (Parham, 2001; Caserto, 2013). Spindle variant of the

RMS is listed as a new entity in the human medicine by WHO (Jo and Fletcher, 2014) and is rarely reported in veterinary medicine (Da Roza et al., 2010). Though alveolar RMS can occur at any age most of the cases of alveolar RMS have been reported in young dogs and both classic alveolar and solid variants have been observed in animals (Murakami et al., 2010; Otrocka-Domagala et al., 2015). There is no specific site of prediction and a variable gross appearance i.e. white, yellow-gray, fish flesh, red-brown, with the presence of necrosis and hemorrhage have been reported (Cooper and Valentine, 2016). The classical alveolar pattern is characterized by an alveolus like arrangement of tumor cells on a dense fibrous septum. The neoplastic cells are generally small, uniform, poorly differentiated cells. Hyperchromatic nuclei are round to oval in shape. In humans, presence of large, round to oval, multinucleated cells with a peripheral nucleus and pale eosinophilic cytoplasm are considered a diagnostic feature of the alveolar variant (Cooper and Valentine, 2016). The solid variant resembles neuroendocrine tumors as the small, round tumor cells are arranged as sheets of closely packed cells with minimum or no fibrovascular stroma (Otrocka-Domagala et al., 2015)

1.3. 3- Methylcholanthrene and Phosphodiesterase 5 Inhibitor Tadalafil

A polycyclic aromatic hydrocarbon 3-Methylcholanthrene (3-MCA), alone and in combination with other toxic agents has been used since long for carcinogenesis studies in experimental animals (Gruenstein et al., 1966). This chemical carcinogen has been used to produce RMS (Inoue and Wu, 2006) and fibrosarcoma (Cohen et al., 2010) in mice. Histological variants of RMS induced by 3-MCA include pleomorphic RMS, embryonal RMS (Inoue and Wu, 2006) and undifferentiated RMS (Seitz et al., 2012). Normally, member of the myogenic regulatory family named MyoD is responsible for pushing out the cell out of cell cycle and differentiate it into myogenic lineage (Tintignac et al., 2001). Hypoxia can inhibit multinucleated myotube formation by degrading MyoD thus preventing expression of myogenic factor like myogenin, p21, pRb (Di Carlo et al., 2004). In human pediatric RMS, myogenin is relatively highly expressed in alveolar RMS than embryonal RMS (Heerema-McKenney et al., 2008; Morgenstern et al., 2008). However, in animals, sufficient

information is not available regarding the use of myogenin in subclassification of RMS variants (Caserto, 2013).

Cyclic nucleotides like cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are intracellular second messenger molecules responsible for smooth muscle relaxation. They are generated by the activity of adenylate and guanylate cyclases, respectively (Carvajal et al., 2000). They exert their effects by subsequent activation of protein kinases, cAMP-dependent protein kinase (PKA) and cGMP dependent protein kinase (PKG) by reducing the intracellular calcium (Lincoln and Cornwell, 1991). The catabolic activity of cyclic nucleotide phosphodiesterase (PDE) regulates the activity of these second messengers by degrading phosphodiester bond in the cAMP and cGMP and converting them to corresponding nucleotides AMP and GMP (Carvajal et al., 2000).

Phosphodiesterases (PDE's) are the enzymes found in almost all cells. So far 11 isozymes have reported in the PDE family. Among these, PDE-4, PDE-7, PDE-8 only effect cAMP while PDE-5, PDE-6, PDE-9 effects only cGMP and PDE-1, PDE-2, PDE-3, PDE-10, PDE-11 hydrolyzes both cAMP and cGMP (Boswell-Smith et al., 2006; Ghosh et al., 2009). Phosphodiesterase-5 (PDE-5) is mainly found in the thrombocytes, smooth muscles of blood vessels, heart, placenta, skeletal muscles and pancreas while to a lesser extent in the brain, liver, and lungs (Kotera et al., 2000; Ghosh et al., 2009). PDE-5 inhibitors include sildenafil, vardenafil, and tadalafil (Das et al., 2015). These therapeutic agents are known for their therapeutic efficacy in the treatment of male erectile dysfunction (Huang and Lie, 2013). In addition to this, these novel compounds have protective effects against myocardial ischemic/reperfusion injury (Ockaili et al., 2002). A literature review describes the potential influence of PDE-5 inhibitors in various pathological conditions like heart diseases, male genitourinary system, cancer, diabetes, cystic fibrosis and CNS related diseases (Ribaudo et al., 2016). C- type natriuretic peptide (CNP) in combination with sildenafil inhibits tumor cell proliferation in RMS cell lines by blocking the Raf/MEK/ERK pathway. Both these compounds act in synergism to inhibit tumor growth *in vivo* without significant side effects (Zenitani et al., 2016). Studies have shown the anti-cancerous potential of these PDE-5 inhibitors either used singly or as chemoadjuvant. Both *in vitro* and *in vivo* studies

demonstrated that sildenafil can induce apoptosis in cancer cells when used as a chemoadjuvant (Keats et al., 2017). Tadalafil is a long-acting selective inhibitor of cGMP specific PDE-5 and has beneficial effects on patients with pulmonary arterial hypertension (Galiè et al., 2009). Tadalafil has shown cardioprotective effects by reducing infarct size, apoptosis and necrosis in cardiomyocytes, and anti-inflammatory effects by ameliorating circulatory inflammatory cytokines like tumor necrosis factor alpha and interleukin1- beta in the diabetic mice, it also improved fasting glucose level (Varma et al., 2012). Another study reports the analgesic and anti-inflammatory effects of tadalafil by promoting antinociception and reducing the leukocyte influx into the synovial cavity in zymosan arthritis (Rocha et al., 2011). Tadalafil attenuates the cardiotoxic effects of doxorubicin without interfering its anti-tumor potential (Koka et al., 2010). In human medicine, myeloid-derived suppressor cells and regulatory T cells are key players in the development of head and neck squamous cell carcinomas. Tadalafil has the potential to reduce these cells in the circulation as well as their influx in the tumor mass and augments the tumor-specific CD8 T cells (Califano et al., 2015; Weed et al., 2015). Supplementation of tadalafil with lenalidomide in patients suffering from myeloma improves the clinical response and enables the patient to tolerate the lenalidomide therapy (Noonan et al., 2014).

1.4. Tyrosine Kinases and Endothelial Cell Adhesion Molecules

Platelet-derived growth factor (PDGF) is a 30 kDa dimeric molecule, composed of 2 disulfide-linked polypeptide chains designated as A and B that dimerizes in all possible combinations: PDGF-AA, PDGF-BB, and PDGF-AB (Andrae et al., 2008). The PDGF isoforms exert their cellular effects by binding to a dimeric tyrosine kinase receptor consisting of α and/or β subunits (Alvarez et al., 2006; Ostman and Heldin, 2007). PDGF ligands and their receptors are of major importance in angiogenesis (Ferrara and Kerbel, 2005; Alvarez et al., 2006; Ostman and Heldin, 2007), cell proliferation, chemotaxis, and survival in neoplastic cells (Heldin and Westermark, 1999). Furthermore, they are also critical for the growth of fibroblast and vascular smooth muscle cells (Heldin et al., 1998). PDGF expression has been implicated in the development of human soft-tissue sarcomas

(Malhotra and Schuetze, 2012; Teyssonneau and Italiano, 2017) in an autocrine manner and stimulates angiogenesis and tumor growth (Montag et al., 2009).

Expression of platelet-derived growth factor receptors (PDGFR) has been observed in canine injection site sarcomas (Jacobs et al., 2017). PDGFR-β expression was observed in feline injection site sarcoma cell lines, non-injection site associated feline fibrosarcoma cell lines, feline fibroblast-derived cells line and an *in vivo* tumor model exhibited expression of PDGF-BB can protect tumor cells from apoptosis induced by serum starvation or doxorubicin (Katayama et al., 2004; Lawrence et al., 2012). In human non-GIST soft tissue sarcomas, the PDGFR-α expression is correlated with the malignancy grade i.e. grade 3 tumors have higher expression compared with grade 1 tumors and PDGFR-β has higher positivity in patients with metastasis compared with non-metastatic patients (Kilvaer et al., 2010). PDGR-α and β expression have been observed in embryonal RMS, alveolar RMS, pleomorphic RMS, and high level of expression have been associated with reduced survival time (Armistead et al., 2007). PDGFR-alpha is suggested as a therapeutic target for alveolar RMS (Taniguchi et al., 2008). Gene expression analysis of 101 human RMS cases revealed increased expression levels of PDGF-C, PDGF-D, and PDGFR-β. Immunohistochemical analysis showed that PDGF-CC and PDGF-DD along with PDGFR-α were expressed in tumor cells while PDGFR-β in the vascular stroma (Ehnman et al., 2013).

Angiogenesis is vital for the growth of solid tumors and mediated by endothelial cell adhesion molecules (ECAM) which are represented by four main groups which are selectins, integrins, cadherins and membrane proteins immunoglobulin superfamily. The selectins consist of three members, L-, P-, and E-selectin expressed by leucocytes, platelets, and endothelial cells, respectively. E-selectin, a 115-kDa cytokine-inducible protein participates in the rolling and adhesion of neutrophils and monocytes along endothelium at sites of inflammation (Lasky, 1992). E-selectin participates in active neovascularization and detected in human melanomas, colon carcinomas (Lauri et al., 1991), and osteosarcomas. Furthermore, the expression of E-selectin was found to be significantly higher in malignancies than in benign tumors in human head and neck cancers (Lieder et al., 2005), breast tumors (Shaker et al., 2006) and lung cancers (Gogali et al., 2010). These findings

indicate E-selectin specifically expressed or secreted by activated endothelial cells, contributes to tumor growth by acting as a proangiogenic factor (Koch et al., 1995). However, E-selectin is not constitutively expressed by endothelial cells its expression is also stimulated by inflammatory molecules such as tumor necrosis factor (TNF-α) and interleukin-1 (IL-1) (Bevilacqua et al., 1989).

Integrins are heterodimeric transmembrane proteins composed of non-covalently attached α and β subunits and function to mediating cell to cell and cell to extracellular matrix adhesions (Hynes and Zhao, 2000). There exist 18 α and 8 β subunits whose combinations can generate 24 different receptors having a different affinity and tissue distribution (Hynes, 2002; Takada et al., 2007; Barczyk et al., 2010). Alpha v integrin is a key molecule contributing to cell proliferation and apoptosis and it is expressed in most cancer cells playing an essential role mediating cell-matrix and cell-cell interactions. Alpha v integrins increase the capacity of tumor cells to degrade the extracellular matrix and migrate within their environment (Silletti et al., 2001). Consequently, alpha v integrin up-regulation at the surface of neoplastic cells is frequently associated with local invasion and metastatic dissemination of cancers (Nejjari et al., 2002). By their angiogenic and adhesion properties, both integrin alpha and E-selectin are involved in tumor growth and metastasis by mediating microvessel neoformation processes and therefore both ECAM influence cancer prognosis and/or response to antiangiogenic therapy (Mannori et al., 1997; Vonlaufen et al., 2001; Hosotani et al., 2002).

1.5. Objectives of the Study

There is a scarce literature in cats and dogs showing the role of PDGFs in the development of feline and canine cancers (Katayama et al., 2004). The previous studies mainly focused on the evaluation of the role of PDGF-B and PDGFR-β in some of the soft tissue sarcomas like in feline injection site sarcomas. There is also no conclusive study investigating the significance of the simultaneous expression of ECAM and PDGFs in feline and canine soft tissue sarcomas. Moreover, knowledge about the complex interactions between growth factors and ECAM is still incomplete as their functional activity is not restricted to angiogenesis alone. A more detailed understanding of the regulatory pathways

and the interactions between growth factors and ECAM in feline and canine soft tissue sarcomas will essentially improve the clinical prognosis of this heterogeneous group of tumors. Therefore, the current study was planned to assess the expression profiles of PDGF-A/PDGFR-α, E-Selectin, Integrin alpha v in feline and canine soft tissue sarcomas.

The experimental component of the study was a multipurpose project aimed at inducing a RMS in mice by 3-MCA administration and investigating the role of PDGF-A, its cognate receptor, E-selectin and integrin alpha v in its pathogenesis and subsequent treatment with tadalafil to evaluate its anti-tumor capability, as information about these aspects was lacking in the literature.

2. REVIEW OF LITERATURE

2.1. Soft Tissue Sarcomas

Skin and soft tissues are the most common site for tumor development in dogs (Dobson et al., 2002). Skin and soft tissue sarcomas (STSs) are a group of heterogeneous tumors of mesenchymal origin having closely resembling histological appearance and biological behavior. They can arise anywhere in the body, but in dogs generally, they develop in the subcutaneous tissues (Bray, 2016) of limbs, trunk including the tail and perineal region and head (Bray et al., 2014). STSs constitute approximately 15% of all canine and 7% of all feline skin and subcutaneous tumors. These tumors are locally expansive and grow between fascial planes (Mauldin, 1997; Liptak and Forrest, 2013). Compression of the peritumoral connective tissue may produce a pseudo-capsule which may contain tumor cells leading to poorly define margins (Dennis et al., 2011). Local recurrence after surgery may occur in 7-75% dogs but there has been considerable improvement in the management of STSs in the past 30 years, but still 1 out of 5 dogs die due to the disease (Bray, 2016).

2.1.1. Nomenclature

Not all tumors arising in the soft tissue are categorized as STSs as there are tumors which are excluded from this group based on their specific microscopic pattern or anatomical location which makes it easy to distinguish them from other tumors and another reason is their malignant behavior as a group. So hemangiosarcoma, fibrosarcoma of the oral cavity, histiocytic sarcoma, lymphangiosarcoma, PNST of brachial plexus (schwannoma, neurofibroma), synovial cell sarcoma and leiomyosarcoma are excluded from STSs (Chase et al., 2009; Dennis et al., 2011) whereas fibrosarcoma, myxosarcoma, liposarcoma, PNST (non-brachial plexus), perivascular wall tumors, malignant mesenchyoma, pleomorphic sarcoma and undifferentiated sarcoma are included in STSs category (Ehrhart, 2005; Dennis et al., 2011). However, some authors do include RMS in the category of STSs (Liptak and Forrest, 2013)

2.1.2. Grading

Grading of soft tissue sarcomas is important from the prognostic point of view and has been correlated with the survival time. Histological grading takes into consideration the degree of differentiation of the tumor cell, mitotic index and percentage of necrosis in the tumor tissue (McSporran, 2009). Higher tumor grade is indicative of grave prognosis and recurrence of neoplastic growth (Bray et al., 2014). Tumor mitotic rate is prognostic for spread to distant organs. Incomplete excision margins favor recurrence (Kuntz et al., 1997) while complete surgical margins are negatively correlated with recurrence. Grade 1 and grade 2 tumors do not substantially affect the overall survival of the patients. Tumor grade is especially important in marginally excised tumors.

2.1.3. Incidence of Tumors in Dogs and Cats

An incidence rate of tumor occurrence 1,437 per 10,000 dogs annually was observed in the United Kingdom. Canine cutaneous histiocytoma was the most common of these skin/subcutaneous tumors with an incidence of 377 per annum followed by lipoma (317), adenoma (153), mast cell tumors (126), and soft tissue sarcomas (122) and miscellaneous tumors (342) cases per year (Dobson et al., 2002).

A study of tumor incidence in Dutch Golden retrievers to judge breed predisposition indicated 2,242 dogs out of 100,000 dogs per year are at risk of tumor development. Analysis of the histological database showed that the most common tumor was mast cell tumor (26%), followed by soft tissue sarcomas (11%) and melanomas (8%) while in cytological archive lipoma (35%), mast cell tumor (21%) and non-Hodgkin lymphoma (10%) were major entities (Boerkamp et al., 2014).

An investigation of the 900 biopsy samples from dogs with skin lesions showed 60% tumors, of which 44.4% were mesenchymal, 39.4% epithelial, 7.4% lymphohistiocytic and 8% melanocytic (Mukaratirwa et al., 2005).

A retrospective study of the data from 1955-2008 in the Swiss canine cancer registry indicate tumors in 55.7% (n= 67,943/121,963) of the animals. Forty-seven percent of these tumors were malignant in nature. The incidence of epithelial, mesenchymal and lymphoid tumors was 38.45%, 35.10%, and 13.23%, respectively. Skin tumors (37.05%) were highest

in number followed by mammary (23.55%) and soft tissue tumors (13.23%) (Gruntzig et al., 2015).

In a study of 350 canine STSs, neurofibromas or neurofibrosarcomas (69.1%) were the most common followed by fibrosarcoma (18.8%), perivascular wall tumors (6.3%), myxoma (3.7%), liposarcoma (1.7%) and giant cell tumors (0.3%) (Bray et al., 2014).

A study involving a large cohort of cats (n=51322) over a span of 43 years revealed tumor development in 34.79% of the animals with epithelial tumors being the highest in number (43.06%) followed by mesenchymal (27.98%) and others. Skin (27.05%) and connective tissue (19.04%) was the most common site diagnosed with a tumor (Graf et al., 2015).

2.1.4. Fibrosarcoma

Fibrosarcoma is a tumor of malignant fibroblasts. In well differentiated, spindle-shaped tumor cells having scant cytoplasm with uniformly sized elongated to oval nuclei are arranged in a herringbone or interwoven pattern. Mitotic figures are rare in well-differentiated fibrosarcomas. While anaplastic variants of fibrosarcoma exhibit anisocytosis and anisokaryosis with the presence of ovoid or polygonal cells having large round to oval nuclei and prominent nucleoli. Multinucleated cells are frequent. Immunohistochemistry and careful examination of several sections may help in differentiation of fibrosarcomas from other neoplasms (e.g., myopericytoma, malignant melanoma, and leiomyosarcoma) which can have regions that resemble fibrosarcoma. (Hendrick, 2016; Mauldin and Peters-Kennedy, 2016).

Fibrosarcoma in Dogs

Fibrosarcoma is more common in dogs and cats as compared to other animal species. The mean age in these animal species is 9 years. Golden retrievers and Doberman pinschers are relatively at high risk. Though tumor can develop in any part of the body but frequently observed in head and limbs.

Maxillary or mandibular fibrosarcoma in dogs though not included in the category of STSs but is an important neoplasm of the oral cavity. These tumors may be anaplastic with cellular pleomorphism (Frazier et al., 2012) or have a benign histological appearance but

aggressive biological behavior. The tumors have low to moderate cellularity but aggressively invade the adjacent tissues and may cause bone lysis (Ciekot et al., 1994). Dogs with maxillary tumors have longer survival time than with mandibular tumors (Gardner et al., 2015).

Fibrosarcoma in Cats

In 1969, a two-year-old, short hair cat was diagnosed with multiple subcutaneous fibrosarcomas with metastasis to different organs. The electron microscopic studies of the tumor revealed the presence of c-type virus particles in the tumor cells. The tumor homogenate was used to reproduce the disease in the kittens (Snyder and Theilen, 1969). The virus was named as feline sarcoma virus (FeSV). FeSV is a defective mutant of feline leukemia virus (FeLV) and lack pol and env genes thus require the assistance of FeLV, to replicate (Hardy, 1981). The mean age for the occurrence of these viral tumors is 3 years in cats, but cats as young as few months can get infected. Tumors are often multicentric with the ability to metastasize (Snyder et al., 1970). Microscopically, tumors are pleomorphic fibroblastic growth ranging from well differentiated to anaplastic cells with the high mitotic count, large multinucleated cells may also be observed. The tumor is infiltrative in nature. (McDonough et al., 1971). However, the only 2% of the feline fibrosarcomas are associated with FeSV. The rapid progression of the tumor is of great aid in the differential diagnosis of this virus-induced sarcoma (Hendrick, 2016).

Feline Injection Site Sarcoma/Vaccine Associated Sarcoma

In the 1987, Scientists at the Laboratory of Pathology at the University of Pennsylvania School of Veterinary Medicine began to note an increase in the number of cases with subcutaneous necrotizing granulomatous inflammatory reaction characterized by the presence of a well-circumscribed subcutaneous firm mass with a necrotic center and peripheral aggregates of macrophages, lymphocytes, plasma cells and eosinophils at the site of vaccine injection in cats (Hendrick and Dunagan, 1991). The increase coincided with the enactment of a law requiring rabies vaccination in cats. Parallel to this, there was an increase (61% between 1987-1991) in the incidence of fibrosarcoma in cats (Hendrick and Goldschmidt, 1991). Most of the tumors were noted at the sites routinely used for vaccination

by veterinarians (dorsal neck/interscapular, dorsolateral thorax, hindlimb, dorsal lumbar regions). In 1991, fifty-one percent of the tumors (101/198) were surrounded by mononuclear inflammatory cells i.e. lymphocytes and macrophages, 42 tumors contained aluminum oxide within the macrophages which was correlated with aluminum-based adjuvants used in rabies vaccines (Hendrick et al., 1992). Comparison of fibrosarcoma at vaccination and non-vaccination sites revealed that cats receiving FeLV and rabies vaccination were at greater risk of developing a tumor at the injection site within one year of the vaccine application (Kass et al., 1993). Though most of the tumors at the vaccine site are fibrosarcomas, the incidence of malignant fibrous histiocytoma, osteosarcomas, RMS, and chondrosarcomas have also been recorded (Hendrick and Brooks, 1994). The vaccine site tumors were observed in younger cats and were large in size, biologically more aggressive as compared to non-vaccine site tumors which were observed in relatively old cats. However, metastasis was not a significant feature of vaccine site tumors (Hendrick et al., 1994). A retrospective study on the feline sarcomas (n=195) submitted to the Indiana Animal Disease Diagnostic Laboratory between years 1988-1994, demonstrated that 87% of these tumors were fibrosarcomas. The median age of the cats with the vaccine site sarcomas was 8 years and non-vaccine site sarcomas were seen in cats with a median age of 11 years. Subcutaneous location, necrosis, cellular infiltration, high mitotic activity, pleomorphism, variability in extracellular matrix density were more characteristic features of vaccine site fibrosarcomas. Lymphocytes were the predominant inflammatory cell population observed in 69.2% vaccine site fibrosarcomas. Macrophages were also observed in vaccine site fibrosarcomas. However, in non-vaccine site fibrosarcomas, only 25% tumors were infiltrated by lymphocytes. Few of the vaccine site fibrosarcomas did show osteoid, chondroid, myxomatous matrix (Doddy et al., 1996). Chronic inflammation and immunological reactions to foreign elements are believed to play an important or pivotal role in the pathogenesis of vaccine site fibrosarcomas in cats by derangement in the fibrous tissue repair process (Hendrick et al., 1992). Necrosis in the vaccine site fibrosarcomas may be ascribable to the infarction/thrombosis, or insufficient oxygen supply to the rapidly growing tumor cells to the fat necrosis and inflammation resulting from vaccine injection (Doddy et al., 1996). After vaccination, 10 out

of 100,000 cats are at risk of neoplastic transformation at the site of injection (Kass et al., 1993).

Though some authors believe that the incidence could be much higher than this (Macy and Hendrick, 1996). A web-based survey enrolling 401 veterinarians in the USA and Canada over a period of two to three years was done to estimate the incidence of post-vaccinal reactions and sarcomas in cats. The post-vaccinal inflammatory reaction was reported in 73 (out of 31,671 cats) cats after administration of a total of 61,747 doses of vaccines. Only 2 cats developed sarcomas while in others, healing occurred without any intervention over a period of 3-4 months (Gobar and Kass, 2002). In 1996, the vaccine-associated feline sarcoma task force published its recommendation for vaccine injection site. They recommended that the administration of rabies vaccine in the distal part of the right hind limb and feline leukemia virus vaccine in the distal part of the left hind limb, and any other vaccine can be administered on the right shoulder avoiding the midline or interscapular space (Romatowski, 1997). Similar instructions have been given by European Advisory Board on Cat Diseases (Hartmann et al., 2015). After the recommendations of the vaccine-associated feline sarcoma taskforce to administer rabies and feline leukemia vaccines in distal limbs, there is a shift in the anatomical location of these injection site sarcomas from interscapular region to limbs (Shaw et al., 2009).

Vaccines are not the only agents linked with injection site sarcomas. Several other drugs like antibiotics, long-acting steroids, microchips used for animal identification, a surgical sponge, orthopedic implants had been associated with tumor formation in different animal species (Esplin et al., 1999; Kass et al., 2003; Srivastav et al., 2012). In 2008, a fibrosarcoma was diagnosed in a 14 years old cat in the interscapular region in the vicinity of a microchip implant. But as the cat had an earlier history of vaccination in the same region, the absolute relationship of tumor development to microchip implant was difficult to establish. (Daly et al., 2008). However, a few years later, the scientist observed a 9 years old cat suffering from a neoplastic mass in the neck at the site of a microchip implant. There was no history of vaccination in this region. The tumor was diagnosed as fibrosarcoma and the histological features were in line with those of vaccine site sarcomas. The tumor contained smooth muscle actin immunoreactive myofibroblasts (Carminato et al., 2011). A recent study

investigated metalloproteinases (MMP2, MMP9) and their inhibitor (TIMP-2) expression in feline injection site sarcomas. MMP2 was mainly observed in mononucleated tumor cells while multinucleated tumor cells were positive for MMP9. TIMP2 was observed in all the tumors and in both mononucleated and multinucleated tumor cells. The authors are of the view that metalloproteinases may play an important role in the behavior of the feline injection site sarcomas. They further added that a threshold value of 3.75 cm for the size of the formalin-fixed tumor (major diameter) and 20 for the mitotic count in an area of 2.37mm^2 can be set to predict the recurrence of the tumor (Porcellato et al., 2017). Any sarcoma in the presumed sites of vaccination should be considered as an injection site sarcoma and should be treated aggressively. The wide surgical excision (3-5 cm of normal tissue around the tumor in all directions and deep fascial planes) is the commonly used method of treatment. But to prevent recurrence, a combination of surgery and radiation therapy is recommended. Though low metastatic rate contends against the use of chemotherapy some oncologist does use chemotherapeutic agents like carboplatin, doxorubicin etc. as there are reports of recurrence even after aggressive surgical and radiation therapy. Early tumor detection and pet owners' education is important in the successful treatment of these sarcomas (Saba, 2017). A combination of neoadjuvant chemotherapy, surgery, and adjuvant chemotherapy improved tumor-free survival rate and disease-free interval (Bray and Polton, 2016).

Injection Site Sarcoma in Dogs

Injection site sarcomas are relatively rare in dogs (Jacobs et al., 2017). However, foreign materials like orthopedic hardware or implants, surgical swabs, microchips and pacemakers' generator implants are linked to neoplastic growths in dogs. Retention of surgical swab resulted in abdominal fibrosarcoma in a 3-year-old Rottweiler female dog (Rayner et al., 2010). Orthopedic implants are usually associated with osteosarcoma (Straw, 2005; Atherton and Arthurs, 2012; Dunn et al., 2012), however, fibrosarcoma, spindle cell sarcoma, and histiocytic sarcoma may also occur. The time duration between implant placement and tumor identification varies between 9 months to 10 years with a median of 5.5 years (Burton et al., 2015) while microchips and pacemaker's generator implants may cause either fibrosarcoma (Vascellari et al., 2006) or RMS (Thieman Mankin et al., 2014).

Histological and immunohistochemical comparison between injection site fibrosarcomas (n=15) and non-injection site fibrosarcomas (n=10) in dogs with 20 feline post-vaccinal fibrosarcomas revealed histological similarities between injection site sarcomas in both species. Injection site tumors were having characteristic infiltrates of lymphocytes at the periphery of tumor. Two non-injection site fibrosarcomas in dogs had inflammatory infiltration around blood vessels within the tumor mass. Vimentin immunoreactivity was observed in all neoplasms. Most of canine (n=10/15) and all feline fibrosarcomas from injection site were of myofibroblastic immunophenotype. Aurintricarboxylic acid technique demonstrated aluminium deposits in canine fibrosarcomas (n=8) from and feline fibrosarcomas from presumed injection sites (Vascellari et al., 2003).

2.1.5. Myxosarcoma

Myxosarcoma is a tumor of fibroblastic origin with abundant mucopolysaccharide rich myxoid matrix. Middle to old aged dogs and cats are generally affected (Liptak and Forrest, 2013). Histologically, the tumor is composed of the spindle to stellate cells loosely arranged in a mucopolysaccharide rich matrix (Headley et al., 2011). This myxoid tumor can occur in the eyes (Dennis, 2008; Campos et al., 2015), brain (Richter et al., 2003), heart (Foale et al., 2003; Adissu et al., 2010), mitral valves (Beal et al., 2014), intrathoracic (Sommerey et al., 2012), lungs (Hill et al., 2008), vertebral column (Kunkel et al., 2007), jaws (Galan et al., 2007), sacroiliac region (Kirby et al., 2014), perineal region (Weeden et al., 2016). Williamson and Middleton (1998), studied 74 cases of canine soft tissue sarcomas and diagnosed myxosarcoma in 6 animals. All cases showed positive staining with vimentin & S-100 positivity was noticed in 3 of these tumors. Cytokeratin, desmin and vWF remained negative. Histological pattern of myxosarcoma has close similarity with myxoid variants of liposarcoma and PNST. Special stains like oil red o and S-100 may help in the differential diagnosis of these variants (Hendrick, 2016). Though tumor is locally invasive, rare cases of metastasis are observed. A 13 years old dog with subcutaneous mass in the right scapular area & a small nodule in the caudal abdomen was diagnosed with myxosarcoma. The tumor showed metastasis to the lungs. Histologically, tumor was located in the subcutaneous area and was poorly capsulated and was composed of plump, haphazardly arranged spindle to

stellate shaped cells dispensed in the mucin-rich matrix. Nuclear pleomorphism was easily recognizable. Vascular invasion by the tumor cells were also observed by the authors. Tumor cells showed strong immunoreactivity for vimentin and limited positivity with smooth muscle actin, S-100 and desmin (Headley et al., 2011).

2.1.6. Liposarcoma

Liposarcoma is a malignant tumor of the adipose tissue with rare occurrence in animals. Though there is no internationally accepted nomenclature for the sub-classification of liposarcoma in animals, it can be divided into well differentiated, anaplastic (pleomorphic) and myxoid variants (Hendrick, 2016). Round cell variants of the myxoid liposarcoma have also been reported in a dog (Plumlee et al., 2016) and a Japanese Macaque (Kwon et al., 2007). Incidence is more in dogs as compared to other animal species. However, sporadic occurrence in other animal species has been reported e.g. intranasal liposarcoma was documented in a cow (Shive et al., 2006) and a cranial mediastinal liposarcoma in horse (Kondo et al., 2012). Liposarcoma occurrence in cats is also relatively well documented (Tanimoto et al., 1987; Cramer et al., 2011; Kabak et al., 2011; Diep and Fleis, 2012) and even has been linked with viral etiology (Stephens et al., 1983). Histologically, most tumors are composed of sheets of round to polygonal cells with minimum to the non-collagenous stroma. Though there is cellular variability in terms of shape and size of the nucleus and cytoplasmic vacuoles, well-differentiated liposarcomas, are composed of cells resembling normal adipocytes with a peripheral nucleus and a single clear fat vacuole. The pleomorphic or anaplastic variant is characterized by highly pleomorphic cells with bizarre multinucleated cells and only a small percentage of cells have fat vacuoles. In myxoid subtype dispersed spindle cells, lipocytes and lipoblasts loosely arranged in the bubbly mucoid stroma. Liposarcoma may occur spontaneously (Kabak et al., 2011) or as a result of some foreign intervention like vaccine injection in cats (Esplin et al., 1996) or microchip implant and glass foreign body in dogs (McCarthy et al., 1996; Vascellari et al., 2004). Metastasis has been reported to lungs (Esplin et al., 1996) and kidney (Cramer et al., 2011) and many other organs (Diep and Fleis, 2012) in cats. Co-occurrence of myxoid liposarcoma and well-differentiated liposarcoma was observed in the ovary of a female dog. The surgical removal of the mass

was carried out but 4 months later dog died due to multiorgan metastasis. The neoplastic cells were positive for S-100, vimentin and adipophilin (Shiwa et al., 2016). Avallone et al. (2017) examined 50 canine liposarcoma cases. Myxoid (n=7), pleomorphic (n=25), dedifferentiated (n=4) and well differentiated (14) subvariants were noticed. Sixty two percent tumors demonstrated high immunoreactivity for fibroblast growth factor-2, 68% for its receptor (FGFR1). PDGFB and its cognate receptor (PDGFRβ) upregulation were noticed in 81.6% and 70.8% tumors, respectively. A relatively low number of cases showed c-KIT expression. Liposarcomas poses a new challenge in their diagnosis by expressing muscle markers. La Douceur et al. (2017) reported alpha-smooth muscle actin (n=7/25), myogenin (n=3/25) and desmin (n=7/25) expression in canine liposarcomas (Avallone et al., 2016).

2.2. Rhabdomyosarcoma

The malignant cancerous growth with the tendency to differentiate to skeletal muscle lineage is termed RMS. The tumor can not only develop from resting myoblasts or satellite cells in the skeletal muscles but also from the primitive mesenchymal cell with the potential to convert into myogenic cells in any part of the body. Skeletal muscle tumors are rarely encountered in animals. In a retrospective study at the college of veterinary medicine, Cornell University, USA, out of 58/83000 tumors previously diagnosed as rhabdomyoma or RMS, only 16 were confirmed by use of recent techniques. Presence of giant cells often misleads to a diagnosis of RMS. The tumor is more common in dogs than in cats (Cooper and Valentine, 2016). In the United States of America, each year 850-900 cases of soft tissue sarcoma are being recorded in the children and adolescent under 20 years of age, out of these approximately 350 are RMS. Soft tissue sarcoma contributes 7.4% of the cases of malignancy under 20 years of age while RMS was most common soft tissue tumor among children under 15 years of age representing 50% of the soft tissue sarcomas for this age group with an incidence rate of 4.6 per million. Embryonal subtype was more common in infants. Young children have higher survival rate than older children and adolescents (Gurney JG, 1999). On the basis of genetic, histologic and clinical differences World Health Organization (WHO) classify RMS into five subtypes (Fletcher et al., 2002). These subclasses include embryonal, alveolar, botryoid, pleomorphic and spindle cell/sclerosing RMS (Caserto, 2013). In humans,

embryonal RMS generally develops in the head/neck, abdomen, a genitourinary tract of infants and young children (Parham, 2001). The histological classification has prognostic significance in humans but not studied in the animals yet. This skeletal muscle malignancy is included in the STSs by some scientists (Caserto, 2013) while not by others, but the common grading scheme used for STSs is not applied for RMS. One major difference between RMS and other soft tissue sarcomas is the age of onset as RMS is mostly observed in young dogs (2 years or less) and STSs in middle to old aged dogs (Dennis et al., 2011; Caserto, 2013). Embryonal subtype of RMS can occur at many sites of the body even those which normally don't have skeletal muscle but commonly observed in the head and neck region e.g. oral cavity, tongue and larynx, in aged dogs. Botryoid type which has a characteristic grape-like appearance is mostly observed in the urinary bladder of young animals (≤ 2years), with a predisposition of female sex and large-breeds like Saint Bernard (Liptak and Forrest, 2013; Cooper and Valentine, 2016). A relatively newly described subtype which is grouped with spindle cell variant of RMS in the WHO classification systems is sclerosing RMS. Until now only 20 cases of pediatric RMS have been reported. Males at a mean age of 9 years develop a tumor in the extremities or head/neck regions (Kumar et al., 2014). Pleomorphic RMS is the least common type in humans and is observed in the adults. The botryoid and spindle cell variants of human RMS are linked with favorable prognosis, embryonal with intermediate prognosis and alveolar with poor prognosis (Newton et al., 1995). Age is considered an adverse prognostic factor of survival in patients with alveolar and embryonal RMS (Van Gaal et al., 2012). Prognosis of RMS in adults is usually worse than children. Adults mostly develop pleomorphic type RMS or RMS not otherwise specified (Sultan et al., 2009). Like humans, pleomorphic RMS is less common in animals as well. Though there have been several reports of pleomorphic tumors which seem to be incorrectly diagnosed as the photographs published are suggestive for embryonal subtype. Some of the pleomorphic sarcomas have been diagnosed as muscle tumors just on the basis of histomorphology and without using any immunohistochemical marker. All RMS show some certain level of pleomorphism and any tumor having an area of embryonal or alveolar differentiation should be categorized as such (Cooper and Valentine, 2016).

2.2.1. Histopathology

In animals, embryonal RMS may be either composed of predominant round cells or primitive myotube-like cells (Cooper and Valentine, 2016). Round cells type usually has intermixed population of small cells with scanty cytoplasm and large round to polygonal cell with abundant cytoplasm (Caserto, 2013). Nuclei are smaller than those in the alveolar RMS and have lighter chromatin. Strap or ribbon-shaped cells, or tadpole cells with bipolar cytoplasmic extension and eccentric nuclei with only one cytoplasmic process or spiderweb cells having Periodic acid Schiff positive vacuoles between thread like strands of cytoplasm, are highly suggestive for embryonal subtype (Newton et al., 1995). Mononuclear giant cells with more than three times large nucleus than the surrounding cells is also observed in some cases of embryonal type RMS (Kodet et al., 1993). In botryoid type, tumor normally adjoins an epithelial surface and projects as multinodular excrescences into the lumen of the organs e.g. bladder, vagina, conjunctiva or bile duct (Parham, 2001). Histologically, there is sub-epithelial condensation of neoplastic cell in several layers (cambial layer of Nicholson), at least focally so the presence of intact epithelium is important in the diagnosis of this subtype. Primitive myotubes are often observed in botryoid RMS (Cooper and Valentine, 2016).

The alveolar type may have classical alveoli like appearance in which tumor cells line or fill the alveolar spaces formed by anastomoses of fibrovascular stroma or as a solid variant with densely packed cells with no or hard to identify alveolar structure, stroma surrounds nests of the tumor cells. Tumor cells are generally round with a small rim of eosinophilic cytoplasm. Nucleus has coarse chromatin with a variable number of nucleoli (Newton et al., 1995; Parham, 2001).

Spindle cell variant consists of fusiform cells with collagen-rich or poor collagen matrix. Those with poor collagen occur in fascicles while collagen-rich adopt storiform or whorls like pattern (Leuschner et al., 1993; Newton et al., 1995).

2.2.2. Incidence, Pathology and Immunohistochemistry of Rhabdomyo-sarcoma in Animals

Rhabdomyosarcoma is relatively a tumor with low frequency in animals and is more common in dogs than cats (Cooper and Valentine, 2016). A review published in 2013

reported only 65 published cases of canine RMS with botryoid variant being the most common (43%), followed by embryonal (23%,) and unclassified RMS (20%) while alveolar (11%) and pleomorphic (3%) was least observed variants (Caserto, 2013). While a study at Cornell university encountered more cases (n=8/16) of embryonal variant (Cooper and Valentine, 2016). Like in human medicine, spindle cell variant of the RMS has been diagnosed (Da Roza et al., 2010). In veterinary oncology, laryngeal RMS is dealt as a separate clinicopathological entity (Yamate et al., 2011). Though RMS can occur at any age but most of the animals diagnosed with this malignancy are less than 2 years (Caserto, 2013). RMS occurring in urogenital/visceral areas are followed by head & neck region and appendicular skeleton are the frequent sites of RMS development (Caserto, 2013).

Sporadic reports of feline RMS are available in the literature occurring in muscle (Miller et al., 2009), liver (Minkus and Hillemanns, 1997), heart (Venco et al., 2001), limb (Simon et al., 2000), eyelids (Spugnini et al., 2010) and interscapular region (Chang et al., 2006). In cats, RMS may be associated with vaccine administration (Hendrick and Brooks, 1994; Chang et al., 2006).

RMS in animals show immunoreactivity to vimentin, desmin, myoglobin, sarcomeric actin, alpha-smooth muscle actin, muscle specific actin, myogenin and MyoD1 and mostly remained negative for GFAP, S-100 and epithelial markers (Mulas et al., 1992; Suzuki et al., 2006; Chapman et al., 2008; Kimura et al., 2013; Park et al., 2016).

2.2.3. Molecular Pathogenesis of Rhabdomyosarcomas

Alveolar RMS development is associated with the translocation involving chromosome 2 and 13 in human (Turc-Carel et al., 1986). This translocation t(2;13) (q35;q14) results in the formation of a novel chimeric protein of two transcription factors PAX3(chromosome 2) and FKHR or FOXO1 (chromosome 13) which is more strong transcriptional activator as compare to PAX3 (Fredericks et al., 1995). Another translocation t(1;13)(p36;q14) has also been reported where PAX3 is substituted by PAX7 (Davis et al., 1994). The PAX3-FOXO1 fusion protein is a much more potent transcriptional activator than PAX3 and may act as an oncogenic transcription factor by enhanced activation of normal PAX3 target genes (Fredericks et al., 1995). The 2:13 translocation is more common than 1:13 translocation (Parham and Barr, 2013). This chromosomal fusion is not necessarily

present in all alveolar RMS patients and 20-25% tumors may be fusion negative (Parham et al., 2007). RMS variants are often associated with gains or loss of chromosomes and genomic amplifications. The frequency of gains is higher in embryonal variant than alveolar, approximately 25-50% of embryonal RMS cases are associated with a gain of 2,8,12, 13 chromosomes (Parham and Barr, 2013).

Embryonal RMS arises from the cells that have lost heterozygosity on chromosome 11at band p15.5-pter (Scrable et al., 1989). A comprehensive genomic analysis of RMS cases has demonstrated that tumor exists in two genotypes, first with PAX3 or PAX7 fusion and second without fusion but containing mutations in crucial signaling pathways. Though overall mutation rate is relatively low, multiple genes like HRAS, KRAS, NRAS, PIK3CA, FBXW7, BCOR, CTNNB1 are often mutated. Approximately, 93% of the RMS have altered receptor tyrosine kinase/RAS/PIK3CA axis. RAS mutations are more common in an embryonal subtype of RMS (Shern et al., 2014). There is no parallel study on the genetics of RMS in animals.

2.2.4. Experimental Models

Intramuscular injection of heavy metals especially nickel and cobalt have been used to induce RMS in rats and rabbits (Gilman and Herchen, 1963; Hildebrand and Biserte, 1979; Zanola et al., 2012). Pyrrolizidine alkaloids can also generate muscle tumor in rats. Administration of dehydroretronecine, a metabolite of monocrotaline in rats induced RMS in more than 50% of the rats (Allen et al., 1975). Subcutaneous injection of benzenediazonium sulfate produced RMS, fibrosarcoma, and osteosarcoma in rats (Toth et al., 1998). Chemical compounds like *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and methylazoxymethanol-acetate (azoxymethane metabolite) produced mesenchymal lineage tumors including RMS in different fish models such as Medaka and Zebrafish (Bunton and Wolfe, 1996; Spitsbergen et al., 2000; Zanola et al., 2012). Polycyclic aromatic hydrocarbons e.g. 7,12-dimethylbenz(a) anthracene and benzo(a)pyrene was used to elicit RMS in rats (Taguchi et al., 2006) and mice (Maenza et al., 1971), respectively. Repeated exposure of CD-1 mice to β-radiation in the back region triggered RMS, squamous cell carcinoma, malignant fibrous histiocytoma (Gupta et al., 1999). Moloney-murine sarcoma virus can

induce RMS in mice (Perk et al., 1968; Nanni et al., 1991). In addition to these chemical and virus-induced tumor models, genetically engineered animal models targeting different molecular pathways e.g. P53 pathway, PAX3-FKHR pathway, RAS/ERK pathway, Hgf/c-MET pathway, Sonic Hedgehog pathway has been used to study the pathogenesis of RMS (Zanola et al., 2012).

3- methylcholanthrene induced tumors in mice and rat has been used since long to study tumor immune surveillance. This chemical carcinogen can produce sarcomas as well as carcinomas (Chamoto et al., 2010). Sarcomas produced by MCA depend on the dose of the carcinogen rather than the immune status of the host (Engel et al., 1997). MCA has the potential to produce fibrosarcomas (Cohen et al., 2010) as well as RMS in animal models (Vogel et al., 1991). A single dose of 1 mg of MCA dissolved in oil is sufficient to produce neoplastic growth in mice (Vogel et al., 1991). Kosmehl et al. (1989) generated RMS in murine models and after removing a part of these tumors allotransplanted to nude mice. Repeated surgical biopsies were taken from both the original tumors and allotransplanted mice tumors to compare the cellular differentiation and immunohistochemical expression of vimentin, desmin, and myoglobin. The results indicated dissimilarity in histological appearance and expression of immunohistochemical markers from repeated submissions. However, the tumors retained the basic criteria to be classified as RMS. This variation is not necessarily reflected dedifferentiation but may also indicate maturation or differentiation.

Though previous studies did not categorize the RMS into different variants based on histomorphology but they described the presence of spindle-shaped cells admixed with polygonal cells and numerous multinucleated and mononucleated giant cells (Vogel et al., 1991). The later investigation reported a mixture of embryonal and pleomorphic subtypes of RMS after injection of MCA in mice (Inoue and Wu, 2006). Another study by the same group of scientists demonstrated that some areas in the MCA induced RMS can show malignant fibrous histiocytoma-like morphology and may express vimentin only. These areas are actually rhabdomyosarcomatous areas. They found that tumor cells of RMS phenotype express vimentin, desmin, myoglobin, and alpha-actinin (Kosmehl et al., 1990). Laminin expression is observed around the giant cells or well differentiated polygonal cells in experimentally induce murine RMS while fibronectin wrap less differentiated spindle cells.

This finding suggests that differentiation of RMS cell have some dependence on extracellular matrix composition (Vogel et al., 1991). There can be cells in the MCA induced RMS which show a positive reaction to cytokeratin. These cells may be arranged in small clusters or dispersed haphazardly in the tumor (Langbein et al., 1989). Aberrant expression of cytokeratin has been recorded in the spontaneous human RMS (Miettinen and Rapola, 1989).

Multinucleated cells in MCA induced RMS rarely show myogenin expression while myoblast-like cells are generally positive for myogenin. p21 play important role in myotube formation and myosin expression (Inoue and Wu, 2006). Pleomorphic RMS show higher reactivity of p53 and PCNA as compared to embryonal RMS.

2.3. Tadalafil

Tadalafil is a phosphodiesterase-5 inhibitor and widely used to treat erectile dysfunction in males. It has a longer half-life than sildenafil. The drug acts as a selective inhibitor of cyclic guanosine monophosphate-(cGMP-) specific phosphodiesterase type 5 (PDE 5), resulting in smooth muscle relaxation, vasodilation, and enhanced penile erection. (Meuleman, 2003). Besides this, tadalafil has been approved by United States Food and Drug Administration for the treatment of pulmonary arterial hypertension in humans (Henrie et al., 2015).

PDE-5 inhibitors do possess some antineoplastic properties. C-type natriuretic peptide secreted by endothelial cells can suppress the growth of many types of mesenchymal cells. In vitro study demonstrated that C-type natriuretic peptide along with sildenafil acted in synergy to inhibit the proliferation of RMS cells by suppressing the Raf/MEK/ERK pathway (Zenitani et al., 2016). Myeloid-derived suppressor cells (MDSC) and T regulatory cells are important players in the progression of head and neck squamous cell carcinoma in humans. Postoperative tadalafil administration significantly decreases MDSC and T regulatory cells in the circulation and in tumor mass. It increased the antitumor CD8+ T cells (Califano et al., 2015; Weed et al., 2015). Tadalafil therapy in a patient with end-stage multiple myeloma suppressed MDSC by reducing IL4Rα. Likewise, reduction in the expression of iNOS, arginase-1, and ROS was observed while IFNγ and TCRζ levels were increased. Tolerance to lenalidomide chemotherapy was enhanced by the addition of tadalafil

in the patient (Noonan et al., 2014). A combination of celecoxib and PDE-5 inhibitor sildenafil killed a vast range of neoplastic cells in ex-vivo. This combination suppressed the growth of mammary tumors in mice model (Booth et al., 2015). Many tumors express high levels of PDE-5 of cancers, e.g. human oral squamous cell carcinoma, non-small cell lung cancer, metastatic breast cancer and urinary bladder tumor. Based on these findings it can be postulated that PDE-5 inhibitors can be of great therapeutic significance in such tumors (Tiwari and Chen, 2013). Synthetic analogs of PDE-5 inhibitors have been evaluated for their anti-tumor effects. Exisulind is a tadalafil analog which can induce apoptosis in colon cancer cell and urinary bladder tumor cells. *In vivo* study in rats showed a dose-dependent decrease in the tumor multiplicity and incidence (Piazza et al., 2001). Two new analogs (names not mentioned in the study) of tadalafil manifested growth suppressing properties in the HT29 colorectal carcinoma cells line (Abadi et al., 2009). *In vitro* apoptotic effect of sildenafil and vardenafil was observed in chronic lymphocytic leukemia cells (Sarfati et al., 2003). The beneficial effects of PDE-5 inhibitors make them a potential candidate for inclusion in the cancer chemotherapy. To the best of our knowledge, therapeutic effects of tadalafil have not been investigated in the 3-MCA induced RMS in the mice model.

2.4. Histochemical and Immunohistochemical Markers

2.4.1 Ancillary Histochemistry

Various ancillary tests became part of surgical pathology soon after the birth of histopathology. In human medicine, Periodic acid–Schiff (PAS) stains were routinely used until the 1990s to detect the abundant glycogen associated with Ewing sarcoma or the crystals associated with alveolar soft part sarcoma. To demonstrate cross-striations in the tumors with myogenic differentiation e.g. RMS, phosphotungstic acid–hematoxylin staining (PTAH) is used (Parham, 2015).

2.4.2. Immunohistochemistry

Muscle Specific Actin, Desmin, and Sarcomeric Actinin

Muscle-specific actin (MSA) is considered a specific and sensitive marker for muscle differentiation. MSA diffusely stains vascular smooth muscle cells while desmin

shows a focal reactivity in vascular smooth, not in all samples (n=100/196) (Rangdaeng and Truong, 1991). Desmin protein is an intermediate filament found in the skeletal muscles, cardiac muscles and smooth muscles (Paulin and Li, 2004). Desmin is a very useful marker for diagnosis of RMS in animals and humans (Caserto, 2013). However, smooth muscles and some non-myogenic tumors may also show variable reactivity to these markers (Rangdaeng and Truong, 1991). Alpha-actinin cross-links two actin filaments, so a positive reaction to it indicate myogenic differentiation (Sjoblom et al., 2008).

Myoglobin

Myoglobin is found exclusively in striated muscle and can be useful in the diagnosis of RMS. There are numerous studies in human oncology emphasizing its usefulness in the diagnostic panel (Leader et al., 1989). Well-differentiated rhabdomyoblastic cells are usually positive for myoglobin staining while poorly differentiated cells are less likely to react with anti-myoglobin antibodies (Seidal et al., 1987). However, there could be exceptional cases in which differentiated cells showing cross striation can remain negative for myoglobin staining (Kagawa et al., 1983; Leader et al., 1989). Canine malignant PNST may also express myoglobin (Chijiwa et al., 2004). A case of myofibroblastic injection site sarcoma in a cat showed a positive reaction for myoglobin and vimentin but not for desmin (Dubielzig et al., 1993).

Myogenin and MyoD1

Myogenin and MyoD1 are nuclear transcriptional factors or myogenic regulatory proteins expressed at early stages of skeletal muscle myogenesis. They are more specific markers for diagnosis of RMS as compare to MSA and desmin (Cessna et al., 2001). Alveolar RMS show more intense nuclear staining than embryonal RMS. In an investigation of myogenin reactivity of human RMS (n=69), 75-100% tumors cells in alveolar RMS showed positive staining while in embryonal RMS, rare to 25% cellular positivity was noticed in embryonal RMS (Kumar et al., 2000). Other mesenchymal tumors or tumor-like conditions e.g. nodular fasciitis, myxofibrosarcoma, leiomyoma, leiomyosarcoma, malignant fibrous histiocytoma, inflammatory myofibroblastic tumor, or alveolar soft part sarcoma did not stain with myogenin (Cessna et al., 2001). There are reports of focal nuclear positive reaction with

anti-myogenin antibody in synovial sarcoma (n=1/10), infantile fibrosarcoma (n=2/10), desmoid (n=2/10), infantile myofibromatosis (n-=2/10), ectomesenchymomas with myogenic differentiation (n=2/2), nephroblastomas (n=2/6) with myogenic differentiation and in one out of one myogenous Wilms' tumor (Wang et al., 1995; Kumar et al., 2000; Cessna et al., 2001). MyoD1 has good reactivity in RMS but cross-reactions with non-muscle tissues, non-specific cytoplasmic and background immunoreactivity may lower its utility in the differential diagnosis of tumors (Wang et al., 1995).

Alpha Smooth Muscle Actin

Smooth muscles contain four isoforms of the actin filament. Alpha and gamma smooth muscle actin isoforms are generally referred as contractile isoforms but are produced by different genes. Alpha smooth muscle actin is mainly present in the vascular smooth muscle cells while gamma is found in the smooth muscle of gastrointestinal tract (Fatigati and Murphy, 1984). The two cytoplasmic or cytoskeletal isoforms of non-muscle actin are β and γ are constituent of all smooth muscle tissues (Drew et al., 1991; Lehman and Morgan, 2012). Myofibroblasts express alpha-smooth muscle actin which is typically present in vascular smooth cells (Skalli et al., 1986; Hinz et al., 2001). Alpha smooth muscle expression observed in feline injection site sarcomas is correlated with myofibroblasts (Hendrick and Brooks, 1994). A recent study highlights the immunoreactivity of alpha-smooth muscle actin in canine liposarcoma. Seven out of twenty-five confirmed liposarcomas showed reactivity to alpha smooth muscle actin antibody (Ladouceur et al., 2017). RMS both in humans and animals also demonstrate immunoreactivity to anti-smooth muscle actin antibody (Fletcher et al., 2002; Cooper and Valentine, 2016). Canine alveolar RMS cases had shown alpha-smooth muscle actin positivity in tumor cells (Kimura et al., 2013; Otrocka-Domagala et al., 2015). Milovancev et al. (2015) made a comparative pathological evaluation of canine soft tissue sarcomas (n=32). They reported smooth muscle expression in the neurofibrosarcoma, myxosarcoma, neurofibroma, fibrosarcoma and hemangiopericytoma.

Vimentin

Vimentin, ubiquitous 57KD type III intermediate mesenchymal filament is observed in cells of different tissues during various stages of development (Coulombe and Wong,

2004). The intermediate filaments function as a cytoskeletal scaffold to maintain the structural and mechanical integrity of the quiescent cells and tissues. Most of the fetal cells also express this intermediate filament during early developmental stages as do mature fibroblasts, endothelium and leukocytes (Coulombe and Wong, 2004; Wick and Hornick, 2011). Vimentin is expressed by different mesenchymal tumors like fibrosarcoma, osteosarcoma, RMS, soft tissue sarcomas and some canine epithelial mammary tumors (Hellmen and Lindgren, 1989). Vimentin contributes to epithelial-mesenchymal transition by cytoskeletal reorganization and focal adhesion stability in cancer cells (Liu et al., 2015).

S-100

S-100 is a family of twenty-one members with structural homology but different functional profiles (Bresnick et al., 2015). S-100 expression is observed in a large number of cancers in humans as well as animals. Once included in the panel for differential diagnosis of soft tissue sarcomas, now considered highly non-specific. Injection site chondrosarcoma, fibrosarcoma, hemangiopericytoma, myxosarcoma, schwannoma, melanomas and canine RMS all may show variable levels of S-100 immunoreactivity (Hendrick and Brooks, 1994; Williamson and Middleton, 1998; Ramos-Vara et al., 2000; Koenig et al., 2001; Ramos-Vara et al., 2002; Chijiwa et al., 2004; Milovancev et al., 2015).

Glial Fibrillary Acid Protein (GFAP)

GFAP is an intermediate filament expressed in the astrocytes, ependymal cells and retinal Muller cells but not in mature oligodendroglia. The antibody against GFAP has no significance in the soft tissue sarcoma diagnostics (Wick and Hornick, 2011). Non-glial tumors like osteosarcoma, soft tissue myoepitheliomas, chondrosarcomas and pleomorphic adenomas in human express GFAP (Santos et al., 2009). GFAP (n=44/59) and S-100 (n=59.59) expression was detected in feline PNST (Schulman et al., 2009). Canine benign PNST also express GFAP reactivity (Chijiwa et al., 2004).

Proliferating Cell Nuclear Antigen (PCNA)

PCNA is a multirole protein found in the nucleus of all the eukaryotes. It plays an important role in DNA replication (Kelman, 1997). So, it is also used as a marker for cell

proliferation. Its expression has been evaluated in 3-MCA induced RMS in mice (Inoue and Wu, 2006) and many other human and animal tumors (Preziosi et al., 1995; Roels et al., 1999).

2.5. ECAM and Tyrosine kinases

2.5.1. PDGF and PDGFRs

The PDGF was discovered in the 1970s as a platelet-derived serum growth factor for smooth muscle cells (Ross et al., 1974), fibroblasts (Kohler and Lipton, 1974) and glial cells (Westermark and Wasteson, 1976). Later in 1979, it was purified and characterized by a group of scientists who reported that this growth factor is a 30 kDa protein comprising of two different polypeptide chains linked by reduction-susceptible bonds (Heldin et al., 1979). Platelet-derived growth factor is a group of signaling molecules and is made up of dimers of polypeptide chains A, B, C and D with the possibility of forming five ligands i.e. PDGFA-AA, PDGFA-AB, PDGF-BB, PDGF-CC, and PDGF-D. The compound is a potent mitogen for cells of mesodermal origin and had a wide range of actions in normal healthy cells as well. The receptors for these ligands are tyrosine kinase receptors consisting of α- or β-chains subunits and forming a combination as PDGFR-αα, PDGFR-αβ, and PDGFR-ββ. The PDGF isoforms have different affinity for these tyrosine kinase receptors, where PDGF-BB binds all PDGFRs, PDGF-AA exclusively interacts with PDGFR-αα. PDGF-AB and -CC has affinity for both the PDGFR-αα and PDGFR-αβ and PDGF-DD binds both PDGFR-αβ and PDGFR-ββ (Fredriksson et al., 2004). Interaction of the ligand with the receptor leads to the autophosphorylation of the receptors on tyrosine residues with a subsequent activation of several signaling cascades (Magnusson et al., 2007). PDGFs/PDGFRs axis plays a key role in various physiological as well as pathological functions in the body like angiogenesis, progression through cell cycle, evading apoptosis, the recruitment and regulation of the stromal elements of the tumor and interstitial fluid pressure in the tumor (Ostman, 2004; Tallquist and Kazlauskas, 2004). The PDGFR-α expression is correlated with malignancy grade, higher the expression more malignant the tumor and PDGFR-β expression is relatively stronger in tumors with metastatic potential (Kilvaer et al., 2010). Cell motility is an essential element of many physiological and pathological phenomena including wound healing,

fibrosis, angiogenesis and tumor cell metastasis. PDGF/PDGFRs are believed to play a pivotal role in cell migration. PDGFR-β is known to promote smooth muscle cell migration while PDGFR-α antagonize it, but both PDGFR-α and -β synergistically act to mobilize fibroblasts (Yu et al., 2003). PDGFs and their receptors (PDGFRs) are one of the important mediators of angiogenesis in tumors. Moreover, they recruit and regulate stromal elements of tumor (Ostman, 2004). Interaction between PDGFR-β and its ligand PDGF-B results in activation of PI3K/Akt signal transduction pathway. It generated proliferation and migration of endothelial progenitor cells. The process culminates in the formation of new blood vessels (Wang et al., 2012). They possess the capability of a transforming factor and push the cells through growth cycle, at same time helping them to avoid apoptosis (Tallquist and Kazlauskas, 2004). Upregulation of PDGF-B and PDGFR-α, especially if they co-express is considered a negatively correlated with the disease-free survival time of patients with non-GIST STS (Kilvaer et al., 2010). PDGF-B can stimulate two types of receptors i.e. PDGFR-α and PDGFR-β while PDGF-A only interact with PDGFR-α. PDGF-α show antagonistic effects on PDGFR-β induced phenotypic transformation (Yu et al., 2000). PDGF-β expression was observed in the feline injection site sarcoma cell lines (n=5) and in one non-injection site fibrosarcoma cell line through immunoprecipitation. Addition of imatinib mesylate in these cells inhibited the ligand-induced autophosphorylation of PDGFR-β in cell lines from injection site sarcomas. Imatinib affected the survival of tumor cells in a dose-dependent manner. *In vivo* experiment established the therapeutic efficiency of imatinib in treating injection site sarcomas (Katayama et al., 2004). Similar effects have been reported by masitinib which is an anti-tyrosine kinase receptor drug. When PDGF-BB stimulated cell line derived from primary and lung metastasis were treated with masitinib, growth inhibitory effects were noted at doses 0.86 μM and 8.6 μM for primary and metastatic cell lines, respectively (Lawrence et al., 2012). *In vitro* studies using human dermal and lung fibroblasts indicated that there is difference in PDGFR stimulation by different PDGF ligands in different tissues e.g. in human dermal fibroblast, PDGF-DD can phosphorylate both PDGFR-α and -β while in lung fibroblasts, all PDGF ligands can stimulate PDGFR-α (Donovan et al., 2013). PDGFR-β is linked with fibroblast migration as its deletion reduces fibroblast migration. Though bovine fetal serum and PDGF-A showed mitogenic and anti-apoptotic

properties but failed to produce migration of dermal fibroblasts in absence of receptor beta (Gao et al., 2005). Jacobs et al. (2017) observed PDGFR, vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR) and stem cell factor (SCF) expression in an injection site sarcoma of a dog. PDGFR-A, its subsequent effector molecules MAPK and Akt are overexpressed in primary and metastatic alveolar RMS in humans (Taniguchi et al., 2008). Gene expression study demonstrated that PDGF-C and PDGF-D are upregulated in all subtypes of human RMS (n=101). Immunohistochemical analysis showed that tumor cells express PDGF-C, PDGF-D, and PDGFR-α while stromal cells expressed PDGFR-β (Ehnman et al., 2013). Canine liposarcoma highly express PDGF-B (n=40/49) and its cognate receptor PDGFR-β (n=34/48) along with other tyrosine kinases (Avallone et al., 2017). Biopsies from canine oral fibrosarcoma cases revealed PDGFR-α and -β overexpression (Milovancev et al., 2016). Both human and canine osteosarcomas express tyrosine kinase receptors (PDGFR-α and PDGFR-β). Expression profiling of tyrosine kinase receptors and their ligands in canine osteosarcoma cases (n=33) by using immunohistochemistry and qPCR indicated an overexpression of these molecules. Through immunohistochemistry, PDGF-A expression was noticed in 42% cases while PDGF-B was positive in 60% tumors. Seventy eight percent tumors expressed PDGFR- α and 81% PDGFR-β (Maniscalco et al., 2013).

2.5.2. E-Selectin

The members of the selectin family are lectin containing glycoproteins. They function to anchor the leucocytes to the endothelial cells and mediate migration of inflammatory cells. The members of the selectin family include E-selectin (C62E), P-selectin (CD62P), and L-Selectin (Lasky, 1992). E-selectin is found on the vascular endothelium, P-selectin on platelets and leucocytes, and L-selectin on leucocytes (Rosen and Bertozzi, 1994; Kramer, 2016). The ligands for the selectin molecules are glycoproteins, or glycolipids or proteoglycans containing sialylated, fucosylated, or, in some cases, sulfated glycans. Under physiological conditions, selectin expression is strictly regulated to control the inflammatory response, however, disruption of this regulation may lead to disorders of inflammatory and thrombotic response, and may also help in tumor metastases (McEver, 1997). The distinctive

property of E-selectin is its ability to pair with numerous sialyl Lewis x bearing ligands and intercede slow rolling of cells. In mouse, ligands for E-selectin include CD44, E-selectin ligand-1 (ESL-1), and P-selectin glycoprotein ligand-1 (PSGL-1) which play a significant role in slow rolling of leukocytes along the endothelium, their attachment to endothelial cells and transendothelial migration (Chase et al., 2012). Scientific literature indicates the potential role of selectins in mediating the adhesion of circulating metastatic cancer cells to the vascular endothelium (Laubli and Borsig, 2010; St Hill, 2011). E-selectin is an important mediator in the metastasis of human prostate cancer to bones. ESL-1 ligand of E-selectin has high expression on normal and local prostate cancer cells but PSGL-1 which also serves as a ligand for E-selectin has relatively high expression on the human bone-metastatic prostate neoplastic cells suggesting its potential role in prostate cancer metastasis to bone (Dimitroff et al., 2005). Mac-2 binding protein is a newly reported ligand of E-selectin expressed on breast cancer cells (Shirure et al., 2012). Likewise, expression and binding of E-selectin ligands of colon cancer cells to E-selectin receptor is linked with metastasis. An *in vitro* study on malignant and non-malignant breast and colon cancer lines and *in vivo* severe combined immunodeficient (SCID) mice xenograft model delineated the overexpression of selectin ligands sialyl-Lewis x (sLe^x, CD15s) and sialyl-Lewis a (sLe^a, CA19-9) in metastatic colon cancer cells as compare to non-metastatic cells (Schnegelsberg et al., 2011). sLe antigens may promote metastasis by forming emboli of cancer cells and platelets, which favor their arrest on endothelia. Based on their pivotal role in tumor metastasis, selectins may be a potential target for anti-cancer therapeutic agents (Trinchera et al., 2017). Contact of human melanoma cells with human umbilical endothelial resulted in increased expression of intercellular cell adhesion (ICAM) molecules and E-selectin. Further investigation revealed that interaction between vascular E-selectin and CD44 on melanoma cells was responsible for high expression of ICAM (Zhang et al., 2014). Contrary to this, E-selectin expression in human Merkel cell carcinoma is associated with the entry of CD8+ T-lymphocytes and a favorable outcome with better survival of the patients (Afanasiev et al., 2013). In human squamous cell carcinoma, the influx of myeloid-derived suppressor cells leads to down-regulation of E-selectin through nitric oxide production. The inhibition of nitric oxide restores E-selectin expression in the vascular endothelium and thus increases T-cell

recruitment in the tumor (Gehad et al., 2012). Thus, E-selectin has a dynamic role in physiological and pathological conditions in the body.

2.5.3. Integrin alpha v

Integrins and their ligands play a very important role in many physiological and pathological processes e.g. immune reaction and white blood cell trafficking, hemostasis. They also contribute to many genetic and autoimmune human diseases. Moreover, they play significant role in some cancers e.g. cutaneous melanoma. They serve as receptors for viruses and bacteria as well (Hynes, 2002). Integrins play an important role in cell migration during e.g. tissue repair, migration of cells responsible for immunity of body, stem cells migration to their target organs. Alteration in integrin assisted adhesion and migration has been linked with many cancers and immunodeficiency in humans (Huttenlocher and Horwitz, 2011). The extracellular domain of the integrins determines the binding specificity and interact with various ligands including collagen (e.g., α1β1, α2β1), fibronectin (e.g., α5β1, αvβ3, α4β1), and laminin (e.g., α2β1, α3β1, α6β1). Integrins also interact with members of Ig superfamily e.g. ICAM-1 (αLβ2, αMβ2) or VCAM-1 (α4β1) (Huttenlocher and Horwitz, 2011).

Alpha v integrin can bind to several different beta subunits (Table-1). Expression of these alpha v integrin molecules has been linked with many types of cancers in humans (Takayama et al., 2005). The interaction of ligands with integrins (especially alpha v) is an essential event in the angiogenesis, limiting leucocytes to sites of repair, or the invasive capability of neoplastic cells (Weis and Cheresh, 2011). Immunohistochemical analysis of human pancreatic cancers showed that stromal tissue upregulation of integrin alpha v was associated with lymph node metastasis of tumor cells while *in vitro* knockdown of alpha v integrin (in pancreatic stellate cells) resulted in reduced growth and movement of cells. Furthermore, gene expression related to ECM and tumor-stromal interaction was decreased (Horioka et al., 2016). Likewise, laryngeal and hyolaryngeal squamous cell carcinoma in humans showed elevated levels of integrin alpha v. The tumors with lymph node metastasis showed increased expression of the integrin alpha v molecule as compared to primary cancer and tumors without metastasis (Lu et al., 2008).

Feline fibrosarcoma express αvβ3 integrin on endothelial cells and tumor cells in the vicinity of these blood vessels (Wenk et al., 2013). Breast cancer cells (MDA-MB-231) expressing a higher level of integrin α5β1 are three times more invasive than their low expressing counterparts. The cells with high integrin expression generate seven times greater contractile forces than cells with low α5β1 (Mierke et al., 2011). Extracellular matrix (ECM)/integrin axis is responsible for resistance of cancer cells to chemotherapy by protecting cells from drug-induced apoptosis (Aoudjit and Vuori, 2012). Alpha vβ3 integrin expression on melanomas is associated with tumor invasion (Seftor et al. 1992). Likewise, the increased levels of α2β1 integrin are associated with metastasis of RMS (Chan et al., 1990). Breast cancer cells with high level of α5β1 show increase in stress fiber formation, focal adhesion density and cell spreading leading to an increase in cell stiffness, remodeling of cytoskeleton and generation of increased contractile force. This enables the cells to the physical barriers of the ECM (Mierke et al., 2011). Expression of αvβ3 integrin in human cutaneous melanomas is associated with more invasiveness and metastatic potential (Seftor et al., 1999). Tumor cells with upregulation of αvβ3 produce matrix metalloproteinase-2 and an elevation in the mRNA levels of MT1-MMP and TIMP-2 (Felding-Habermann et al., 2002). Likewise, an immunohistochemical analysis of melanocytic tumors of dogs indicated integrin αvβ3 expression in cutaneous (72%) and oral melanomas (88%). The expression was limited to endothelial cells and melanocytes and their vicinity (Rawlings et al., 2003). Canine lymphoma cells lack integrin αvβ3 which make it resistant to any viral vector-based gene therapy (O'Neill et al., 2011).

Table 1. Different integrin alpha v receptors and their ligands

Integrins	**Human A chain characteristics**	**cleavage**	**Alpha I**	**Prototypic ligands/recognition sequences**	**Additional ligands**
α5 β1(CD49c, VLA5)	1049aa	x		Fibronectin (RGD)	Endostatin
α5 β1(CD51)	1048aa	x		Fibronectin, vitronectin (RGD)	
α5 β3		x		Vitronectin, Fibronectin, Fibrinogen (RGD)	Tenascin, Fibrillin, Osteopontin
α5 β5		x		Vitronectin RGD	
α5 β6		x		Fibronectin, TGF- β-LAP (RGD)	
α5 β8		x		Vitronectin, TGF- β-LAP (RGD)	

RGD is tripeptide found in various proteins e.g. fibronectin, vitronectin, table adopted from (Barczyk et al., 2010)

3. MATERIALS AND METHODS

Current research project comprised of two components. The first component was a retrospective analysis of mesenchymal tumors submitted to Department of Veterinary Pathology, Faculty of Veterinary Medicine, Ondokuz Mayis University Samsun. The archive of the department was screened (2004-2017) and tumors belonging to mesenchymal lineage were selected for further investigations. The second part of the project was the experimental induction of RMS in mice and investigating effects of PDE5 inhibitor Tadalafil.

3.1. MATERIALS

3.1.1. Specimens for Retrospective Study of Tumors of Cats and Dogs

The departmental archive was screened for all the tumors diagnosed as soft tissue sarcoma/or of mesenchymal origin. A total of 51 cases of feline and canine soft tissue sarcoma cases were shortlisted for further examination. Formalin-fixed paraffin-embedded blocks were sectioned into 5µm thick slices using microtome. Additionally, control tissues were collected from cats (n =5; four female and one male), aged 3 months to 6 years (mean 2.85 years) and adult male (n=5) dogs, aged 1-8 years (mean: 5.4 years) from the interscapular area which is a common site for occurrence of soft tissue sarcomas in these species. Control tissues were collected during routine necropsies of cats and dogs died from diseases apart from endocrine diseases and diseases affecting the skin. Sections were deparaffinized as per standard histological procedure and stained with hematoxylin and eosin (HE) and after histological analysis of the tumors, 25 specimens with features of soft tissue sarcomas were enrolled in the study (Table 2 & 3).

3.1.2. Mice for Experimental induction of Tumor

The study was conducted on 12-week old male Swiss albino mice having 25-30 g body weight. Mice were obtained from legally authorized experimental animal producers & suppliers. The mice underwent routine health checks and body weight measurement. The animals were reared at the Laboratory of the experimental animal production and research center of Kırıkkale University. A period of two weeks was provided for the acclimatization to the animals, during which proper ventilation, a temperature of 21±1 ºC and relative

humidity proportion of 50±5% and 12-hour light/dark cycle was maintained. During this period, animals were not subjected to any experimental intervention. Animals were provided with *ad libitum* feed (pellet) and water.

T**able 2.** Clinical characteristics of 14 cat tumor cases selected for study

Tumor No	**Case No**	**Age (years)**	**Sex**	**Breed**	**Tumor Site**
1	4712	9	M	Van	Interscapular
2	1812	NA	NA	NA	Interscapular
3	8411	5	M	Turkish Angora	Back/Dorsum
4	2111	10	M	Tabby	Right gluteal
5	3009	11	F	DSH	Left thoracic
6	8215	9	M	Turkish Angora	Back/Dorsum
7	892	NA	NA	NA	Dorsolumbar
8	5207	13	M	DSH	Interscapular
9	6375	12	F	DSH	Dorsal region
10	8231	10	M	NA	Scapular region
11	6217	12	F	NA	Interscapular
12	3109	6.5	M	Angora x Van	Left thoracic
13	8377	9	F	DSH	Left thoracic wall
14	8604	8	F	DSH	Interscapular

M: Male; F: Female, DSH: Domestic short hair, NA: Not available

Table 3. Clinical characteristics of 11 dog tumor cases selected for study

Tumor No	Case No	Age (years)	Sex	Breed	Tumor Site
1	3615	4	M	Mix	Submandibular
2	1915	9	M	Kangal	Neck
3	114	10	M	Mix	Prepuce
4	822	14	F	NA	Left Cheek
5	6754	7	M	NA	Ear
6	9664	NA	NA	NA	Flexor carpi tendon
7	2706	NA	NA	NA	Metatarsus
8	8087	4	M	Boxer	Left Back/dorsum
9	8101	NA	NA	NA	Dorsal cervical
10	1120	10	F	Mix	Left Flank
11	1239	5	M	German Shepherd	Right shoulder

M, male; F, female; NA: not available

3.1.3. Equipment

Leica TP-1020 automated tissue processor was used for processing of tissues for histopathological analysis. The paraffin-embedded blocks were prepared by using Leica embedding station and tissues were cut by using microtome (Leica, RM2125RT). Slides were analyzed by using a Nikon microscope (Nikon, Eclipse E600, Nikon Corporation, Japan). For antigen retrieval during immunohistochemistry, a microwave oven was used.

3.1.4. Chemicals

For tumor induction in mice, 3-MCA was procured from Sigma-Aldrich, St. Louis, Montana, USA. Tadalafil (25mg/ml) used as a theraputic agent in mice was obtained from The Chemo Depot, USA.

Histochemical Stains

Haematoxylin and eosin, Masson's trichrome stain, and Alcian blue/Periodic acid Schiff (AB-PAS) and PTAH were used for the evaluation of various components of tumor tissues.

3.1.5 Antibodies

The antibodies used in the study for immunohistochemistry (IHC) are listed in table 4 and 5

Table 4. Panel of antibodies used for histogenic characterization of tumor in dogs, cats & mice

Antibody	Dilution in dog cat	Dilution in mice	Host/isotype/immunogen	Source & product codes
Myoglobin	1:10,000	1:500	Rabbit, PAb, IgG, Human	Abcam, ab74213
Alpha sarcomeric actinin	1:10,000	1:2000	Mouse, MAb, IgG1, rabbit	Sigma Aldrich, A7811
Alpha Smooth Muscle Actin	1:10.000	1:2000	Mouse, MAb, IgG2akappa	Thermo, MS-113-P
Desmin	1:10,000	1:2000	Mouse, MAb, IgG2b, Chicken	Santa-Cruz, sc-23879
Myogenin	1:50	1:50	Mouse, MAb, IgG1 Kappa	MGN185(F5D), NBP2-29431
MyoD1	1:50	ND	Mouse, MAb, IgG1 Kappa	NB100-56511, Novus Biologicals
Muscle Specific Actin	Ready to use	Ready to use	Mouse, Mab, IgG1 kappa	Neomakers, Ab-4, MS-742-R7
Vimentin	1:10,000	1:1000	Mouse, MAb, IgG2a, Bovine	Abcam, ab28028
S-100	1:10.000	ND	Mouse, MAb, IgG2a, Bovine	Thermo, MS-296-P
GFAP	1:4000	1:4000	Mouse, MAb, IgG1	Merck Millipore, MAB3402
PCNA	ND	1:100	Rabbit, PAb, IgG	Thermoscientific, PA5-27214

ND; Not determined, PAb; Polyclonal antibody, Mab; Monoclonal Antibody

Table 5. Panel of antibodies used to study ECAM and tyrosine kinases expression

Antibody	Dilution	Host/Isotype/Immunogen	Source & Product codes
E-Selectin	1:100	Rabbit, PAb, IgG	Biovision:3631-100
Integrin alpha v	1:500	Rabbit, PAb, IgG,	Merck: AB1930
PDGF-A	1:100	Mouse, MAb, IgG1	Santa Cruz (E-10): sc-9974
PDGFR-alpha	1:400	Rabbit, PAb, IgG,	Santa Cruz (C-20): sc-338

PAb; Polyclonal antibody, MAb; Monoclonal antibody

3.2. METHODS

3.2.1. Histopathological Examination of Tumors of Cats and Dogs

The diagnosis of the tumors was reviewed after histological analysis and subsequent immunolabelling and a total of 25 animal (cats=14, dogs=11) was confirmed as STSs and processed for further studies. Histological grading of the tumors was done by following the criteria described for soft tissue sarcomas by Federation Nationale des Centers de Lutte Contre le Cancer (FNCLCC) for humans but is also widely accepted in the animal oncology (Dennis et al., 2011; Meuten, 2016). The tumors were graded on the basis of the mitotic rate, presence or absence of necrosis and degree of differentiation (Trojani et al., 1984; Meuten, 2016) (Table 6). Mitotic count was determined in an area of 2.37mm^2 at 40x (10 high power field) by using a microscope assembled with an ocular of field number (FN) 22mm (Meuten et al., 2016).

Table 6. Criteria used for grading of soft tissue sarcomas

Degree of Differentiation	Score 1: Tumor cells closely resembling normal adult mesenchymal tissue e.g. well-differentiated liposarcoma Score 2: Sarcomas for which histologic type is certain e.g. myxoid liposarcoma Score 3: Embryonal and undifferentiated sarcoma, Sarcomas of doubtful types, synovial sarcoma, osteosarcoma,
Mitotic Count:	Score 1: 0-9 mitotic figures in an area of 2.37mm^2 Score 2: 10-19 mitotic figures in an area of 2.37mm^2 Score 3: more than 20 mitotic figures in an area of 2.37mm^2
Tumor necrosis:	Score 0: No necrosis Score 1: Less than 50% tumor necrosis Score 2: More than 50% tumor necrosis
Histological grade:	Grade 1: Total score 2, 3 Grade 2: Total score 4,5 Grade 3: Total score 6, 7, 8

3.2. 2. Experimental Induction of Rhabdomyosarcoma Using 3-MCA in Mice

A total of 60 mice were divided into 6 groups each containing 10 animals. Experimental setup details are given in Table 7. Briefly, Group 1 (normal skin: NS) was not subjected to any treatment and served as control. Group 2 (3-MCA) was subcutaneously injected in the interscapular region with 1mg/0.2ml 3-MCA (in the sesame oil). Group 3 (Tad IP) was injected intraperitoneally with 10mg/kg body weight tadalafil at the same time when group 5 was started. (Dose of the tadalafil was determined from the previous studies in laboratory animals by Sanna et al., 2009 & Arikan et al., 2010). Group 4 (Tad Sc) was given a subcutaneous injection of Tadalafil 10 mg/kg in the interscapular region at the time of start of group 6. Group 5 (IP) was injected with a single dose of 1 mg/0.2ml 3-MCA (in sesame oil) in the interscapular region subcutaneously and Tadalafil 10mg/kg intraperitoneally (after the tumor has developed to a 10 mm size). Group 6 (IT) was treated with a single dose of 1mg/0.2ml 3-MCA in sesame oil in the interscapular region and after the development of tumor to a size of 10 mm was given a intratumoral injection of Tadalafil 10mg/kg. Tadalafil treatment was continued for a period of 14 days with an interval of 24 hours.

Table 7. Experimental design for the induction rhabdomyosarcoma using 3-MCA and its treatment with tadalafil

Group	*n*	Treatment	Dose	Route of administration	Duration
Group 1	10	No treatment	0	No treatment	0
Group 2	10	3-MCA	1 mg/0.2ml	*SC/ISR	Single dose
Group 3	10	Tadalafil	10 mg/kg	**IP	14 days
Group 4	10	Tadalafil	10 mg/kg	SC/ISR	14 days
Group 5	10	3-MCA	1 mg/0.2ml	SC/ISR	Single dose
		Tadalafil	10 mg/kg	IP tumor size >10 mm	14 days
Group 6	10	3-MCA	1 mg/0.2ml	SC/ISR	Single dose
		Tadalafil	10 mg/kg	IT, tumor size >10 mm	14 days

*SC/ISR: Subcutaneous/interscapular region; **IP: intraperitoneal, IT: intratumoral

Mice from all the groups were sacrificed by cervical dislocation after 24 hours of the last treatment. A systemic necropsy was performed and samples from tumor and regional skin were collected for histopathological examination. Histopathological analysis of the tumor was carried out and later tissues were stained with the immunohistochemical markers.

3.2.3. Histochemistry

Masson's trichrome staining was performed for the evaluation of collagen as described by Bancroft and Layton (2013) and was graded 1 for very mild staining of the stromal tissue, 2 for moderate and 3 for fair positivity. AB-PAS staining was carried out as per method of the Mowry (1956; 1963) for the evaluation of mucopolysaccharides and PTAH was performed on all the specimens under study in order to look for any cross striations in the tumor cells (Bancroft and Layton, 2013).

3.2.4. Immunohistochemistry

Immunohistochemistry (streptavidin-biotin peroxidase method) was performed on the formalin fixed paraffin embedded tissue sections (5µm) obtained from the neoplastic tissues of dogs, cats, and mice. A panel of antibodies (Tables 6-8) was used to classify the tumors as well as investigating their role in tumor propagation and pathogenesis. Proliferating cell nuclear antigen (PCNA) was used to assess the proliferating potential of the tumor cells.

Serial sections were dewaxed in xylene and hydrated through graded alcohols. Endogenous peroxidase activity was blocked with H_2O_2 (3%) in absolute methanol for 10 minutes. The sections were rinsed with phosphate buffered saline (PBS, pH 7.4) and subsequently heated in citrate buffer (pH 6.0) in a microwave oven (600 W) for 10 min for antigen retrieval except for myogenin and MyoD1, for which tris EDTA (pH 9) was used for 25 minutes. All sections were pre-incubated in blocking solution (Zymed Laboratories, Inc.; San Francisco, CA, USA) at room temperature for 10 minutes to block nonspecific binding of the second-step antibody (Zymed Laboratories). After washing with PBS, the sections were then incubated with each of the primary antibodies for 1-2 hour at room temperature or overnight at 4°C (Tables 6-8). Sections were briefly rinsed with PBS 2-3 times and incubated with biotin-conjugated second-step antibody for 20 minutes at room temperature. After

further washing with PBS, all sections were treated with streptavidin-biotin-peroxidase complex (SABC, Zymed Laboratories) for 20 min at room temperature. After brief rinsing with PBS labeling was ‘‘visualized’’ with 3-amino-9-ethylcarbazole (AEC substrate kit; Invitrogen, Camarillo, Canada) as the chromogen. Sections were counterstained briefly with Harris hematoxylin for 1 min, rinsed with tap water, and mounted with an aqueous mounting medium (Vision Mount; Lab Vision, USA). Primary antibodies were omitted from negative control sections, which were incubated with either PBS or diluted normal serum from the species in which the primary antibody was raised.

3.2.5. Evaluation of Immunostaining

Tissue sections were scored semiquantitatively for immunolabeling. The percentage of the positive cells and intensity of staining were taken into consideration during the evaluation and a score between 0-300 was given depending on both factors. H-score (Histo score) was calculated by the formula 1x (% cells weak positive), +2x (% cells moderately positive), +3x (% cells strong positive). For calculation of proliferation index photographs of 10 high power (objective 40x) microscopic fields were captured and cells were manually counted.

3.2.6. Double Immunofluorescence Staining

Selected cases were stained for PDGF-A and PDGFR-alpha with double immunofluorescent staining method. The protocol used is as follow:

1. Sections were deparaffinized and rehydrated through descending grades of alcohol.
2. Endogenous peroxidase was blocked by using H_2O_2 3% in absolute methanol for 10 minutes at room temperature.
3. Antigen retrieval was performed in the citrate buffer solution placed in a microwave oven adjusted at 600 watts for 10 minutes.
4. Non-specific protein blocking was done by using a commercial blocking solution Super Block® (ScyTek Laboratories, Logan, Utah, USA, Ref. AAA125, Lot number 39918) for 10 minutes.
5. Tissue sections were covered with primary antibody against PDGFR-alpha (Rabbit polyclonal; 1/400 dilution) and an overnight incubation was performed at 4°C.

6. Sections were washed with PBS.
7. Sections were incubated with a FITC conjugated anti-rabbit secondary antibody (Sheep anti-rabbit IgG, F7512, Sigma, 1:128 dilution) for 30 minutes at room temperature in the dark.
8. Sections were briefly rinsed with PBS.
9. It was followed by the application of second primary antibody incubation i.e. PDGF-A (Mouse monoclonal, dilution 1:100 in PBS) at room temperature for 1 hour.
10. Sections were briefly rinsed with PBS.
11. Rhodamine-linked goat anti-mouse (AP124R, EMD Millipore, dilution 1:128 in PBS) was applied to the section and incubated for 30 minutes at room temperature in the dark.
12. Sections were briefly rinsed with PBS.
13. Nuclear staining was performed by incubating sections with DAPI (Sigma D9542, 10 mg) for 30 Seconds at room temperature. The stock solution was prepared by dissolving 1.250 mg of DAPI powder into 1 ml distilled water. This stock solution was further diluted at a ratio of 1:125 in distilled water.
14. Sections were washed with distilled water for 5 minutes.
15. Sections were covered with mounting medium (Sigma, Fluoromount Medium, F4680-25ml) and a coverslip was applied.
16. Slides were examined using a fluorescent microscope (Nikon Eclipse, E6)

3.2.7. Statistical Analysis

GraphPad Prism® 5 program was used for the statistical analyses of the data. The data (H-score) obtained from the immunohistochemical analysis of different grades of tumors of dogs and cats analyzed by descriptive statics and also compared for the difference in their median values by using Kruskal Wallis test. Furthermore, Spearman's correlation was used to assess any relationship between the expression of PDGF-A, PDGFR-alpha, Integrin alpha v and E-selectin among the tumors in dogs and cats. The data obtained from the mice were first checked for normality of distribution by D'Agostino-Pearson normality test along with measurement of kurtosis and skewness and the data which followed a normal distribution

was analyzed by one-way ANOVA and the data which did not fulfill the assumptions of normality was subjected to analysis by Kruskal Wallis test for measuring the difference in medians. The $p < 0.05$ was considered as significant.

4. RESULTS

4.1. Signalment and History in Cats

The majority of the tumors in the cats were fibrosarcomas (n=13/14), except one case of a myxoid liposarcoma (Table 8). The tumors included in this study occurred at sites commonly used for injections or vaccine administration. No information was available about the use of any vaccine or drug. The age range of the cats varied from 5 to 13 years (median 9.5 years). Seven of the cats were male and five were female while gender information was missing about two cats.

4.1.1. Histopathological Findings in Cat Tumors

The tumors were located beneath the dermis. Pseudocapsule was observed in few cases. Peripheral foci of lymphocytic infiltration were noticed (Figure 1a). However, histiocytic cells or macrophages were scant in the cases under study (observed in 3 cases). The tumor cells were comprised of mesenchymal spindle-shaped cells. Most of the tumors were having anaplastic features with the presence of multinucleated cells while classical herringbone pattern was rarely observed (Figure 1b, 1c). Variably sized areas of necrosis were also present (Figure 1a). Mucin containing areas were observed in some of the tumors.

A case of myxoid liposarcoma was observed in the interscapular area. The tumor was composed of cells with a peripheral nucleus having a vacuolar cytoplasm. Neoplastic cells exhibited marked anisocytosis and anisokaryosis. Many giant cells were scattered through the tumor stroma. AB-PAS staining showed mucin secretion in the myxoid areas leading to the diagnosis of myxoid liposarcoma (Figure 2).

4.2. Signalment and History in Dogs

Eleven dogs with neoplastic growth were included in the study. The age of the animals varied from 4 to 14 years (median, 7 years), information about the age of 3 animals were not available. Six of the dogs were male while 3 were female, information about the rest was not available from the archive. No history about vaccination or any medication was submitted by the veterinarians.

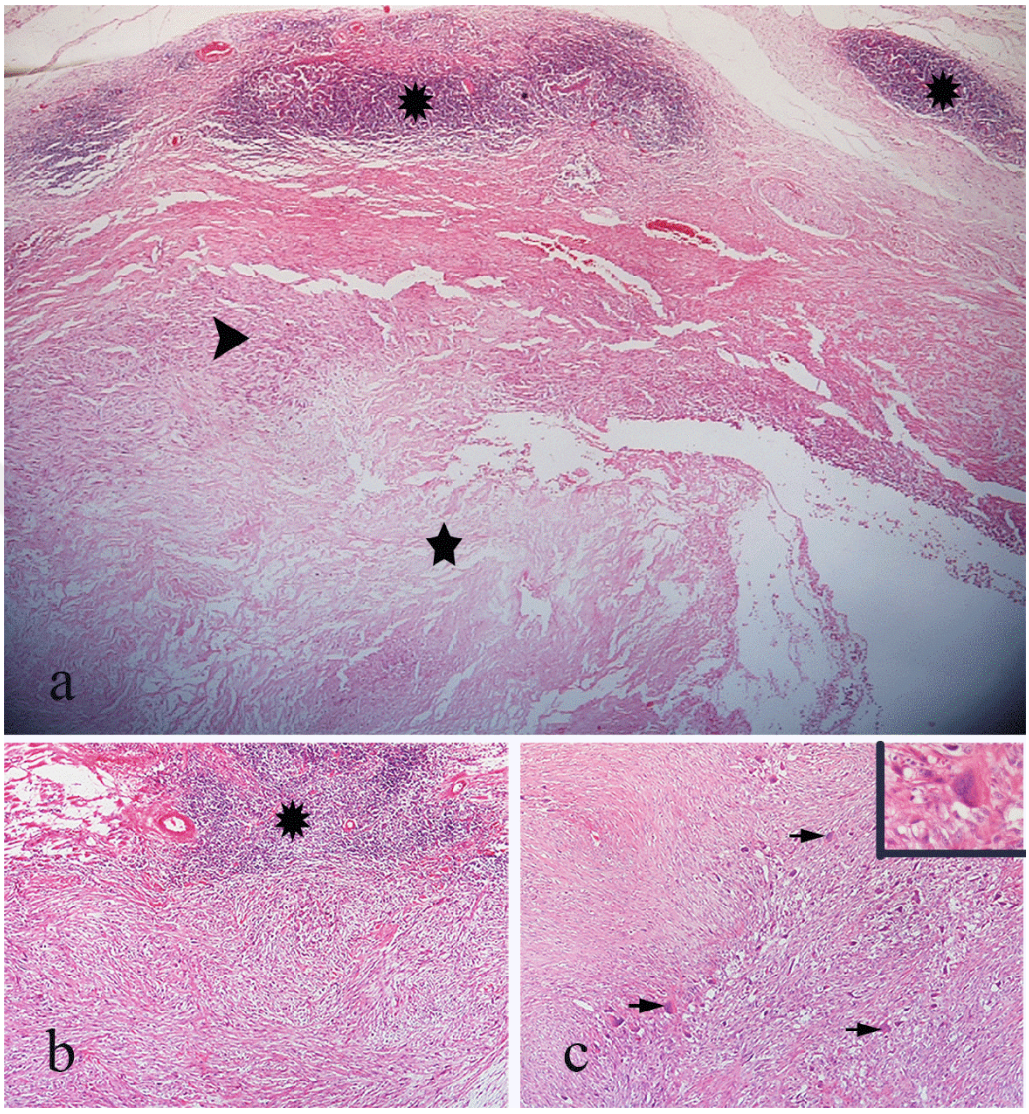

Figure 1. a: Photomicrograph of an injection site fibrosarcoma from a cat. Note the peripheral foci of inflammatory cells (asterisks), tumor cells (arrowhead), and necrotic areas (star), objective 2x. b: A grade 2 fibrosarcoma with peripheral lymphocytic infiltration (asterisk), objective 4x. c: A highly anaplastic grade 3 fibrosarcoma with many multinucleated tumor giant cells (arrows) objective 4x, inset: high magnification view of tumor giant cell, objective 40x.

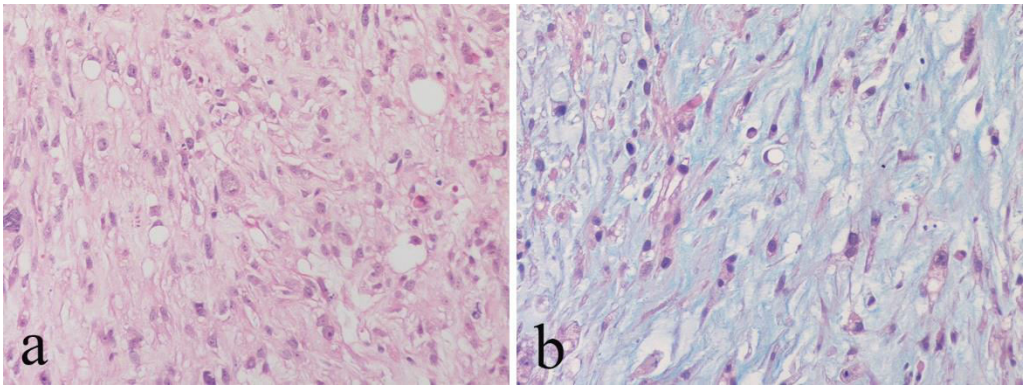

Figure 2. a: Photomicrograph of a myxoid liposarcoma showing neoplastic cells with cytoplasmic vacuolation pushing nucleus to periphery of cell, objective 20x, b: AB-PAS staining of the same tumor showing mucinous matrix, objective 20x

4.2.1. Histopathological Findings in Dog Tumors

Two tumors showed the characteristics of injection site sarcoma, peripheral lymphocytic focal infiltration and central areas of necrosis (Figure 3d). One tumor showed features of myxosarcoma, rest were classified as spontaneous fibrosarcomas. The spontaneous fibrosarcomas were located beneath the epidermis and composed of spindle-shaped cells arranged in interwoven bundles (Figure 3a, 3b, 3c).

Myxosarcoma was characterized by low cellularity and composed of loosely arranged spindle to stellate cells suspended in the mucinous matrix. Mitotic figures were infrequent (Figure 4)

4.3. Histochemical Analysis of Tumors in Cats

Myxoid or mucinous areas stained positive in (8/14; 57%) cat tumors with AB-PAS staining (Figure 5a). In cats, 12 out of 14 tumors showed mild to fair staining of the stromal collagen with trichrome stain (Figure 6 d, 6e. 6f), 4 tumors in each category i.e. 1, 2, and 3, and two tumors were negative (Table 10). PTAH was negative in all the tumors under study in both species.

4.4. Histochemical Analysis of Tumors in Dogs

One myxosarcoma and 4 fibrosarcomas showed positivity for AB-PAS staining in dogs (Figure 5b). Five tumors in dogs showed minor reactivity with trichrome stain, 2 cases

stained moderately and 1 with relatively more collagenous stroma was given a score of 3 (Figure 6a, 6b, 6c; Table 11).

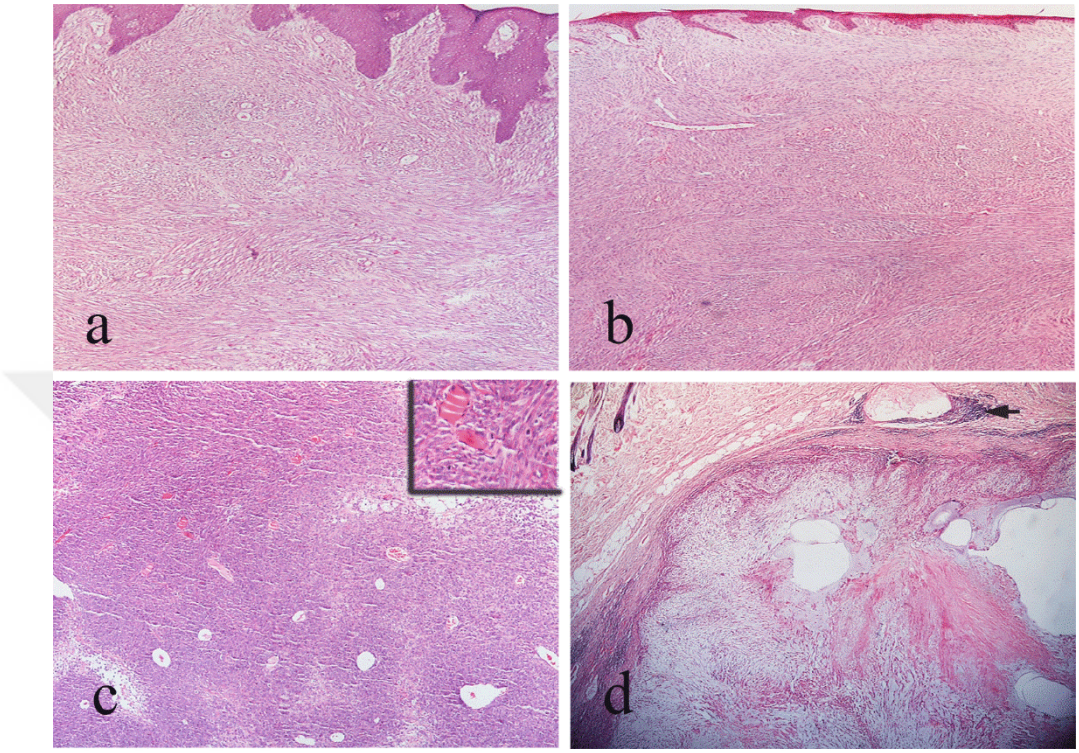

Figure 3. a: A grade 1 canine fibrosarcoma, objective 4x, b: Grade 2 canine fibrosarcoma, objective 4x, c: Grade 3 canine fibrosarcoma, objective 4x, inset: mitotic figures in grade 3 tumor objective 40x, Note the difference in cellularity between different grades, d: Injection site fibrosarcoma with peripheral lymphocytic infiltration (arrow), central necrosis and cavitation, objective 2x

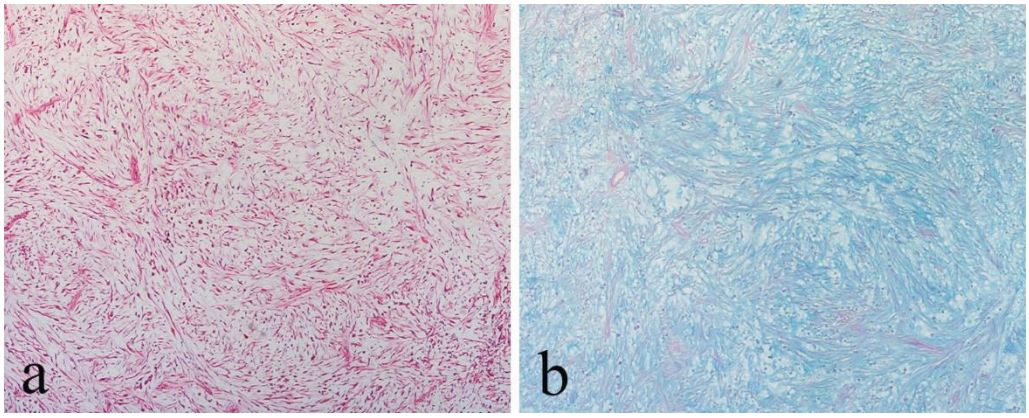

Figure 4. a: Photomicrograph of myxosarcoma in a dog showing loosely arranged fusiform cells, objective 4x, b: AB-PAS stained tissue section showing mucin-rich tumor neoplastic cells, objective 4x

Table 8. Clinical and histopathological characteristics of tumors in 14 cats

Tumor No	Case No	Age (years)	Sex	Breed	Tumor Site	Diagnosis	Mitosis[a]	Necrosis	Differentiation[b]	Tumor Grade
1.	4712	9	M	Van	Interscapular	Fibrosarcoma	1	2	2	2
2.	1812	NA	NA	NA	Interscapular	Fibrosarcoma	2	1	1	2
3.	8411	5	M	Turkish Angora	Back/Dorsum	Fibrosarcoma	1	2	2	2
4.	2111	10	M	Tabby	Right gluteal	Fibrosarcoma	1	2	1	2
5.	3009	11	F	DSH	Left thoracic	Fibrosarcoma	2	1	2	2
6.	8215	9	M	Turkish Angora	Back/Dorsum	Fibrosarcoma	1	1	2	2
7.	892	NA	NA	NA	Dorsolumbar	Fibrosarcoma	1	1	2	2
8.	5207	13	M	DSH	Interscapular	Myxoid Liposarcoma	2	1	2	2
9.	6375	12	F	DSH	Dorsal region	Fibrosarcoma	2	1	2	2
10.	8231	10	M	NA	Scapular region	Fibrosarcoma	2	1	2	2
11.	6217	12	F	NA	Interscapular	Fibrosarcoma	3	2	2	3
12.	3109	6.5	M	Ang. X Van	Left thoracic	Fibrosarcoma	3	2	2	3
13.	8377	9	F	DSH	Left thoracic wall	Fibrosarcoma	3	1	2	3
14.	8604	8	F	DSH	Subcutaneous nodule	Fibrosarcoma	3	1	3	3

M: Male, F: Female, NA: Not available, DSH: domestic short hair, Ang. X Van: Angora X Van, [a]Mitotic count was determined in an area of 2.37mm^2 at high-power field (40x objective); low (1) = 0-9, moderate (2) = 10-19 or high (3) = >20; [b](1) well differentiated, (2) moderately differentiated, (3) poorly differentiated

Table 9. Clinical and histopathological characteristics of tumors in 11 dogs

Tumor No	Case No	Age (years)	Sex	Breed	Tumor Site	Diagnosis	Mitosis[a]	Necrosis	Differentiation[b]	Tumor Grade
1.	3615	4	M	Mix	Submandibular	Fibrosarcoma	1	1	1	1
2.	1915	9	M	Kangal	Neck	Myxosarcoma	1	0	1	1
3.	114	10	M	Mix	Prepuce	Fibrosarcoma	2	0	1	1
4.	822	14	F	NA	Left Cheek	Fibrosarcoma	1	0	1	1
5.	6754	7	M	NA	Ear	Fibrosarcoma	1	0	1	1
6.	9664	NA	NA	NA	Flexor carpi tendon	Fibrosarcoma	2	0	2	2
7.	2706	NA	NA	NA	Metatarsus	Fibrosarcoma	3	0	1	2
8.	8087	4	M	Boxer	Left Back/dorsum	Fibrosarcoma	3	0	2	2
9.	8101	NA	NA	NA	Dorsal cervical	Fibrosarcoma	3	1	2	3
10.	1120	10	F	Mix	Left Flank	Fibrosarcoma	3	1	2	3
11.	1239	5	M	German Shepherd	Right shoulder	Fibrosarcoma	3	1	2	3

M, male; F, female; NA: not available; [a]Mitotic count was determined in an area of 2.37mm^2 at high-power field (40x objective); low (1) = 0-9, moderate (2) = 10-19 or high (3) = >20; [b](1) well differentiated; (2) moderately differentiated; (3) poorly differentiated

Table 10. Histochemical characteristics of tumors in 14 cats

Tumor No	Case No	Diagnosis	AB-PAS	Trichrome*	PTAH
1.	4712	Fibrosarcoma	Positive	+	Negative
2.	1812	Fibrosarcoma	Positive	++	Negative
3.	8411	Fibrosarcoma	Positive	+	Negative
4.	2111	Fibrosarcoma	Positive	++	Negative
5.	3009	Fibrosarcoma	Positive	+++	Negative
6.	8215	Fibrosarcoma	Negative	+++	Negative
7.	892	Fibrosarcoma	Positive	+	Negative
8.	5207	Myxoid Liposarcoma	Positive	+++	Negative
9.	6375	Fibrosarcoma	Negative	-	Negative
10.	8231	Fibrosarcoma	Negative	-	Negative
11.	6217	Fibrosarcoma	Negative	+++	Negative
12.	3109	Fibrosarcoma	Positive	+	Negative
13.	8377	Fibrosarcoma	Negative	++	Negative
14.	8604	Fibrosarcoma	Negative	++	Negative

*Trichrome staining; + (Very low amount of collagenous stroma), ++ (Mild to moderate collagenous stroma), +++ (Fair amount of collagenous stroma).

Table 11. Histochemical characteristics of tumors in 11 dogs

Tumor No	Case No	Diagnosis	AB-PAS	Trichrome*	PTAH
1.	3615	Fibrosarcoma	Negative	-	Negative
2.	1915	Myxosarcoma	Positive	+	Negative
3.	114	Fibrosarcoma	Positive	+	Negative
4.	822	Fibrosarcoma	Negative	+	Negative
5.	6754	Fibrosarcoma	Negative	+	Negative
6.	9664	Fibrosarcoma	Positive	++	Negative
7.	2706	Fibrosarcoma	Negative	-	Negative
8.	8087	Fibrosarcoma	Negative	-	Negative
9.	8101	Fibrosarcoma	Positive	+++	Negative
10.	1120	Fibrosarcoma	Positive	++	Negative
11.	1239	Fibrosarcoma	Negative	+	Negative

*Trichrome staining; + (Very low amount of collagenous stroma), ++ (Mild to moderate collagenous stroma), +++ (Fair amount of collagenous stroma).

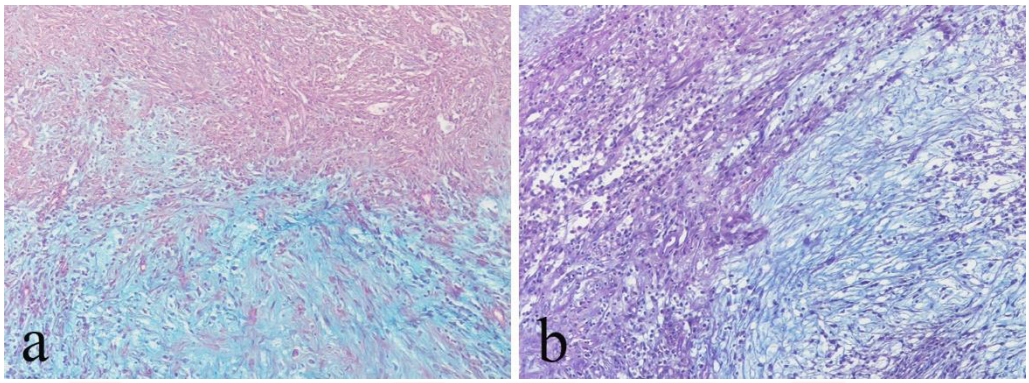

Figure 5. Photomicrograph of Alcian blue/Pas staining objective, 10x. a: mucinous area in an injection site fibrosarcoma of a cat, b: a mucinous area in an injection site fibrosarcoma of a dog

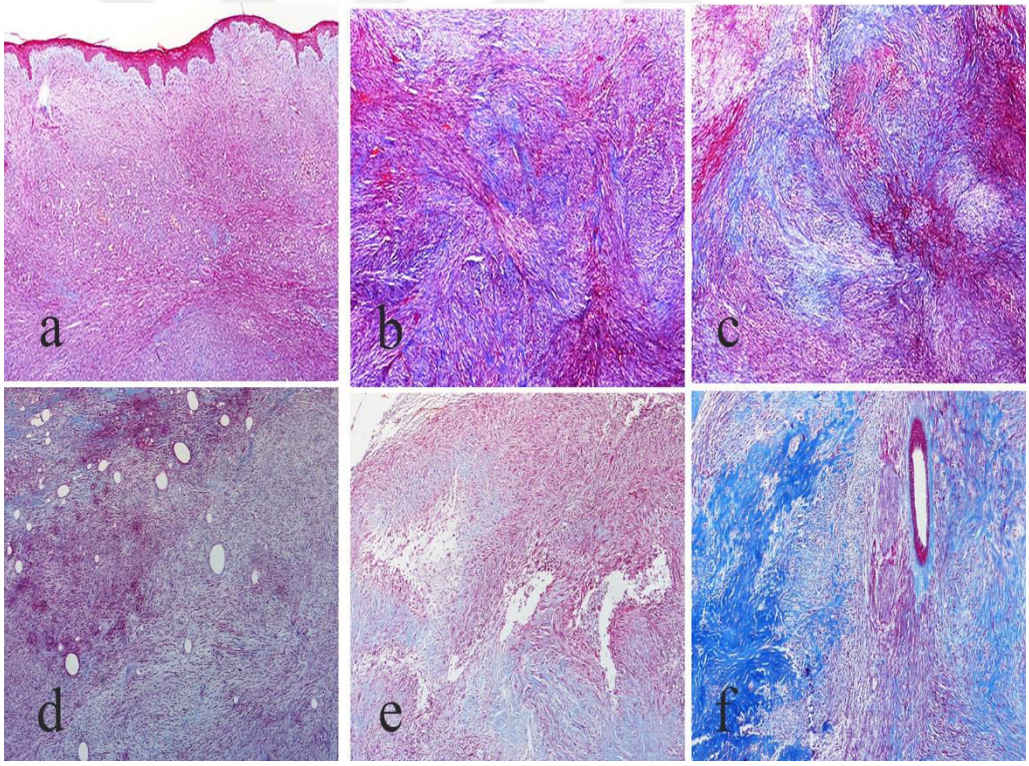

Figure 6. Photomicrograph of trichrome staining in dog and cat tumors objective 4x. a, b and c show low, moderate and fair amount of collagenous stroma in dog tumors, respectively while d, e and f show low, moderate and fair amount of collagenous stroma in cat tumors, respectively

4.5. Immunohistochemical Analysis of Tumors in Cats and Dogs

4.5.1. Immunohistochemical Staining Results for the Histogenic Characterization of Tumors

Vimentin reactivity was observed in all the tumors of the cats (Figure 7a) and dogs (Figure 7b). Four tumors of cats showed smooth muscle actin positivity (Figure 7c) and two for muscle actin. A focal area of mild myoglobin reactivity was noticed in one case of cat (Cat case no: 3109) which was categorized as injection site sarcoma. Tumor sample from two dogs diagnosed with injection site sarcoma showed reactivity to smooth muscle actin at one peripheral area of the tumor located beneath the inflammatory foci. A stromal septum extending from the periphery to inward of the tumor was also stained positive with smooth muscle actin (Dog case no: 1120 & 8101; Figure 7d). GFAP, S100, desmin, muscle actin, sarcomeric actin, myoglobin, myogenin and myoD1 were negative. The detail of the immunoreactivity is presented in the table no. 12 & 13

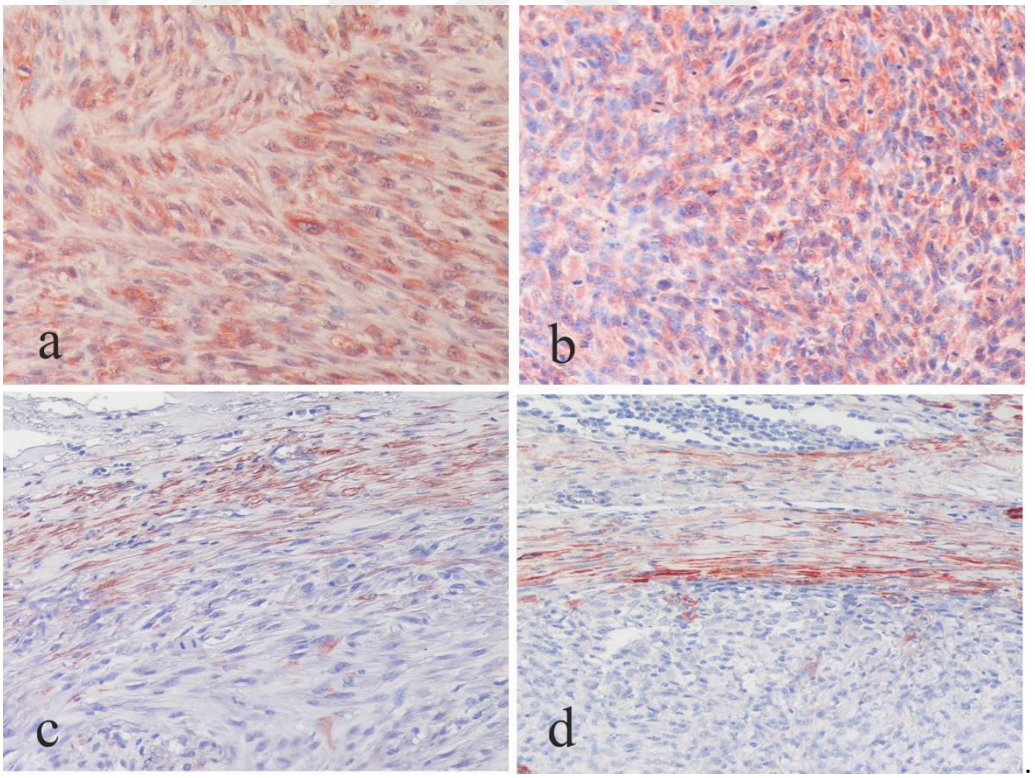

Figure 7. a & b: Vimentin immunoreactivity in tumors of cat and dog respectively. c & d: Alpha-smooth muscle staining at the tumor periphery in a cat and dog, respectively, objective 20x

Table 12. H-score of antibodies used for histogenic characterization of injection site fibrosarcomas and liposarcoma in cats

Tumor no.	Case no	Vimentin	src actinin	SMA	Myoglobin	Desmin	Pancytokeratin	S100	GFAP	Myogenin	MyoD1	Muscle Actin
1.	4712	90	0	1	0	0	0	0	0	0	0	2
2.	1812	90	0	0	0.05	0	0	0	0	0	0	0
3.	8411	180	0	0	0	0	0	0	0	0	0	0
4.	2111	170	0	5	0	0	0	0	0	0	0	0
5.	3009	110	0	5	0	0	0	0	0	0	0	40
6.	8215	70	0	0.5	0.5	0	0	0	0	0	0	0
7.	892	70	0	0	0	0	0	0	0	0	0	1
8.	5207*	35	0	0	0	0	0	0	0	0	0	0
9.	6375	90	0	0	0	0	0	0	0	0	0	0
10.	8231	20	0	2	0	0	0	0	0	0	0	0
11.	6217	97.5	0	0	0	0	0	0	0	0	0	0
12.	3109	210	0	25	30	0	0	0	0	0	0	0
13.	8377	15	0	0	0	0	0	0	0	0	0	0
14.	8604	90	0	0	0	0	0	0	0	0	0	0

*Myxoid liposarcoma, Src. Actinin: Sarcomeric actinin, SMA: Smooth muscle actin, GFAP: Glial fibrillary acidic protein, H-score ≤ 1 was considered negative

Table 13. H-score of antibodies used for histogenic characterization of canine fibrosarcomas and myxosarcoma

Tumor no.	Case no	vimentin	src actinin	SMA	myoglobin	Desmin	Pancytokeratin	S100	GFAP	Myogenin	MyoD1	Muscle Actin
1.	3615	90	0	0	0	0	0	0	0	0	0	0
2.	1915*	110	0	0	0	0	13	0	0	0	0	0
3.	114	90	0	0	0.2	0	0	0	0	0	0	0
4.	822	110	0	0	0	0	0	0	0	0	0	0
5.	6754	195	0	0	0	0	0	0	0	0	0	0
6.	9664	160	0	0	0	0	0	0	0	0	0	0
7.	2706	145	0	0	0	0	0	0	0	0	0	0
8.	8087	65	0	0	0	0	0	0	0	0	0	0
9.	8101**	140	0	2	0	0	0	0	0	0	0	0
10.	1120**	200	0	30	0	0	0	0	0	0	0	0
11.	1239	63	0	0	0	0	0	0	0	0	0	0

* Myxosarcoma, ** Injection site sarcomas, Src. Actinin: Sarcomeric actinin, SMA: Smooth muscle actin, GFAP: Glial fibrillary acidic protein

4.5.2. Expression Profiles of ECAM and Tyrosine Kinase and its Receptor in Tumors of Cats and Dogs

PDGFA and PDGFR-Alpha

PDGFA expression was noticed in all the tumors of cats (Figure 8a). Similarly, in dogs, all tumors revealed upregulation of PDGFA (Figure 8c). Muscle cells, blood vessel walls and epithelium of hair follicles showed positive reactivity for PDGFA. Histiocytic cells with cytoplasmic immunoreactivity were noticed in few cases. PDGFR-alpha expression was observed in 14 out of 14 (100%) cat tumors (Figure 8b) while10 out of 11 (90.9%) dog tumors showed immunoreactivity to PDGFR-alpha (Figure 8d). Tumor cells showed both nuclear and cytoplasmic staining for PDGFR-alpha. Variable staining reaction was observed in peripheral lymphocytes and macrophages for PDGFR-alpha in both cat and dog tumors. Other than tumor cells, tumorous and non-tumorous vessels, muscles were positive for PDGFR-alpha. The semiquantitative analysis of immunoreactivity score is given in table 14 & 15.

Double immunofluorescence staining in the selected tumor samples revealed co-expression of PDGFA and PDGFR-alpha in the neoplastic cells (Figure 9).

Integrin Alpha V

Membranous as well as cytoplasmic staining was observed in the feline (n=13/14) and canine tumors (n=11/11) as shown in figure 10 & 11. Multinucleated tumor giant cells and normal skeletal muscle cells also showed cytoplasmic reactivity for the integrin alpha v. However, infiltrating lymphocytic foci were negatively stained. The mean score of immunoreactivity of integrin alpha v is presented in table 14 & 15.

E-Selectin

Mild to moderate E-selectin expression was observed in vascular endothelium and cytoplasm of tumor cells in canine (n=9/11; Figure 12a, 12b) and feline (n=5/14) tumors (Figure 12c, 12d). But the percentage of immunoreactive blood vessels in tumors was variable. Though blood vessels with luminal inflammatory cells were often reactive, few of the vessels without inflammatory cells were also stained (Figure 12d). The detail of the E-selectin immunopositivity is given in table 14 & 15.

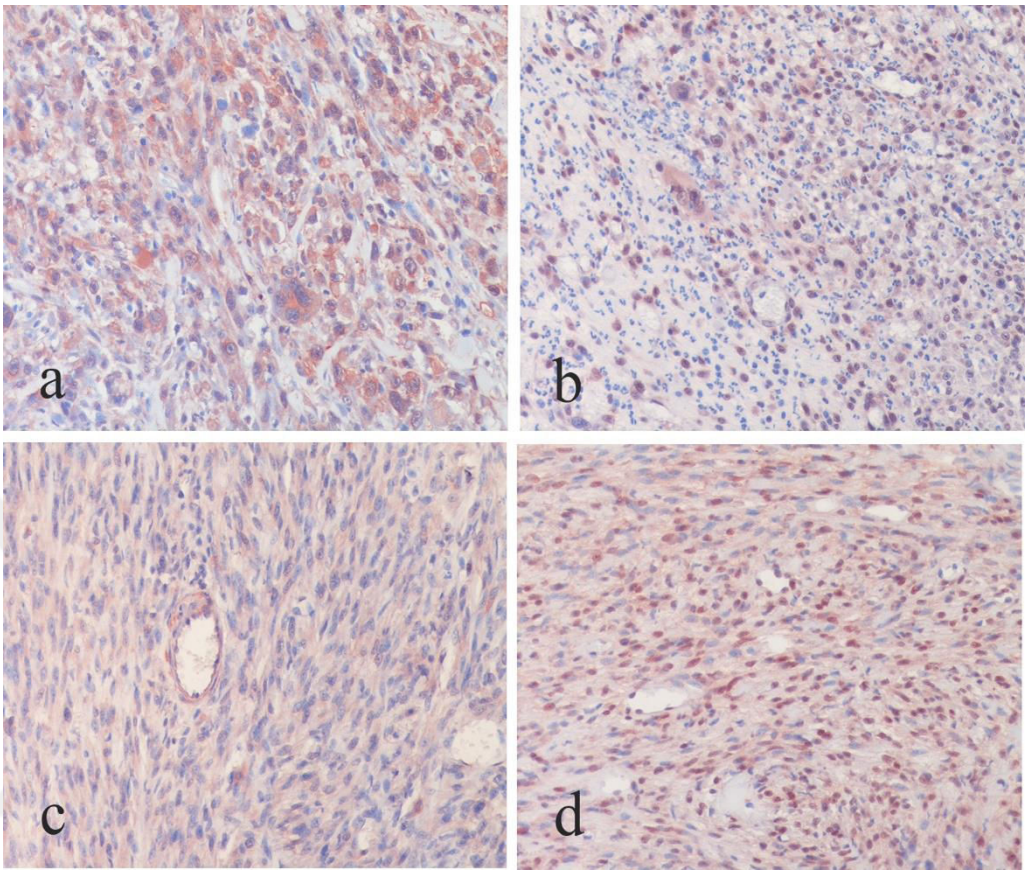

Figure 8. a: PDGFA immunoreactivity in an injection site fibrosarcoma of cat, b: Neoplastic cells from the same cat tumor showing nuclear and cytoplasmic staining for PDGFR-alpha, c: PDGFA staining in a canine fibrosarcoma, d: Nuclear and cytoplasmic reactivity of canine fibrosarcoma cells to PDGFR-alpha, objective 20x

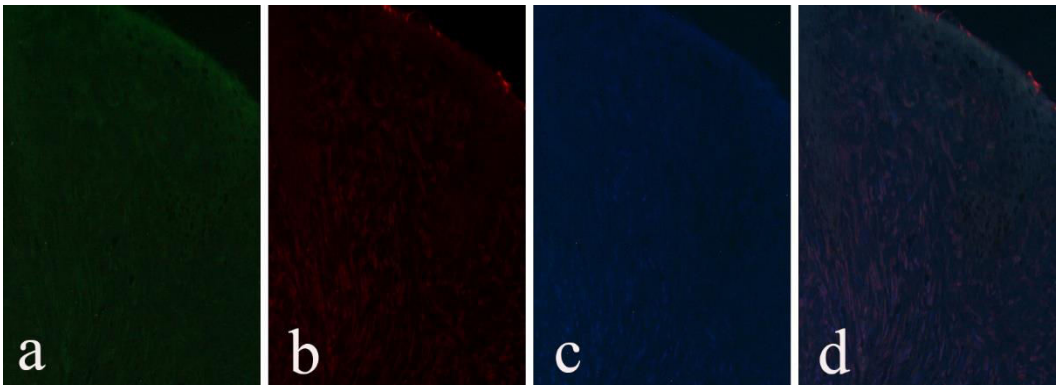

Figure 9. Double immunofluorescence staining with PDGFA and PDGFR-alpha in a dog, a: FITC, b: Rhodamine, c: DAPI and d: merged, objective 20x

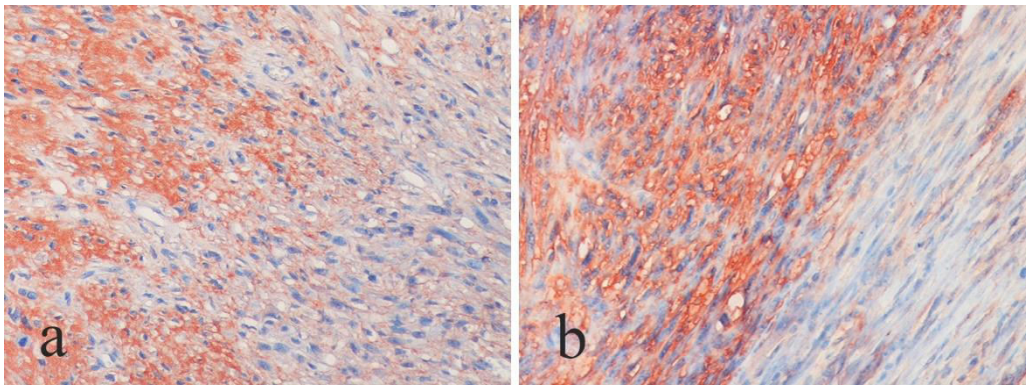

Figure 10. Integrin alpha v expression in a grade 2 (a) and grade 3 injection site fibrosarcoma (b) of a cat, objective 20x

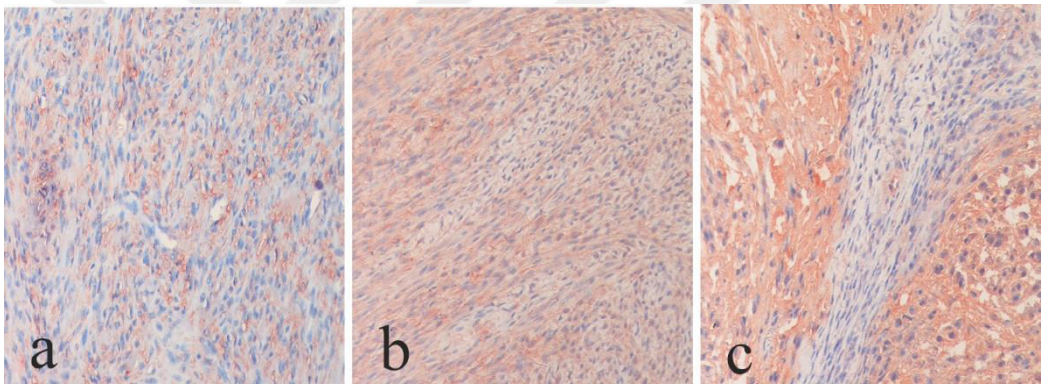

Figure 11. Integrin alpha v expression in a grade 1 (a), grade 2 (b) and grade 3(c) canine fibrosarcoma, objective 20x

Table 14. Comparison of H-score of ECAM and tyrosine kinase and its receptor in feline fibrosarcoma

Tumor Grade	Integrin alpha v	PDGFA	PDGFR-alpha	E-selectin
2	88.33±18.31	108.4±24.91	121.4±19.95	15.28±9.82
3	112.5±33.82	140.0±24.15	78.25±29.01	6.87±4.25

Data presented as Mean ± SEM, $p>0.05$, H-score did not show any significant difference between different grades of tumors in cats

Table 15. Comparison of H-score of ECAM and tyrosine kinase and its receptor in canine fibrosarcoma

Tumor Grade	Integrin alpha v	PDGFA	PDGFR-alpha	E-selectin
1	95±17.08	127.5±41.96	117.5±41.31	17.5±7.77
2	70±25.00	98.67±36.45	70.67±2.96	30.00±25.00
3	62.67±20.83	105.3±44.20	95±31.75	21.67±1.66

Data presented as Mean ± SEM, $p>0.05$, H-score did not show any significant difference between different grades of tumors in dogs

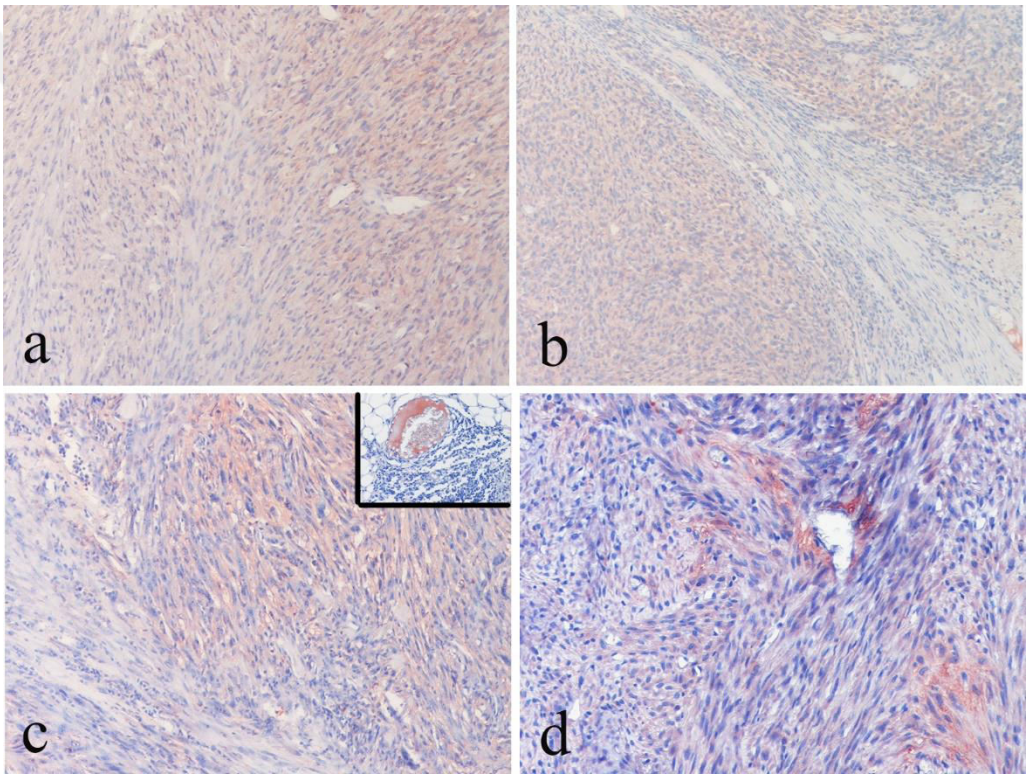

Figure 12. E-selectin expression in the vascular endothelium and tumor cells in canine spontaneous fibrosarcoma (a) objective 10x, an injection site canine fibrosarcoma (b), objective 10x; feline injection site fibrosarcomas (c & d) objective 10x (c) and (20x), while inset in c demonstrate endothelial cells immunoreactivity, objective 40x

4.5.3. Spearman's Correlation Statistical Analysis Results for the ECAM and Tyrosine Kinase and its Receptor in Tumors in Dogs and Cats

Spearman's Correlation in Cats

In cats, PDGFA and its receptor (r= -0.162, n= 13, p= 0.595), and PDGFA and integrin (r= -0.173, n= 13, p= 0.570), PDGFA and E-selectin (r= -0.059, n= 13, p= 0.846), integrin alpha and E-selectin (r= 0.255, n= 13, p= 0.399), did not show any significant correlation. Liposarcoma in a cat and myxosarcoma in a dog was not included in the computing of correlation coefficient due to the different lineage of these tumors. The computation is presented in table 16.

Spearman's Correlation in Dogs

No significant correlation was observed between PDGFA and PDGFR-alpha expression (r= - 0.103, n= 10, p= 0.785), and PDGFA and E-selectin expression (r = 0.030, n = 10. p= 0.946), integrin and E-selectin (r = 0.0061, n = 10. p= 1.00), integrin and PDGFR-alpha (r= -0.376, n= 10, p= 0.278) while PDGFA and integrin alpha v showed significant correlation (r = 0.729, n = 10. p = 0.020). Results are presented in the table 17.

Table 16. Correlation of PDGFA, PDGFR, Integrin alpha v and E-selectin in feline fibrosarcomas (n=13)

	PDGFA	**PDGFR-alpha**	**Integrin alpha v**	**E-selectin**
PDGFA		r=-0.162, p=0.595	r=-0.173, p=0.570	r=-0.059, p=0.846
PDGFR-alpha	r=-0.162, p=0.595		r=-0.184, p=0.546	r=0.264, p=0.382
Integrin alpha v	r=-0.173, p=0.570	r=-0.184, p=0.546		r=0.255, p=0.399
E-selectin	r=-0.059, p=0.846	r=0.264, p= 0.382	r=0.255, p= 0.399	

Myxoid liposarcoma was excluded from the correlation analysis

Table 17. Correlation of PDGFA, PDGFR, Integrin alpha v and E-selectin in canine fibrosarcomas (n=10)

	PDGFA	**PDGFR-alpha**	**Integrin alpha v**	**E-selectin**
PDGFA		-0.103, p=0.785	0.729. p=0.020*	0.030, p=0.946
PDGFR-alpha	-0.103, p=0.785		-0.376, p=0.278	0.208, p=0.560
Integrin alpha v	0.729. p=0.020*	-0.376, p=0.278		0.006, p=1.00
E-selectin	0.030, p=0.946	0.208, p=0.560	0.006, p=1.00	

Myxosarcoma was excluded from correlation analysis, *PDGFA and integrin alpha v showed significant correlation

4.6. 3-MCA Induced Sarcomas in Mice

There was no significant gross or microscopic lesion in the three tumor-free control groups (n=30), while out of 30 animals subjected to 3-MCA treatment for tumor induction, 28 animals developed neoplastic growth. Three animals in intraperitoneally treated tumor group (IP) died during the course of the experiment, and samples for histological analysis were not collected due to severe autolytic/postmortem changes in the tissues. One tumor in the intratumorally treated group (IT) showed complete necrosis of the tumor tissue so was excluded from subsequent immunohistochemical analysis**.** Because of excluding criteria depicted above only 24 mice were subjected to further histochemical and immunohistochemical analysis.

4.6.1. Weight of the Animals

No significant difference ($p>0.05$) was observed in the weight of the mice between all the groups studied (Figure 13).

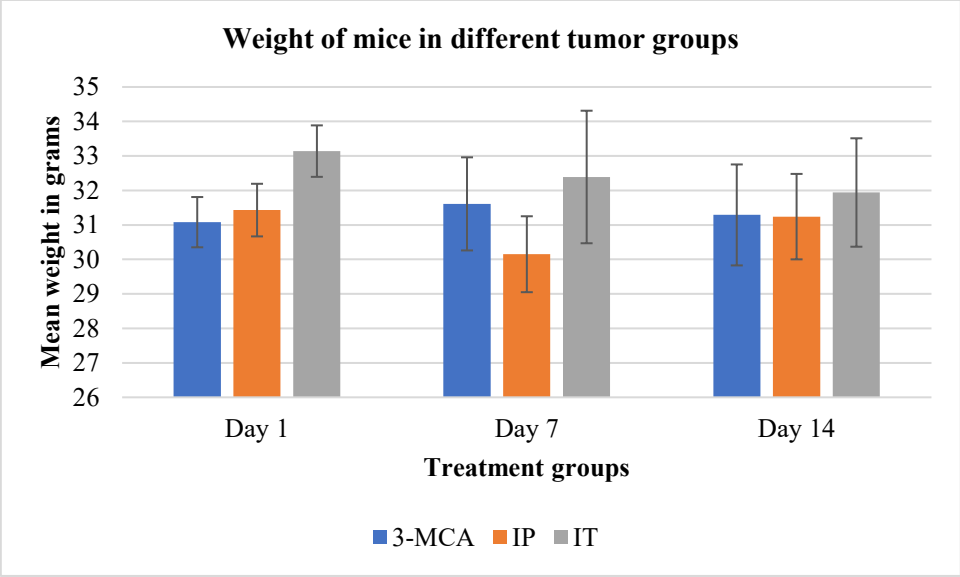

Figure 13. Comparison of the weight of mice in 3 tumor groups during the course of the experiment. No significant difference was observed among the treatment groups.

4.6.2. Volume of the Tumor in Different Groups

Tumor volume did not differ ($p>0.05$) between the 3-MCA induced control tumor group, tumor groups treated intraperitoneally and intratumorally with tadalafil when measured weekly over the course of the experiment after the start of treatment with tadalafil (Table 18).

Table 18. The median volume of the tumors of mice in the three tumor groups with & without tadalafil treatment at week 1, 2 and 3

Groups	Volume day 1 (mm^3)	volume day 7 (mm^3)	Volume day14 (mm^3)
3-MCA	1021(26.14-5679)	5006(36.94-12614)	4945(55.23-16417)
IP	1392(39.75-7818)	2681(122.2-17096)	4783(120.6-27219)
IT	1807(222.2-4811)	3894(358.2-10005)	6194(224.3-10436)

Values in parenthesis indicate range, $p>0.05$, No significant difference was noticed between groups

4.6.3. Gross Examination

Grossly the tumors were variable in size and the superficial surface of some tumors contained crusts. Tumors in general were solid in consistency with variable areas of necrosis. Cut surface was white in the majority of the tumors and in one animal was reddish black (due to severe necrosis and hemorrhage) in the intratumorally treated group.

4.6.4. Histopathology

The two animals in 3-MCA group which did not develop tumor, showed cystic dilatation at the injection site in the subcutaneous tissue. Histopathological examination of the neoplastic growths revealed highly cellular and anaplastic tumor cells. A heterogenous population of neoplastic cells was evident with small round to pleomorphic cells with little cytoplasm to large cells with abundant eosinophilic cytoplasm along with spindle-shaped cells having plump nuclei. Elongated multinucleated cells (strap-like cells) with abundant cytoplasm were also present in most of the tumors (Figure 14d & 14f). Cells were arranged in the form of sheaths. Mitotic figures were frequent. The nucleus contained one to two nucleoli. Necrosis with the influx of neutrophils and few lymphocytes were noticed predominantly in the group treated with intratumoral tadalafil injection (Figure 15a). Many multinucleated and mononucleated giant cells were scattered throughout the tumors (Figure

15b & 15c). The only difference in the histological appearance between groups was the presence of more rhabdomyoblasts like cells in the group treated with intraperitoneal injection of tadalafil (Figure 14). No statistically significant difference was present in terms of mitosis, necrosis, and differentiation between these tumor groups. One tumor from 3-MCA group and 2 from IT group demonstrated areas of squamous cell proliferation along with rhabdomyosarcomaotus differentiation. As the tumor-free control groups did not reveal any histopathological change they are not presented in the table. An overview of the microscopic changes in tumor groups are presented in table 19.

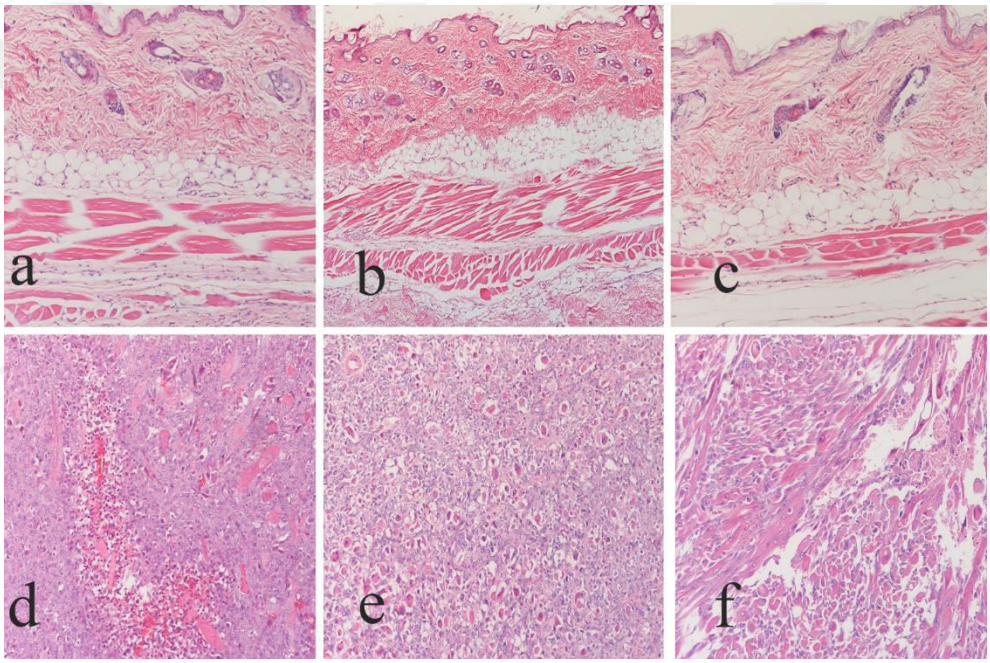

Figure 14. a, b, and c are the photomicrographs from 3 non-tumorous groups i.e. normal skin group, tadalafil subcutaneous group and tadalafil intraperitoneal group, respectively, objective 10x**.** d: 3-MCA group, an area of necrosis and pleomorphic cells are present, objective 10x, e: IT group, many multinucleated and poorly differentiated tumor cells are observable, objective 10x, f: IP group, relatively more differentiated strap-like and round rhabdomyoblastic tumor cells are present, objective 10x

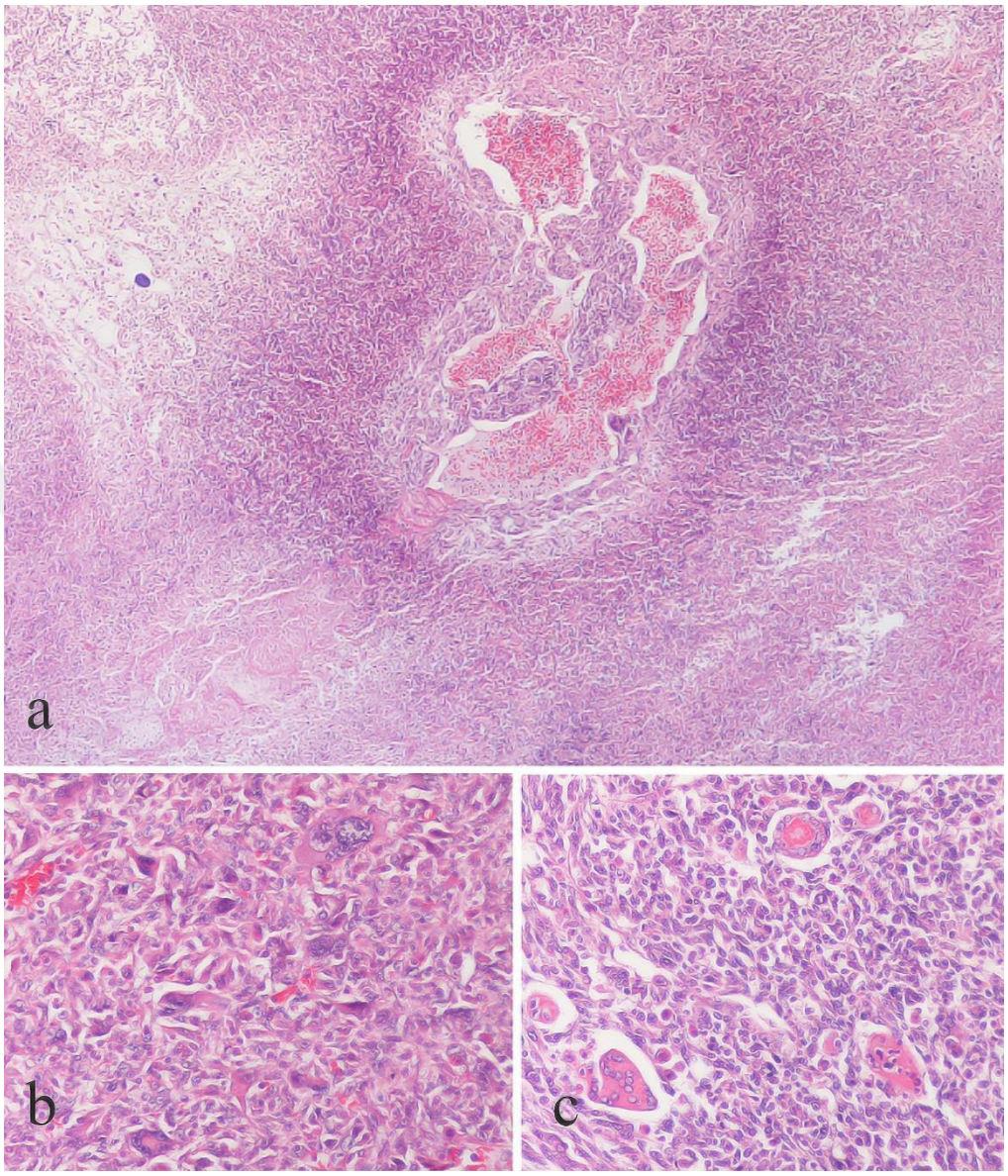

Figure 15. a: Massive area of tumor necrosis in IT group, objective 2x, b &c: Multinucleated tumor giant cells in IT group and 3-MCA group, respectively, objective 20x.

Table 19. An overview of the microscopic changes and diagnosis of 3-MCA-induced tumors in mice

Groups	Mitosis	Necrosis	Differentiation	Giant cells	Inflammation	Diagnosis
3-MCA	0	0	0	0	0	Cystic dilatation
3-MCA	0	0	0	0	0	Cystic dilatation
3-MCA	3	2	2	yes	yes	RMS
3-MCA	3	3	2	yes	yes	RMS
3-MCA	3	2	2	yes	yes	RMS
3-MCA	3	2	3	yes	yes	RMS+SCC
3-MCA	3	2	3	yes	yes	RMS
3-MCA	3	2	3	yes	yes	RMS
3-MCA	3	2	2	yes	yes	RMS
IP	3	2	2	yes	yes	RMS
IP	3	2	3	yes	yes	RMS
IP	2	2	2	yes	yes	RMS
IP	3	2	2	yes	yes	RMS
IP	2	2	2	yes	yes	RMS
IP	3	2	2	yes	yes	RMS
IP	2	1	2	yes	No	RMS
IT	3	3	3	yes	yes	RMS
IT	3	3	3	few	yes	RMS
IT	3	3	3	yes	yes	RMS
IT	2	2	3	yes	yes	RMS
IT	3	3	2	yes	yes	RMS
IT	1	3	2	yes	yes	RMS
IT	2	2	2	yes	yes	RMS
IT	2	2	2	yes	yes	RMS
IT	3	1	2	No	yes	RMS+SCC
IT	2	1	2	Yes	yes	RMS+SCC

3-MCA: Methylcholanthrene induced tumor group, IP: Intraperitoneal injection in mice with tumors, IT: Intratumoral injection, NA: Not applicable, RMS: Rhabdomyosarcoma, SCC: Squamous cell carcinoma

4.7. Histochemical Analysis of 3-MCA Induced Sarcomas in Mice

Two animals in the 3-MCA group and 3 in the IP group showed cross striations with PTAH staining (Figure 16).

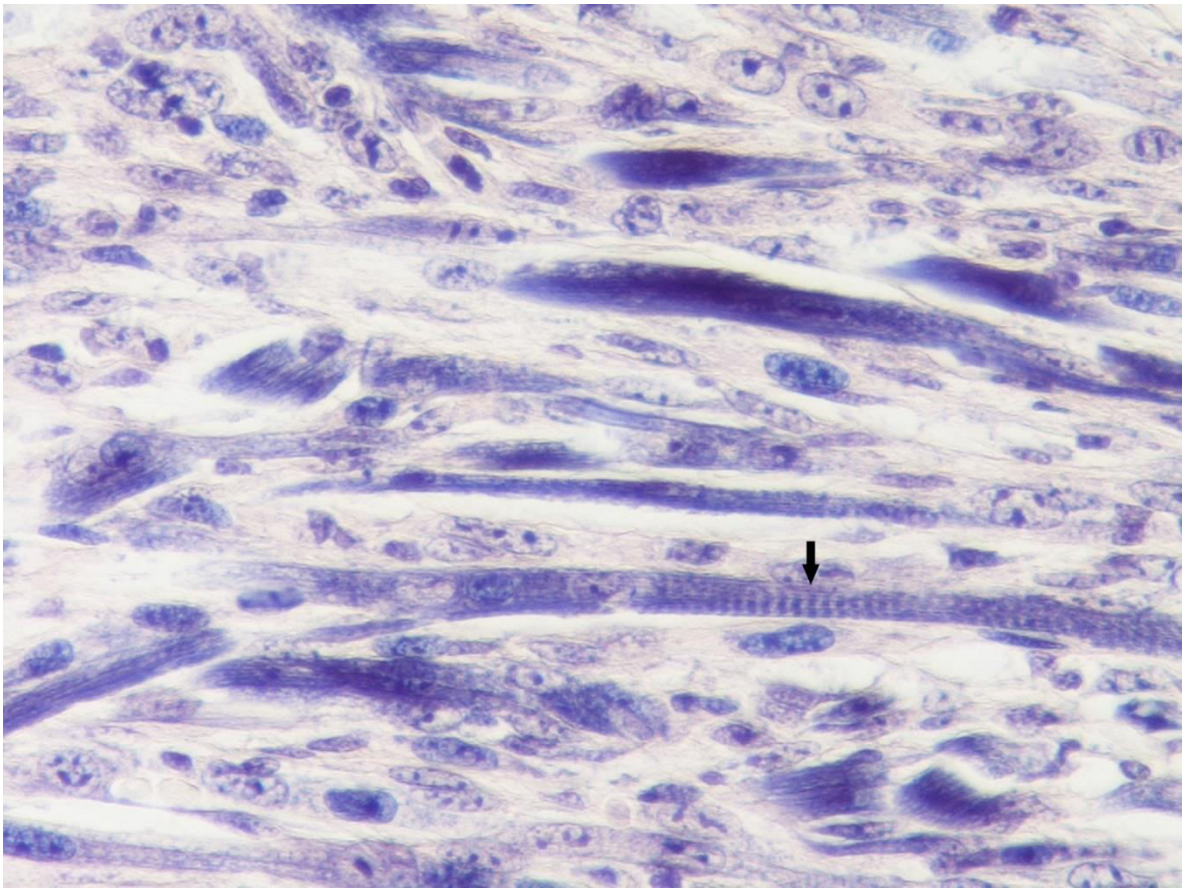

Figure 16. Cross striations (arrow) are visible in a PTAH stained tumor section from IP group of mice, objective 40x

4.8. Immunohistochemical Staining Results for the Histogenic Characterization of 3-MCA Induced Tumors in Mice

H-score ≤1 was considered as negative.

4.8.1. Vimentin

Although 87 % (21/24) of the animals showed cytoplasmic staining with an anti-vimentin antibody, the overall immunoreactivity was low in all the tumor groups. The pattern of reactivity was variable in different animals ranging from small foci to large areas. Most of the multinucleated giant cells and strap-like cells were negative for vimentin, while stromal elements, pleomorphic to round cells and spindloid cells were positively stained (Figure 17). The H-score in IT group ranged from 0-45 (Median=12), in IP group from 0-18.50 (Median=5) and in 3-MCA group from 2-68 (Median=25). Microscopic examination revealed relatively less number of immunoreactivity cells in IP group as compared to other two tumor groups but statistically, there was no difference in vimentin expression between different groups ($p>0.05$).

4.8.2. Desmin

The desmin showed positive staining in 71 % (17/24) of the animals with tumorous growth. Though no consistence staining pattern was noticed but strap cells, multinucleated giant cells and large round cells with ample cytoplasm were predominant desmin positive cells (Figure 18). The H-score of the desmin immunostaining varied between 0-90 (Median=7.50) in IT group, 0-39 (Median=13.50) in 3-MCA group, and 0-110 (Median=32.50) in IP group. Comparison of median between different groups yielded statistically no significant difference.

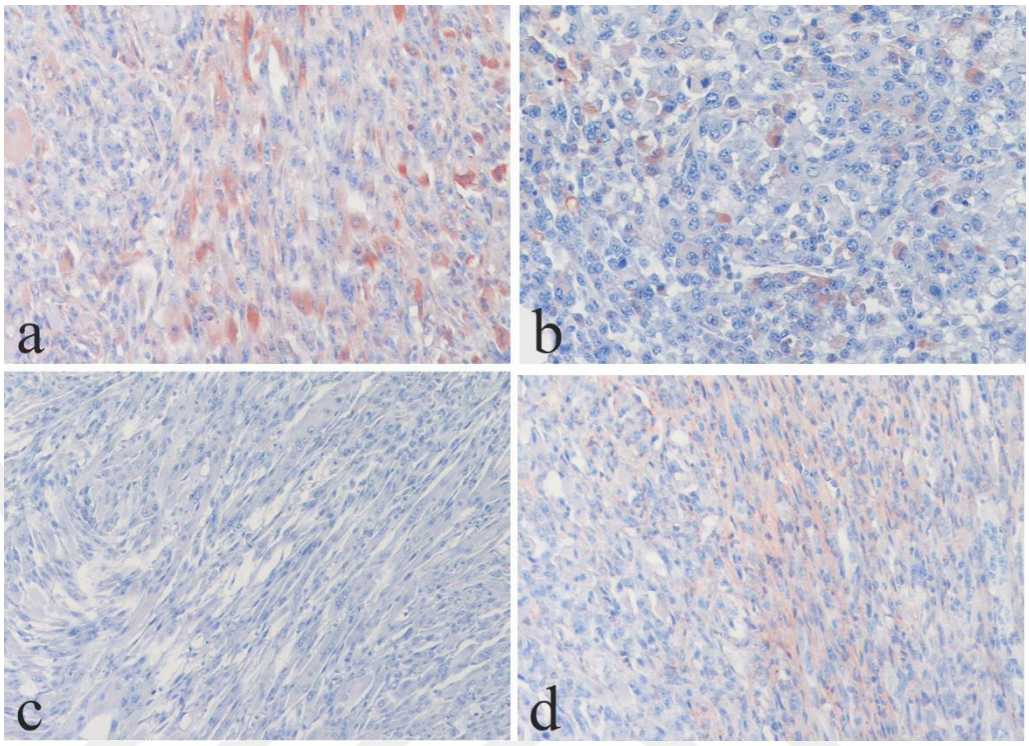

Figure 17. a: Photomicrograph from 3-MCA group showing vimentin immunoreactivity in different cells, objective 20x, b: Tissue section from IP group showing round or pleomorphic cells positive for vimentin, objective 20x, c: More differentiated strap like cells are negative for vimentin (IP group), objective 20x, d: spindle-shaped cells immunoreactive to vimentin (IP group) objective 20x.

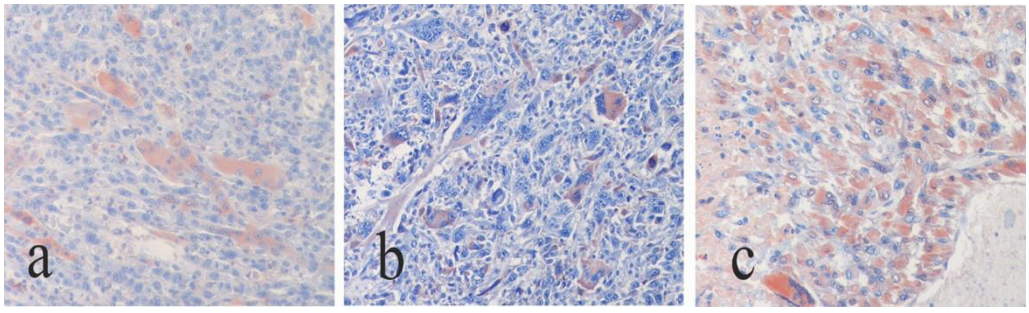

Figure 18. a: Photomicrograph from 3-MCA group, pleomorphic or strap-like cells are positive for desmin, objective 20x, b: Multinucleated cells in a tissue section from IT group showing desmin positivity, objective 20x, c: Tissue section from IP group, round to pleomorphic cells relatively showing more intense reaction to desmin than other groups, objective 20x

4.8.3. Sarcomeric Actinin

All the tumors stained positively with sarcomeric actinin (24/24) (Figure 19). The cellular staining pattern was not much different from the desmin but overall more intense staining reaction was observed in all the groups and relatively IP group showed staining in a greater number of cells but statistically, this difference was non-significant ($p>0.05$). The H-score for immunoreactivity was between 20-200 (Median=100) in IT group, 42.5-150 (Median=105) in 3-MCA group and 85-300 (Median=160) in IP group.

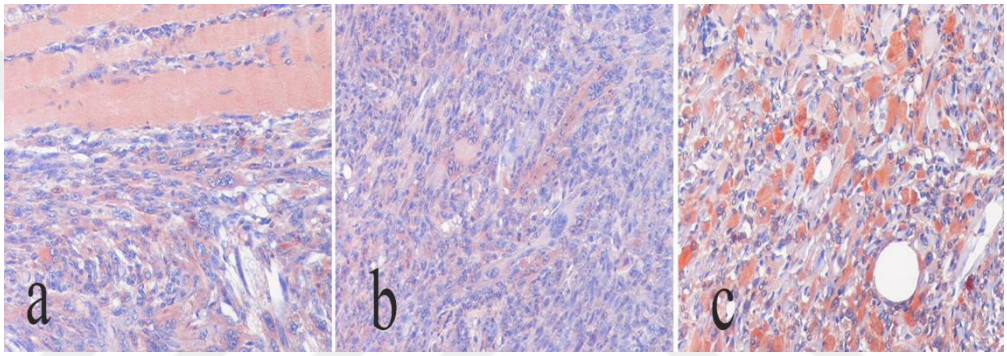

Figure 19. a: Photomicrograph from a tumor in 3-MCA group, normal skeletal muscle cells and tumor cells are positive for sarcomeric actinin, objective 20x, b: Tissue section from IT group, strap-like cells and multinucleated tumor cells are immunopositive, objective 20x, c: Tumor cells from an animal in IP group are showing relatively stronger immunoreactivity for sarcomeric actin, objective 20x

4.8.4. Myoglobin

Myoglobin expression was noticed in 92% (22/24) of the tumors. The inconsistent staining pattern was observed for myoglobin where mostly large strap-like cells with abundant cytoplasm along with other mononucleated and multinucleated cells showed the positive reaction of variable intensity (Figure 20). However, some multinucleated cells were negative too. The myoglobin expression measured in terms of H-score varied between IT (0-135, Median=57.50), 3-MCA (5-110, Median=32), and IP groups (1-70, Median=20). No significant difference was noticed among these treatment groups ($p>0.05$).

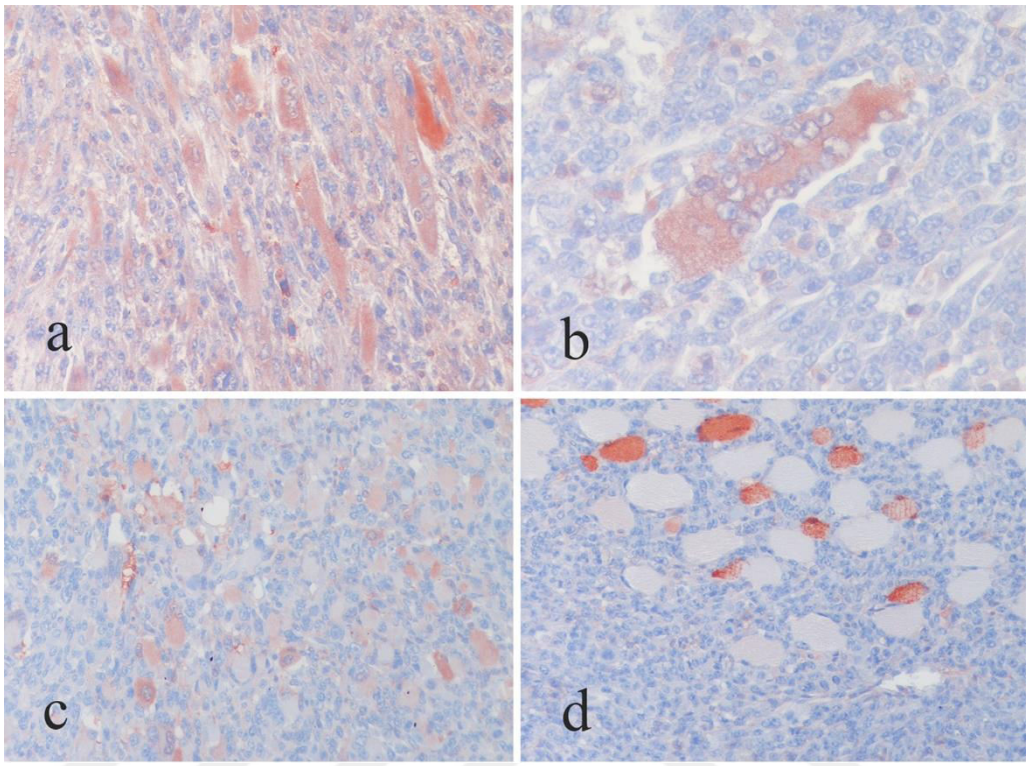

Figure 20. a: tissue section from 3-MCA group, strap-like cells showing positive reaction to anti-myoglobin antibody objective 20x, b: Multinucleated tumor cell from a tumor in IP group is positive for myoglobin, objective 20x, c: Another section from 3-MCA group with round to pleomorphic cells immunoreactivity to myoglobin, objective 20x, d: Tissue section from IT group, relatively less differentiated tumor cells are negative for myoglobin while normal skeletal muscle cells are showing positive cytoplasmic staining, objective 20x

4.8.5. Smooth Muscle Actin

SMA positive cells were seen in 88% (21/24) tumors. Mostly small to large round or pleomorphic cells with variable cytoplasm and spindle-shaped cells revealed immunoreactivity with SMA. Few straps like cells, mono, and multinucleated giant cells also stained positive (Figure 21). However, staining was more intense and common in round cells as compared to large strap or giant cells. SMA H-score in IT group ranged between 0-115 (Median=13.50), in MCA group between 20-65 (Median=50), and in IP group 8-90 (Median=45). No significant difference between these groups ($p>0.05$).

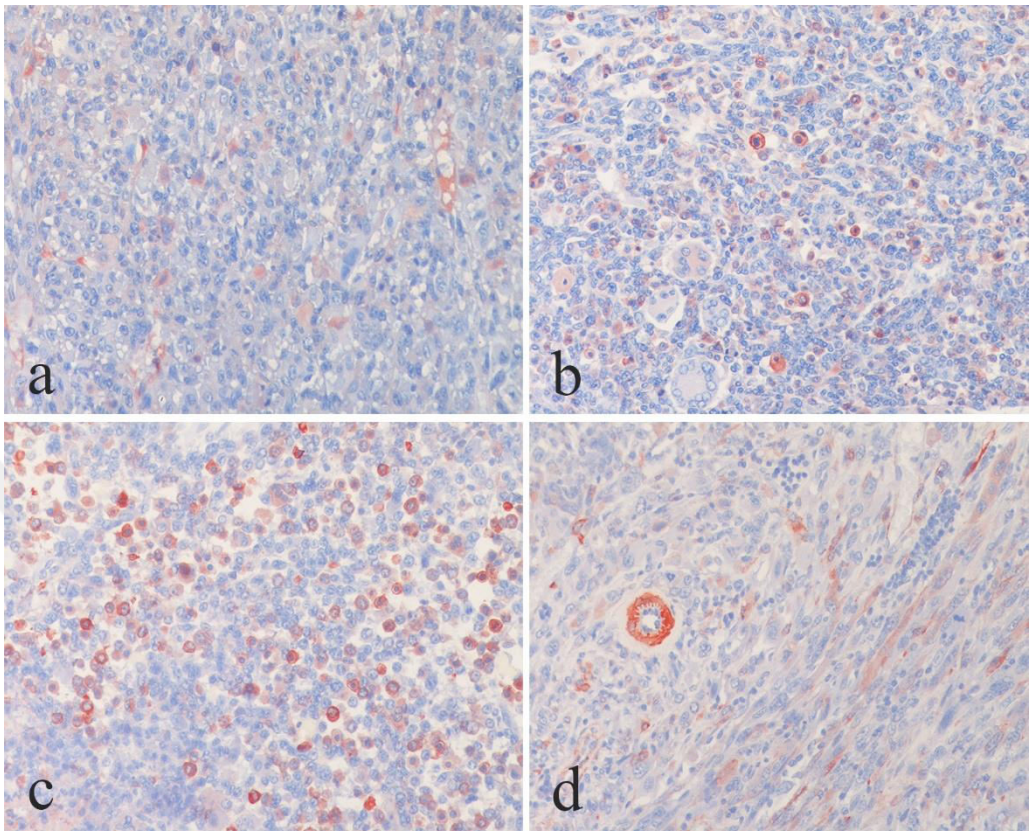

Figure 21. a: Tissue section from IT group, round to oval cells are showing cytoplasmic immunoreactivity to alpha SMA, objective 20x, b: 3-MCA group, round to pleomorphic cells are immunopositive for alpha SMA while multinucleated giant cell is negative, objective, 20x, c: IP group, mostly round cells to pleomorphic cells are positive for alpha SMA, objective 20x, d: IP group, spindle-shaped cells and a strap like cells are positive for alpha SMA staining, objective 20x

4.8.6. Myogenin

Nuclear reactivity for myogenin was observed in 83% of the tumors (20/24). Most of the multinucleated cells and strap-like cells were negative while round cells were predominant positive cells (Figure 22). The H-score for immunoreactivity in IT group varied between 0-75 (Median=8), MCA 0-90 (Median=55), IP 1-90 (Median=27). However, a non-significant difference in the immunoreactivity was observed between the groups ($p>0.05$).

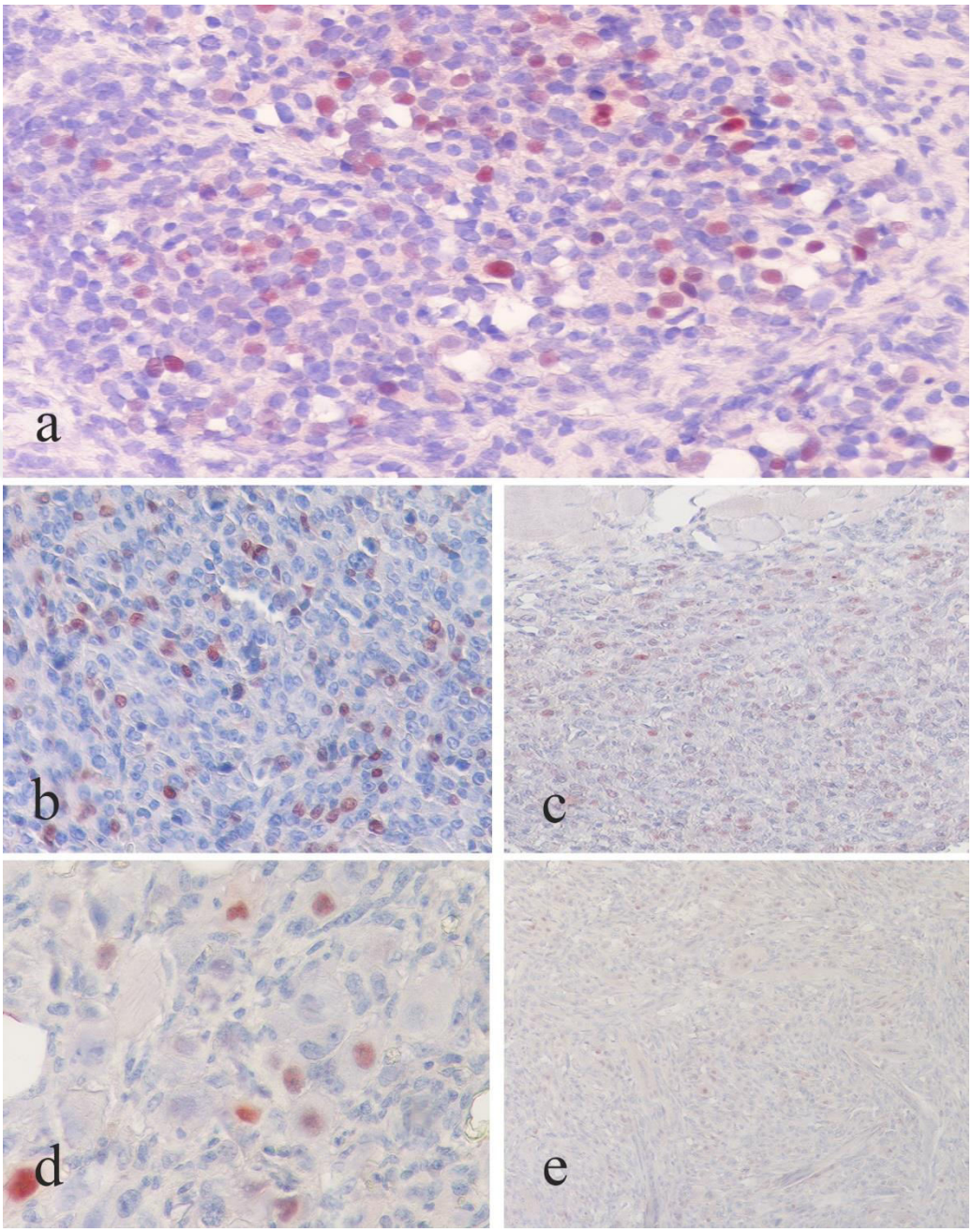

Figure 22. a: A tissue used as a positive control from a human ovarian cancer with myogenic differentiation, objective 20x, b: IT group, Tumor cells are showing nuclear reactivity to myogenin, objective, 40x, c: 3-MCA group, Normal skeletal muscle are negative while neoplastic cells are showing positive nuclear reaction, objective 20x, d: IP group, few large rhabdomyoblastic cells are stained positive, objective 40x, e: IP group, strap-like cells are negative for myogenin, objective 10x

4.8.7. Cytokeratin

Pancytokeratin expression was noticed in 8 tumors, out of which in five cases only a few cells were positive while in 3 cases an area of squamous cell carcinoma was present (Figure 23). The H-score for these three cases were 30, 60 and 120.

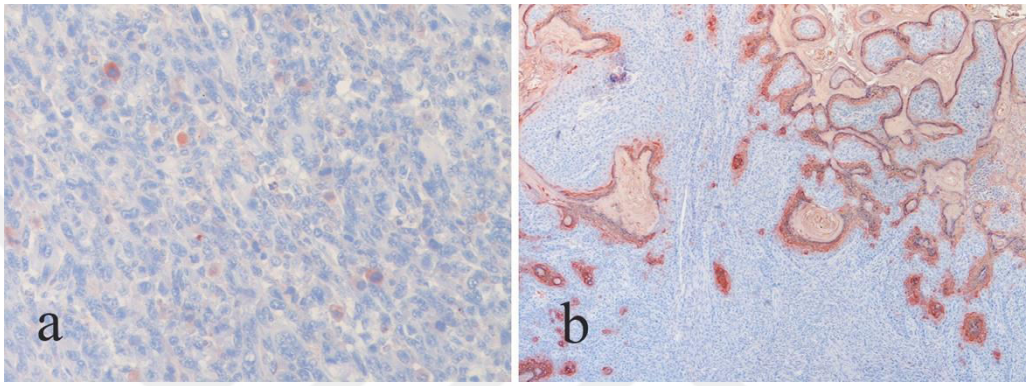

Figure 23. a: A tissue section from a tumor in IP group showing cytokeratin immunoreactivity in only a few cells, objective20x, b: A tumor with mixed rhabdomyoblastic and squamous cell proliferation which are positive for cytokeratin in IT group, objective 4x

4.8.8. Muscle Actin

Tumors from 23 out of 24 animals stained positively with muscle actin (96%) though variation in immunoreactivity was present among different animals. One animal in IP group did not show staining. Strap cells, multinucleated cells, and round cells were often stained positive (Figure 24). Cross striation in some tumors became visible upon staining with muscle actin (Figure 24d). H-score in IT group varied between 3-225 (Median=90), in 3-MCA group between 55-200 (Median=120), and in IP group between 0-270 (Median=165). Comparison of median between different groups did not reveal any significant difference among three tumor treatment groups ($p>0.05$).

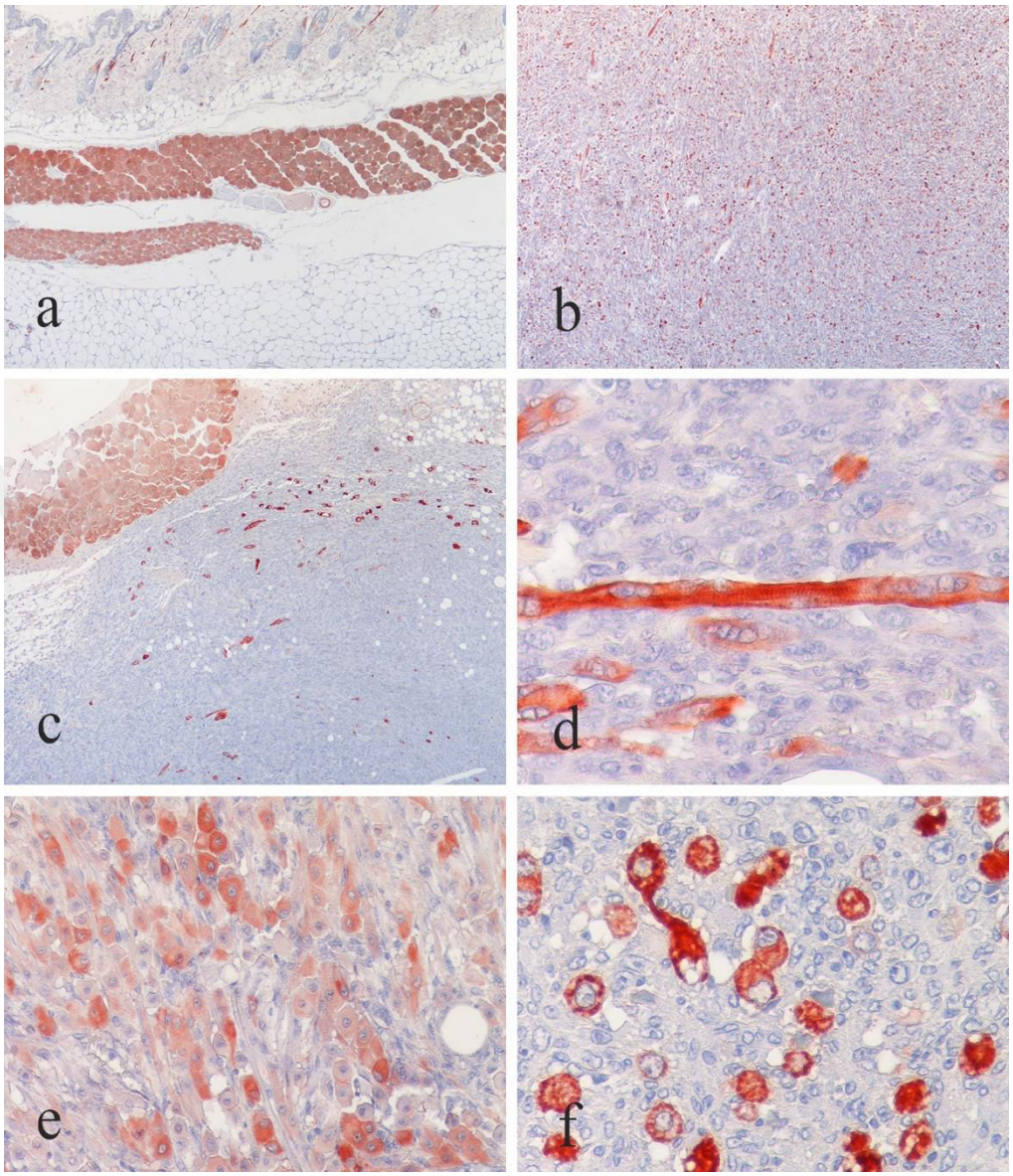

Figure 24. a: Tissue section from NS group, skeletal muscles and smooth muscles of blood vessels are positively stained for muscle specific actin, objective 4x, b:A low magnification view of a tumor from 3-MCA group showing a large number of immunopositive cells, objective 4x, c: IT group, a tumor with relatively low immunoreactivity to muscle specific actin, objective 4x, d: 3-MCA group, cross striations are visible in a strap like cell, objective 40x, e: IP group, large rhabdomyoblastic cells are positively stained for muscle specific actin, objective 20x, f: IT group, round to pleomorphic cells are intensely stained, objective 40x

Table 20. H-scores of Immunohistochemical staining results for the histogenic characterization of 3-MCA-induced tumors

Sr. No.	Groups	Vimentin	Desmin	Src Actinin	Myoglobin	SMA	Myogenin	Actin	cytokeratin
1.	3-MCA	68	39	42.5	32	65	20	135	7.5
2.	3-MCA	63	30	105	90	59	0	200	2.5
3.	3-MCA	13	0.5	110	110	20	70	90	0
4.	3-MCA	60	13.5	95	10	50	55	130	30
5.	3-MCA	5	10	59.5	5	35	24	55	5
6.	3-MCA	25	0	150	55	50	60	120	0
7.	3-MCA	2	16	125	30	65	90	75	0
8.	IT	5	0	20	5	115	75	105	0
9.	IT	20	50	125	5	0	0	8	2
10.	IT	10	0	50	90	14	15	125	0
11.	IT	12	5	110	30	75	55	115	0
12.	IT	7,5	90	30	90	35	0	5	0
13.	IT	0	0	60	35	1	0	75	0
14.	IT	45	10	100	80	13	13	225	0
15.	IT	40	60	130	120	6	2	3	0
16.	IT	20	22.5	100		0	3	9	120
17.	IT	2	2	200	135	60	20	180	60
18.	IP	0	34.5	160	70	8	27	270	0
19.	IP	5	110	91	60	60	1	0	12.5
20.	IP	5	0	300	3	90	25	165	0
21.	IP	4	40	146	15	55	90	155	10
22.	IP	12	32.5	164	1	29	33	230	0
23.	IP	18.5	0	177.5	47.5	45	45	165	0
24.	IP	0	6	85	20	15	15	36	0

Src Actinin: Sarcomeric actinin, SMA: Smoooth muscle actin

4.9. Expression Profiles of ECAM and Tyrosine Kinase and its Receptor in Tumors and PCNA in 3-MCA Induced Sarcomas

4.9.1. PDGFA

The upregulation of PDGFA was observed in 88% (21/24) of the tumor cases. Multinucleated cells, strap-like cells, spindle-shaped cell and round cells all showed variable immunoreactivity in different animals (Figure 25a, 25b, 25c). The H-score for IT group was between 0-220 (Median=48), for 3-MCA group between 0-145 (Median=75), and for IP

group between 0-200 (Median=12.50). Comparison of groups did not show any significant difference between groups for PDGFA expression (p>0.05).

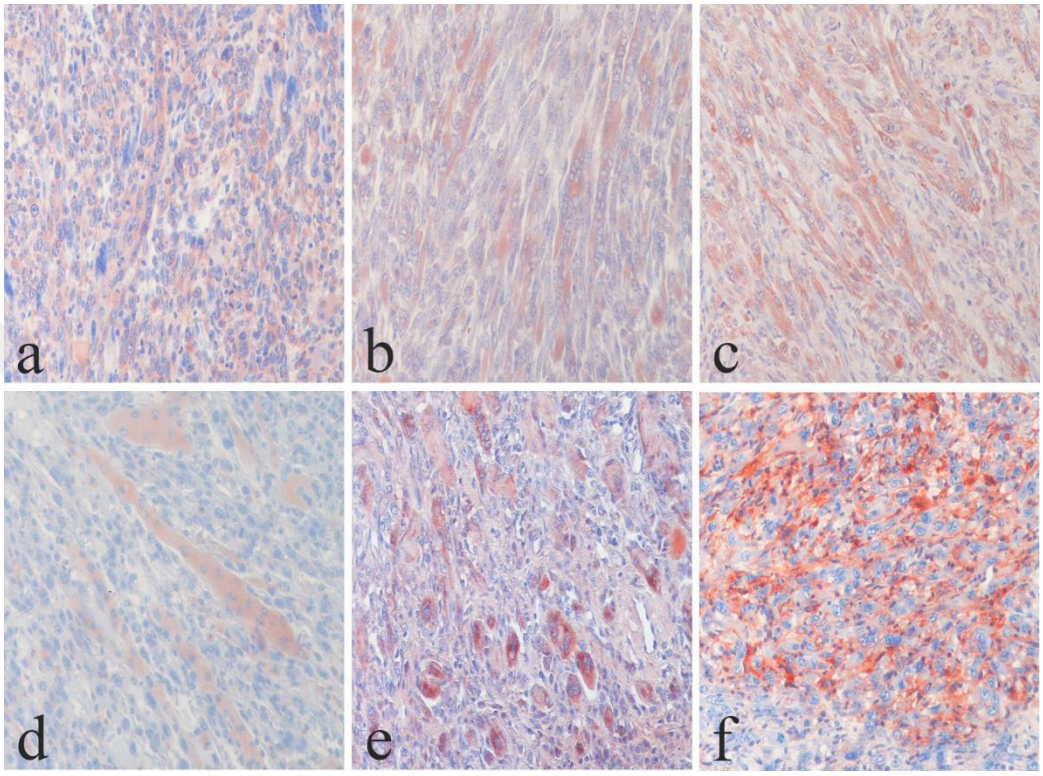

Figure 25. a, b, c: Tissue sections from 3-MCA, IP, and IT groups, respectively, stained with PDGFA, cytoplasmic immunoreactivity is present in the neoplastic cells, objective, 20x; d e, f: Micrographs from 3-MCA, IT and IP group, respectively stained with PDGFR-alpha, objective 20x

4.9.2. PDGFR-Alpha

PDGFR-alpha immunostaining was recorded in 58% (14/24) of the tumors. Tumor cells including strap like multinucleated cells (Figure 25d, 25e, 25f) and blood vessels endothelium showed positive reactivity. The pattern of reactivity varied from scattered individual staining cells to focal and irregular areas of positivity. Values of H-score for IT group was 0-90 (Median=6.25), 3-MCA 1-65 (Median=5), IP 0-76 (Median=1). There was no difference between groups in terms of immunoreactivity (p>0.05).

4.9.3. E-Selectin

E-selectin expression was observed in vascular endothelium and tumor cells in 46% (11/24) of the mice tumors (Figure 26). Generally, the H-score was low and varied between 0-30 (Median=10.28) for IT group, 0-12 (Median=0.5) in 3-MCA group and 0-10.25 (Median=0) in IP group. Statistical analysis did not reveal any difference in the medians of the treatment groups ($p>0.05$).

4.9.4. Integrin Alpha V

Integrin alpha v was expressed in all tumors except one animal in IP group which showed very weak reactivity. Membranous as well as cytoplasmic and extracellular matrix immunoreactivity was noticed (Figure 27). Most of the multinucleated and strap-like cells were negative while spindle cells and round cells showed positivity. The H-score values for IT group were between 14-180 (Median=47.75). 3-MCA 6-166 (Median=16), IP 1-170 (Median=20). Statistically, no significant difference was observed between different groups ($p>0.05$).

4.9.5. PCNA

Nuclear PCNA expression in tumor cells was observed in all groups (Figure 28). A significant difference was observed in the nuclear expression of PCNA between IP group and other two groups where IP group showed low expression ($p=0.0015$) as shown in figure 29.

Table 21. Median H-scores of ECAM and tyrosine kinase and its receptor in 3-MCA induced sarcomas

Treatment Groups	PDGFA	PDGFR-alpha	Integrin alpha v	E-selectin
3-MCA	75.00 (0-145)	5.00 (1-65)	16.00 (6.25-166)	0.50 (0-12)
IP	12.50 (0-200)	1.00 (0-76)	20.00 (1-170)	0.00 (0-10.25)
IT	48.00 (0-220)	6.25 (0-90)	47.75 (14-180)	10.28 (0-30)

Values in parenthesis indicate range, $p>0.05$

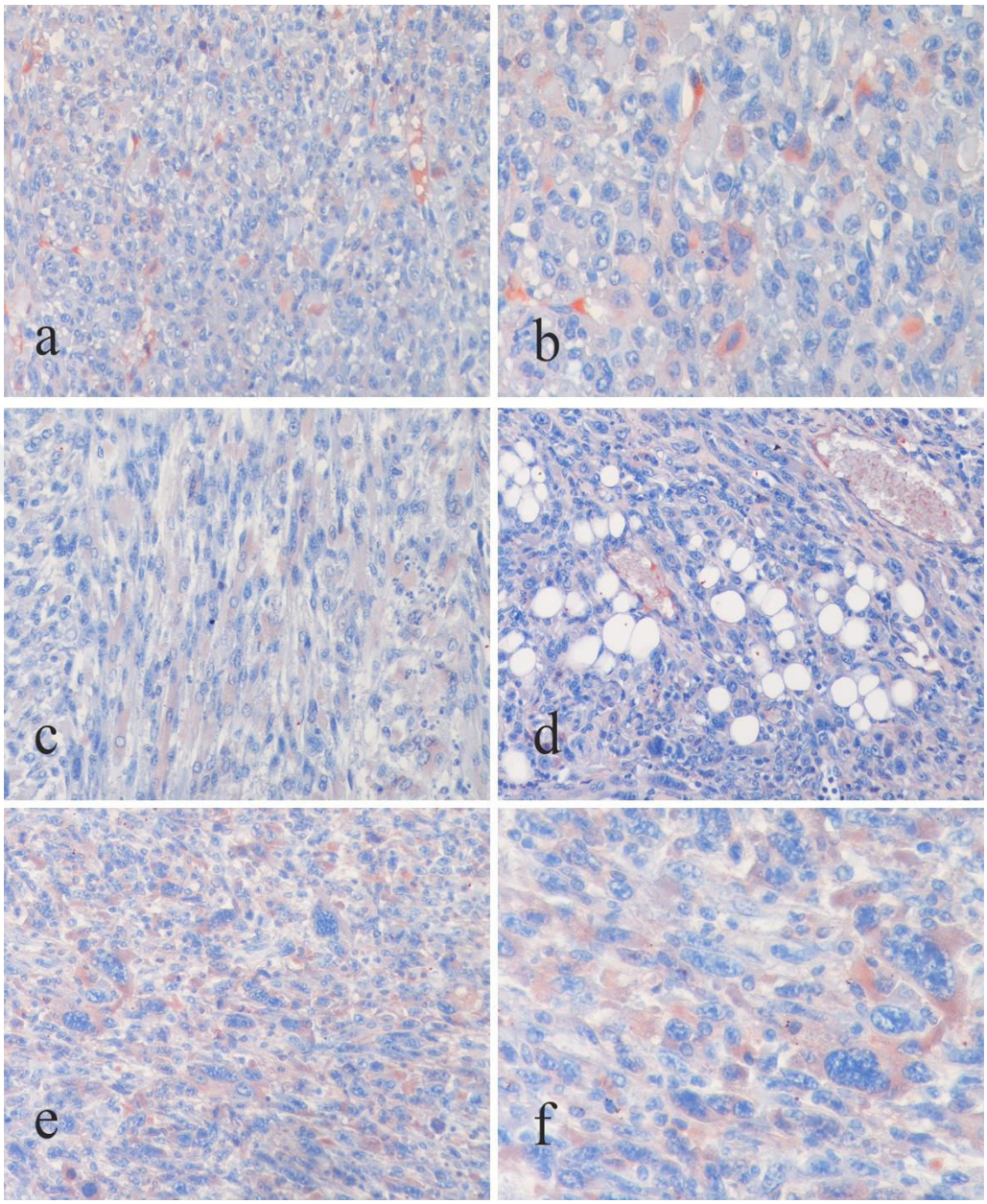

Figure 26. a: E-selectin expression of vascular endothelium and tumor cells in 3-MCA group, objective 20x, b: higher magnification view of the same case, objective 40x, c, d: Tissue sections from two different tumors from IP group note the immunoreactivity of neoplastic cells in c and vascular endothelium and tumor cells in d, objective 20x, e, f: IT group, A low (e, objective 20x) and high (f, 40x) magnification view of tumor cells with cytoplasmic immunoreactivity to E-selectin

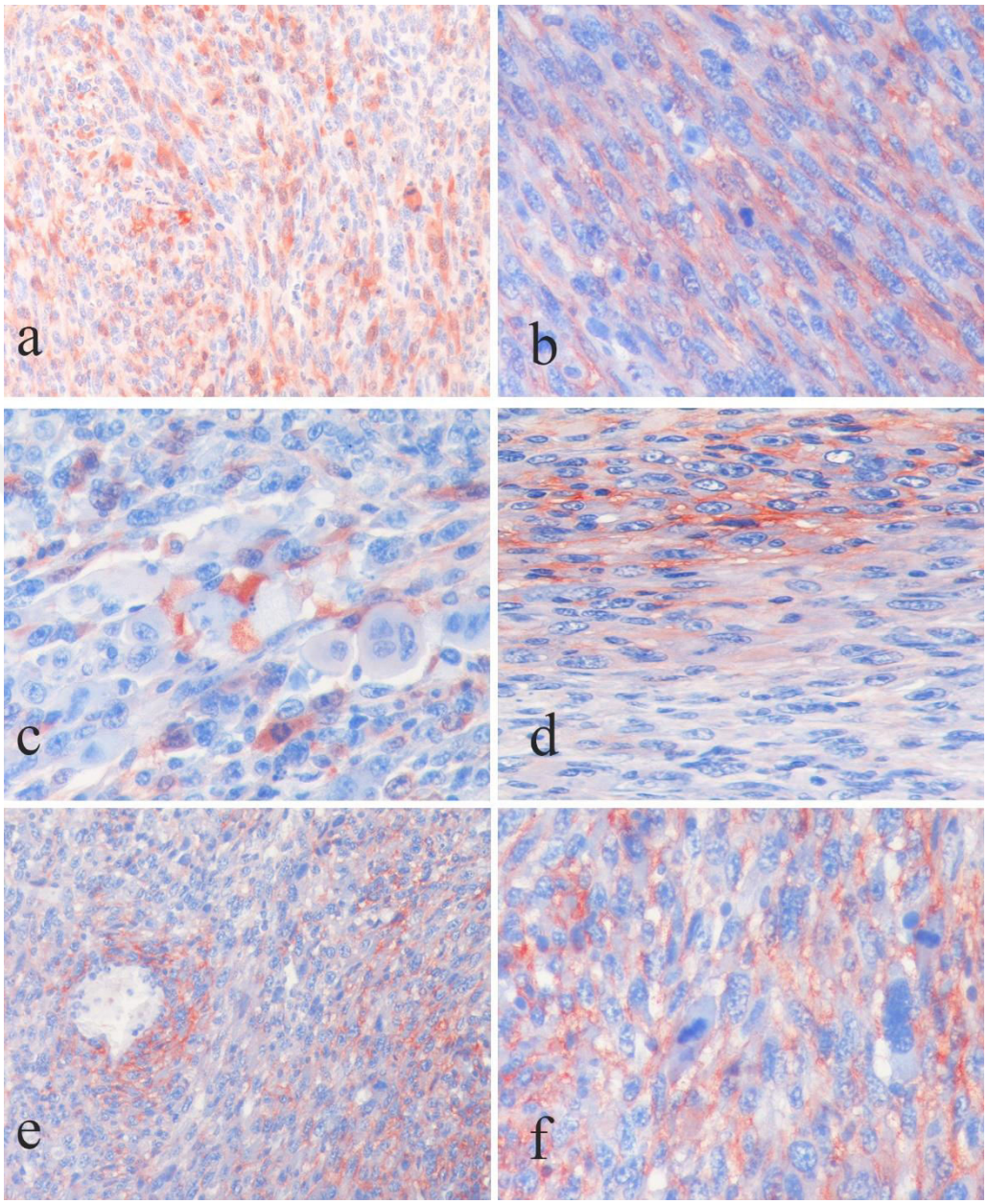

Figure 27. a, b, c: Photomicrographs of tissue sections from three different tumors in 3-MCA group, note cytoplasmic staining (a, objective 20x), membranous staining (b, objective 40x) and extracellular matrix positivity (c, 40x) for integrin alpha v, d: Membranous staining of neoplastic cells in a tumor from IP group, objective 40x, e, f: IT group, two different areas from the same tissue section demonstrating perivascular and membranous immunoreactivity objective 20x (e) and 40x (f)

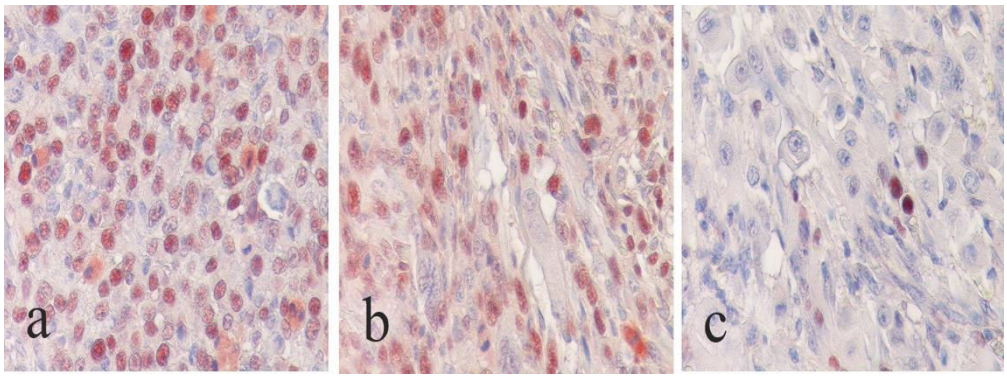

Figure 28. PCNA expression in 3-MCA group (a), IT group (b) and IP group (c), note significantly less number of cells are immunoreactive in IP group, objective 40x

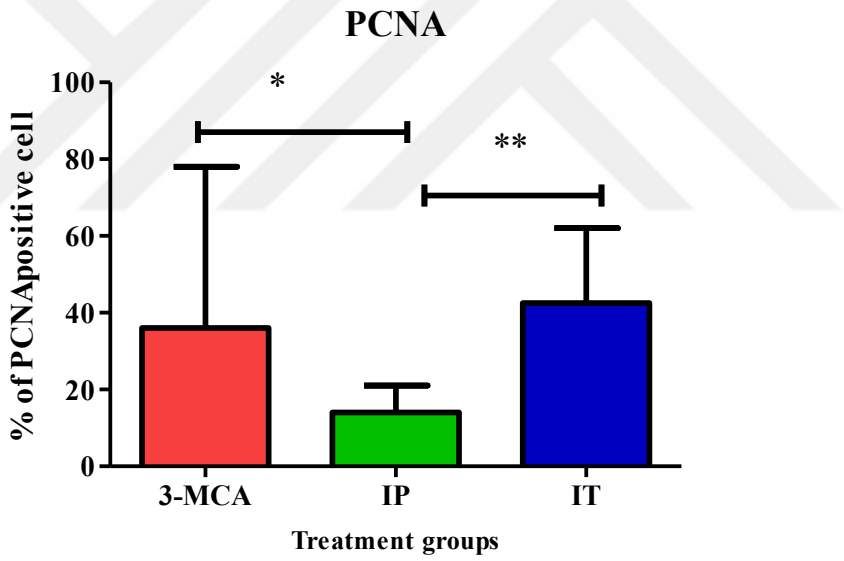

Figure 29. Comparison of PCNA expression between different treatment groups. IP group has significantly less number of cells expressing PCNA (p=0.0015)

5. DISCUSSION

STSs are tumors of mesenchymal origin and share similarities in histological appearance and biological behavior. Fifteen percent of the skin and subcutaneous tumors of the dog and 7% of cat fall in this category (Liptak and Forrest, 2013).

In the current study, 14 cases of neoplastic growth were observed at anatomical sites used for injection or vaccine administration in cats. Out of which 13 were fibrosarcoma and one was a myxoid liposarcoma. As observed in current study fibrosarcoma is the most common histological variant occurring at injection sites in cats, although reports of other tumors e.g. chondrosarcoma, RMS, malignant fibrous histiocytoma, osteosarcoma, and liposarcoma do exist (Hendrick and Brooks, 1994; Esplin and Campbell, 1995; Esplin et al., 1996). Similar to previous studies, (Hendrick et al., 1992; Kass et al., 1993; Hershey et al., 2000), interscapular region (n=6), dorsal/back (n=4), thoracic (n=3), right gluteal region (n=1) were the common sites for occurrence of tumors in cats. These sites are still commonly used for injection or vaccine administration in some parts of the world despite the guidelines by the vaccine-associated feline sarcoma task force. Generally, injection site fibrosarcoma is of moderate to high-grade invasive tumors (Hendrick et al., 1994; Aberdein et al., 2007). We observed grade 2 (n=10; 71.4%) and grade 3 (n=4; 28.6%) tumors in cats and it was in partial agreement with the Couto et al. (2002) who reported higher number of grade 2 (47.7%) and grade 3 (27.3%) fibrosarcomas along with 25% grade 1 tumors. The median age of the cats in the current project was 9.5 years which is in agreement with the findings of Couto et al. (2002) but slightly higher (1.5 years) than that observed by Doddy et al. (1996) but lower (1.9 years) than reported by a scientist from New Zealand (Aberdein et al., 2007). The less number of animals and missing information about age in the current study could be a contributing factor in this difference. Frequency and nature of vaccine or injection administration may also be a factor responsible for this variation in the age of tumor development.

Histological examination of the feline tumors revealed a subcutaneous location of tumors with peripheral lymphocytic infiltration and few histiocytes. This feature has been consistently observed since the very first reports of the injection site sarcomas and is considered pathognomonic for diagnosis (Hendrick et al., 1992; Kass et al., 1993; Hendrick

and Brooks, 1994; Hendrick et al., 1994; Esplin and Campbell, 1995; Doddy et al., 1996). However, they also observed macrophages with engulfed foreign material which they called aluminum-based adjuvant used in the vaccine, which was not a predominant feature in our study. But it is not a mandatory feature of all the injection site sarcomas as Couto et al. (2002) observed large foamy macrophages in only 22.5% and 25% of primary and recurrent fibrosarcomas, respectively. Moreover, there have been reports of the feline fibrosarcomas at the presumed injection site with inflammatory reaction without foamy macrophages (Aberdein et al., 2007; Diep and Fleis, 2012). The presence or absence of the macrophages with intracytoplasmic basophilic granular material may be related to the presence or absence of adjuvants in the vaccine (Aberdein et al., 2007). The persistence of inflammatory cells is a major factor contributing in the pathogenesis of injection site sarcomas (Hendrick and Goldschmidt, 1991; Doddy et al., 1996; Macy and Hendrick, 1996; Woodward, 2011). Necrosis was observed in all the feline tumors under study. Necrotic areas are more frequently observed in the injection site tumors than non-injection site sarcomas (Doddy et al., 1996). The necrosis may develop as a consequence of damage induced by inflammatory cells or due to the deficient blood supply of the rapidly growing tumor cells (Doddy et al., 1996; Couto et al., 2002).

Tumor cells were pleomorphic to spindle-shaped with lack of characteristic herringbone pattern of fibrosarcomas. Multinucleated giant cells are another histological characteristic of injection site sarcomas (Couto et al., 2002; Aberdein et al., 2007) which was also recorded in the present study. Tumor samples from eight cats demonstrated myxomatous/ mucin containing areas with loosely arranged spindle-shaped or stellate cells. Injection site sarcomas with myxomatous matrix were also observed in previous studies and injection site sarcomas have the greater tendency to produce myxomatous stroma as compared to non-injection site feline sarcomas (Doddy et al., 1996; Srivastav et al., 2011).

All the feline and canine tumors in this study showed no reactivity for desmin and were positive for vimentin. This finding is in agreement with previous studies (Couto et al., 2002). One sample of cats showed reactivity to muscle actin and alpha-smooth muscle reactivity was noticed in 4/14 tumors of cats. This number is quite low as compared to previously reported 28/44 and (Couto et al., 2002) and 7/10 (Aberdein et al., 2007). Smooth

muscle reactivity indicates the presence of myofibroblasts in the tumors which are considered a transitory phase through which fibroblasts and macrophages pass during wound healing (Martano et al., 2011). Though their precise role in injection site sarcomas is unclear (Madewell et al., 2001), some authors describe it as an atypical response to traumatic injury (Martano et al., 2011).

Desmin, S-100, sarcomeric actinin, pan-cytokeratin, GFAP, myogenin, and myoD1 was negative in the current study. One case showed focal reactivity to myoglobin and two for muscle actin. Previously, S-100 positivity was observed in a chondrosarcoma at the injection site but not in fibrosarcoma (Hendrick and Brooks, 1994). Myoglobin reactivity in one animal in the current study may indicate regenerating muscle cells in the areas invaded by tumor cells. Myogenin and myoD1 are highly specific and sensitive markers for tumors showing skeletal muscle differentiation (Cessna et al., 2001). Negative reaction to these antibodies in all feline tumors helped in exclusion of rhabdomyosarcomatous differentiation in the tumors under study.

PDGF/PDGFRs axis play an important role in the pathogenesis of many humans and animal tumors including soft tissue sarcomas (Kilvaer et al., 2011; Jacobs et al., 2017). Upregulation of PDGFA and its receptor alpha was present in all the (100%) feline tumors. Co-expression of PDGFA and PDGFR alpha was confirmed by double staining by immunofluorescence method. However, no significant correlation in terms of H-score was observed between this tyrosine kinase receptor and its ligand. Other than tumor cells, muscle cells, blood vessel walls and epithelium of hair follicles showed positive reactivity. The results of the current study are partially in line with the previous reports of immunoreactivity of PDGF and its cognate receptor in feline injection site sarcomas (Hendrick, 1998). He also reported immunopositivity of peripheral lymphocytes and macrophages and strong staining in the tumor cells near these inflammatory cells which were partially observed in the current study. The difference may be due to use of antibodies against a different isoform of PDGFA and PDGFR. The two other studies investigating the role of PDGF/PDGFRs axis in feline injection site sarcomas were carried out targeting PDGF-BB and PDGFR-β but not α (Katayama et al., 2004; Lawrence et al., 2012). They used feline injection site sarcoma cell lines and a xenograft murine tumor model and evaluated imatinib (Katayama et al., 2004)

and masitinib (Lawrence et al., 2012) anti-tyrosine kinase drugs to inhibit tumor cell growth by blocking expression of PDGFR-β. However, in human medicine, co-expression of PDGFR-alpha and its ligands has been implicated in several tumors and an anti-PDGFR-α antibody named olaratumab is being evaluated as a therapeutic candidate (Teyssonneau and Italiano, 2017).

Integrin alpha v binds to vitronectin and fibronectin which are its principal ligands and contribute to local invasion and distant spread of the neoplastic cells (Nejjari et al., 2002). The role of integrin alpha v is well established in a number of human cancers e.g. melanoma, breast and pancreatic tumors (Felding-Habermann et al., 2002; Hosotani et al., 2002; Baum et al., 2007). Integrin alpha v expression was observed in 93% of the feline tumor samples. Statistical analysis did not reveal any correlation of integrin alpha v expression with E-selectin and tyrosine kinases. Literature is scarce on the role of integrin alpha v expression in feline injection site sarcomas and we find only one previous report describing the expression of integrin alpha vβ3 in the tumors what authors described as "highly infiltrative spontaneous fibrosarcomas". The main objective of the study was to test a NIR-dye labeled nanoprobe targeting the integrin alpha vβ3 in the near-infrared optical guided excision of the tumors. They observed integrin expression on the endothelial cells and on the surface of the tumor cells in the proximity of the blood vessels (Wenk et al., 2013). Our results are in partial agreement with the findings of the aforementioned study. The main differential points between the current and previous study are as follow;

1. Though one of the image in the published worked from the previous study had peritumoral inflammatory foci mostly observed in injection site sarcoma but it is not clear whether the enrolled subjects were having spontaneous fibrosarcoma or injection site fibrosarcoma as the pathogenesis of both is different. While the current was focused on injection site sarcoma.
2. The authors described the positive staining in the vascular endothelium and tumor cells in the vicinity of the vessels, but we observed a widespread staining reaction throughout the tumor tissue, including multinucleated tumor cells, and normal skeletal muscles.

E-selectin expression was noticed in the vascular endothelium and tumors cells in 36% feline cases enrolled in the present study. However, the H-score was generally low except in a single fibrosarcoma which showed moderate immunoreactivity. We did not find any literature regarding E-selectin expression in the feline injection site sarcomas. As E-selectin is not expressed on endothelial cells until stimulated by inflammatory mediators (Tremblay et al., 2001), vascular expression observed in the current study can be correlated with the presence and persistence of inflammatory cells in these sarcomas. This hypothesis is supported by the published literature which highlights the pivotal role played by E-selectin and other adhesion molecules in the recruitment of lymphocytes and other leucocytes in the areas of inflammation or tissue damage (Kulidjian et al., 2002; Wiese et al., 2009; Angiari, 2015). Furthermore, E-selectin play a very dynamic role in tumor pathogenesis, in some cases like in human squamous cell carcinoma of head and neck and Merkel cell carcinoma, its downregulation by myeloid-derived suppressor cells is responsible for tumor development due to inability of immune cells to reach at tumor periphery (Gehad et al., 2012; Afanasiev et al., 2013) while in some cases e.g. in breast cancer, its upregulation promotes tumor metastasis (Tözeren et al., 1995). As found in the present study, E-selectin expression is not only restricted to vascular endothelium and tumor cells may also express this glycoprotein like observed in human prostate cancer epithelial cells with high levels of E-selectin expression (Bhaskar et al., 2003). Additionally, E-selectin expression is elevated in hypoxic endothelium (Zünd et al., 1996). Therefore, the role of E-selectin expression in the feline injection site sarcomas is possibly multifactorial involving hypoxic environment along with inflammatory mediators released by tumor-infiltrating inflammatory cells.

In dogs, we diagnosed 10 cases of fibrosarcoma and one case of myxosarcoma. The average age of the animals observed in this study (median=7) was slightly less than that (8.4 years & 9 years) reported by Vascellari et al. (2003) and Hendrick (2016), respectively. This variation may be explained by the differences in breeds and number of subjects in these studies. The fibrosarcomas at non-injection sites were mostly located in the dermis and were of grade 1 (n=4), grade 2 (n=3) and grade 3 (n=1). The two samples from left cheek and submandibular area showed intratumoral lymphocytic infiltration. Inflammatory cell infiltration in few of the non-injection site fibrosarcomas has been reported in dogs

(Vascellari et al., 2003) and cats (Doddy et al., 1996). The precise role of these inflammatory cells in these non-injection site sarcomas is not clear but may be a host immune response to tumor antigens. Two of the tumors of the dog was diagnosed as injection site sarcoma occurring in the dorsal cervical region and left flank and were of grade 3. Like in cats, injection site sarcomas have been reported in dogs, though incidence is less than cats (Yardimci et al., 2011; Jacobs et al., 2017). They share the same histological characteristics as the feline injection site sarcomas i.e. subcutaneous location, peritumoral inflammation composed of lymphocytes, macrophages, plasma cells, necrosis and cellular pleomorphism (Vascellari et al., 2003) but multinucleation, a prominent feature in cat sarcoma is a rare finding in dogs (Jacobs et al., 2017). The histological findings of two cases of injection site sarcoma were quite similar to the previous studies (Vascellari et al., 2003; Vascellari et al., 2006) as peritumoral inflammation predominantly containing lymphocytes, necrosis, absence of multinucleated giant cells was also evident in the present study but differs partially with the observations of Jacob et al. (2017) who reported binucleated and multinucleated giant cell and with Vascellari et al. (2003) in terms of the presence of myxoid areas in the current study. Presence or absence of multinucleated giant cells may be related to the degree of cellular differentiation (Couto et al., 2002).

Vimentin showed a positive reaction in all the tumors of the dogs. It is considered a marker for the mesenchymal cells and its immunoreactivity in canine fibrosarcoma and myxosarcoma and other mesenchymal tumors has been well documented in the literature (Williamson and Middleton, 1998; Vascellari et al., 2003). Smooth muscle actin immunoreactivity was noticed in two tumors diagnosed as injection site sarcomas, mainly at the periphery of the tumor. This indicates the presence of myofibroblasts in the tumor and has been reported in both canine (Vascellari et al., 2003) and feline injection site sarcomas (Couto et al., 2002). Some authors are of the view that myofibroblasts are a part of tumor stroma and function to prevent the entry of the immunocompetent cells into the tumor (Lieubeau et al., 1999). One case of myxosarcoma showed positivity for cytokeratin in a small number of cells. Myxosarcomas are reportedly negative for cytokeratin (Williamson and Middleton, 1998; Headley et al., 2011). The positive cells observed in the current study may be remnants of epithelial adnexal structures

As already mentioned, PDGF and PDGFRs have a dynamic role in tumor pathogenesis by promoting cell proliferation, tumor angiogenesis, evading apoptosis (Ostman, 2004; Tallquist and Kazlauskas, 2004) and their expression in tumors has been correlated with higher potential for malignancy and metastasis (Kilvaer et al., 2010). In the current study, PDGFA expression was noticed in all the tumors of dogs while PDGFR-alpha was expressed in 10 out of 11 (90.9%) cases. Myxosarcoma was negative for PDGFR-alpha. We did not find any literature regarding the expression of PDGFA and PDGFR-alpha in the canine cutaneous fibrosarcomas. However, canine oral fibrosarcoma and canine injection site sarcoma cases were reported to express PDGFR-alpha and PDGFR-beta and their high expression has been correlated with increased tumor cells proliferation (Milovancev et al., 2016; Jacobs et al., 2017). Both nuclear and cytoplasmic staining was observed for PDGFR-alpha in the current study. However, there are conflicting reports in the literature about the specificity of nuclear reactivity of PDGFR-alpha e.g. Milovancev et al. (2016) reported both cytoplasmic and nuclear positivity for PDGFR-alpha in the canine oral fibrosarcomas using the same clone of the antibody as in the current study (Sc-338, Santa Cruz Biotechnology, Dallas, TX) whereas another study describes nuclear localization observed with this antibody as a non-specific reaction due to its polyclonal nature (Holzer et al., 2016), but nuclear localization of PDGFR-alpha was also observed in human alveolar RMS (Aslam et al., 2013) with a different clone and commercial source of the antibody. Other than canine oral fibrosarcoma, canine osteosarcomas also overexpress PDGFA and PDGFR-alpha (Maniscalco et al., 2013). Based on the expression profiling of these tyrosine kinases and their receptors, tyrosine kinase inhibitors are being used for the treatment of various soft tissue sarcomas in dogs (Milovancev et al., 2016; Jacobs et al., 2017).

Integrin expression was observed in all the canine tumors including myxosarcoma. We observed membranous as well as cytoplasmic reactivity in the tumor cells. Multinucleated tumor giant cells and normal skeletal muscle cells also showed cytoplasmic reactivity for the integrin alpha v. To the best of our knowledge, there is no report of integrin alpha v expression in canine cutaneous fibrosarcomas. However, a study reports a high expression of integrin alpha v beta 3 in canine cutaneous and oral melanomas (Rawlings et al., 2003). We did not observe any significant difference in integrin expression between

tumors belonging to different grades. Various heterodimers of integrin alpha v play a crucial role in tumor angiogenesis (Friedlander et al., 1995), invasiveness & metastasis (Seftor et al., 1999; Mierke et al., 2011) as observed in various human cancers. These findings may indicate that integrin alpha v may induce tumor angiogenesis and microvessel proliferation. However, further studies are required to define the exact role of integrin alpha v in the pathogenesis of canine cutaneous fibrosarcomas.

A significant correlation was found between integrin alpha v and PDGFA expression. To the best of our knowledge, there has not been any report about this synergism in the canine fibrosarcomas. However, literature exists in human medicine explaining the interaction between different integrins and PDGF and PDGFRs. An *in vivo* experimental study has demonstrated a synergism between integrin alpha v beta 3 and PDGFR-β resulting in neovascularization and cell migration (Woodard et al., 1998). Additionally, it has been reported that PDGFA and PDGFR-alpha both can activate alpha v beta 3 integrin and promote oligodendrocyte progenitor proliferation (Baron et al., 2002).

In the present study, a low expression of E-selectin was observed in the vascular endothelium and cytoplasm of the tumor cells in (82%) canine tumors. The literature review did not reveal any canine neoplasm with tumor cells' reactivity to E-selectin antibody. In human medicine, prostate cancer cells were reported to express E-selectin where its blockage by monoclonal antibody did not influence the cell proliferation, however, E-selectin helped in the entry of the anti-cancerous drug into the tumor cells (Bhaskar et al., 2003). E-selectin overexpression assists tumor cells' intravasation and extravasation leading to metastasis in human cancerous colon tissues (Maurer et al., 1998). So, E-selectin expression has a versatile role in different tumors. Further studies are required to establish its biological implication in canine soft tissue sarcomas.

The experimental part of the current study was aimed to induce a sarcoma by 3-MCA administration in mice model and its subsequent treatment with tadalafil, a selective phosphodiesterase 5 inhibitor through the intraperitoneal and intratumoral injection. The tumor was induced in 93% (28/30) animals treated with 3-MCA. A similar success rate (91.4%) of tumor induction by 3-MCA was observed in a previous study (Inoue and Wu, 2006). No gross or microscopic lesion was observed in the tumor-free control group. There

was no difference in the weight and volume of the different tumor groups with or without tadalafil application. As three animals in the IP group died during the experiment and one animal from 3-MCA group was excluded because of severe necrosis, 24 animals were available for the histological and immunohistochemical analysis.

Histopathological examination revealed that 3 animals showing areas of squamous cell proliferation along with the presence of rhabdomyosarcomatous areas. As observed in the current study, previous reports also indicate that 3-MCA may induce heterogenous tumors e.g. squamous cell carcinoma mixed with rhabdomyosarcomatous components (Inoue and Wu, 2006). Tumors in the current study were comprised of small round to pleomorphic cells, large rhabdomyoblasts like cells with abundant cytoplasm, many mononucleated and multinucleated giant cells and elongated strap-like cells. These findings are in partial line with the previous reports (Kosmehl et al., 1989; Vogel et al., 1991; Inoue and Wu, 2006). However, Kosmehl et al. (1989) and Vogel et al. (1991) did not categorize the tumors into any of the WHO accepted category while Inoue and Wu (2006) reported embryonal and pleomorphic RMS in their experiment. We followed the criteria described by the Cooper and Valentine (2016) for categorizing tumors into subvariants of RMS and classified all tumors as an embryonal variant of RMS. The RMS is a rare tumor in animals but very important childhood and adolescent malignancy in humans (Gurney JG, 1999; Cooper and Valentine, 2016).

The only difference in the histological appearance between groups was the presence of more rhabdomyoblastic or differentiated cells in the group treated with intraperitoneal injection of tadalafil.

Vimentin positivity was observed in the 87% of the tumor samples, though reactivity was low. No cellular specificity was noticed for vimentin expression and nearly all type of cells showed some sort of staining. During normal myogenesis vimentin is expressed at early stages of cell differentiation and is gradually replaced by other intermediate filaments, however tumor cells in RMS may continue to express this mesenchymal marker during different stages of maturation or differentiation (Wijnaendts et al., 1994) indicating that the tumor may have originated from a vimentin positive undifferentiated mesenchymal cells of

non-muscle origin (Altmannsbergeri et al., 1982; Wijnaendts et al., 1994). RMS in human is reported to show a low vimentin immunoreactivity (Altmannsbergeri et al., 1982) where it is limited to primary and secondary myotubes/myofibers (Wijnaendts et al., 1994). In the present study, although there was no statistical difference between groups, IP group showed less number of cell positive to vimentin. This may be attributable to the presence of more differentiated cells in this group as reported by Kosmehl et al. (1989).

In the current study, tumor cells showed positivity to more than one muscle markers and all the samples were at least reactive to one of these antibodies indicating a clear muscular differentiation. But we did not observe any statistically significant difference for any of the marker in the current study between different groups. Seventy-one percent of tumor samples were positive for desmin, 100% for sarcomeric actinin, 92% for myoglobin, 96% for muscle actin, 88% for smooth muscle actin, while nuclear reactivity for myogenin was observed in 83% tumor samples. Current results are in partial agreement with the Wijnaendts et al. (1994) who reported the expression of muscle actin (96%), desmin (95%), sarcomeric actin (71%), smooth muscle actin (13%) in human RMS samples.

Muscle actin (HHF35) is an antibody capable of detecting all muscle cells i.e. cardiac, skeletal and smooth muscles (Tsukada et al., 1987) and as observed in the current study, is expressed in by tumor cells of human and animal RMS during all stages of differentiation and is similar in specificity to desmin, however smooth muscle tumors needs to be ruled out (Wijnaendts et al., 1994; Caserto, 2013).

Desmin positivity was noticed in multinucleated giant cells, strap-like cells and rhabdomyoblastic cells with abundant cytoplasm. Similar results were reported from a 3-MCA induced RMS in a murine model by Vogel et al. (1991). In human RMS, desmin is expressed during all the stages of tumor cell differentiation. However, the percentage of cells stained vary in primary and relapsed tumors (Wijnaendts et al., 1994).

Alpha-actinin is a cytoskeletal protein which cross-links actin filaments in the cell. It has both muscle and non-muscle isoforms. Muscle isoforms include cardiac, skeletal and smooth muscles actinins (Sjoblom et al., 2008). Alpha-actinin is expressed early than actin in the RMS cell lines (Prados et al., 1993). We used an alpha-actinin (sarcomeric) antibody with the potential to detect cardiac and skeletal actinins. It showed a strong positive reaction

in all the tumors across different groups. Though statistically insignificant among groups but relatively high positivity was observed in group IP. Microscopic examination correlated it with the presence of relatively more differentiated rhabdomyoblasts in this group as also observed by Scupham et al (1986) in human RMS.

Myoglobin appears last in the sequence of appearance of diagnostic muscle protein or intermediate filaments. Therefore, its expression is observed in more differentiated muscle tumors (Caserto, 2013). Similar results were obtained in the present study where 92% of the tumors showed positivity for this protein mainly in the strap like cells, mononucleated and multinucleated giant cells.

A low to moderate cytoplasmic reactivity for smooth muscle actin was noticed in 88% of the tumors. However, Wijnaendts et al. (1994) reported smooth muscle positivity in only 13% from human rhabdomyosarcoma samples. Aberrant expression of smooth muscle actin is reported in both animal and human rhabdomyosarcomas (Wijnaendts et al., 1994; Caserto, 2013; Cooper and Valentine, 2016). Moreover, rat and mouse embryos express alpha-smooth muscle actin during myogenesis (Babai et al., 1990) which is absent in the human embryos (Wijnaendts et al., 1994). Likewise, a nickel sulfide induced rhabdomyosarcoma in rats showed smooth muscle actin expression in 69% of the tumors (Babai et al., 1988). Considering these reports, in rat or mice, expression of smooth muscle actin may represent a resemblance to embryonic myogenesis.

Myogenin is a nuclear transcriptional factor which regulated myogenesis and is expressed early during skeletal muscle development. It is considered a sensitive and specific marker for RMS (Cessna et al., 2001) and has been used as a diagnostic marker both in animals and humans (Caserto, 2013). In the current study, 83% of the tumors showed nuclear positivity to myogenin. The reaction was mainly limited to round cell and absent from strap-like cells and multinucleated giant cells. Our results are in line with the findings of Inoue and Wu (2006) who observed variable myogenin reactivity in 3-MCA induced embryonal and pleomorphic RMS. In their study, multinucleated cells and myogenic precursor cells remained negative for myogenin and only myoblasts like cells stained positive. In humans,

alveolar RMS expresses significantly higher levels of myogenin expression than embryonal variant (Kumar et al., 2000).

Sporadic cells positive for cytokeratin were observed in 21% of tumors other than 3 cases showing both squamous and rhabdomyosarcomatous differentiation. Similar results were reported in a previous study on 3-MCA induced RMS in mice (Langbein et al., 1989). This may indicate that tumors induced by 3-MCA arise from stem cells with capability for heterogeneous differentiation. A transient cytokeratin expression is observed during normal skeletal muscle development in mice. This would, in general, be comparable to an expression of fetal genes in malignant tumors, a phenomenon commonly called retro-differentiation (Uriel 1979; Weinhouse 1980). Human RMS also show aberrant expression of cytokeratin (Miettinen and Rapola, 1989) and various neuroendocrine markers (Coindre et al., 1988; Bahrami et al., 2008).

PCNA expression was significantly low ($p=0.0015$) in the IP group as compared to other groups. Expression of this multirole protein is correlated with the proliferation of the cell (Strzalka and Ziemienowicz, 2011). PCNA expression in 3-MCA induced RMS was also evaluated in a previous study (Inoue and Wu, 2006) but they did not use any therapeutic agent unlike in the present project. Anti-proliferation potential of tadalafil has been observed against pulmonary artery smooth muscles in idiopathic pulmonary arterial hypertension by inhibiting high levels of PDE5 mRNA in these smooth muscle cells and inducing apoptosis (Yamamura et al., 2017), similar effects have been reported in human breast cancers (Barone et al., 2017). Myeloid-derived suppressor cells and regulatory T cells which can cause suppression of T- and NK-cell show their presence in 3-MCA induced sarcomas (Ligtenberg et al., 2015) and tadalafil has the potential to inhibit these cells as observed in human head and neck squamous cell carcinomas (Califano et al., 2015; Weed et al., 2015). As tadalafil show anti-tumor or anti-proliferative activity in different ways, further research is required to elucidate the exact mode of action in 3-MCA induced sarcomas.

The upregulation of PDGFA was observed in 88% (21/24) of the tumor cases. Multinucleated cells, strap-like cells, spindle-shaped cell and round cells all showed variable immunoreactivity. Generally, median H-score in IP group (H-score: 12.50) was less than 3-MCA (H-score: 75) and IT group (H-score: 48) but the statistical analysis did not show any

significant difference. To the best of our knowledge, previous studies did not evaluate the expression of PDGF in 3-MCA induced RMS (Kosmehl et al., 1989; Langbein et al., 1989; Inoue and Wu, 2006). Moreover, any of these experiments did not use tadalafil as a therapeutic agent. In humans, PDGFA, PDGFB, PDGFR-alpha and beta expressions is increased in pulmonary artery hypertension in remodeled arteries, in lungs and in small arteries in the endothelium and in the smooth muscles (Perros et al., 2008) while tadalafil has the antiproliferative and apoptotic effects on the smooth muscles in idiopathic pulmonary artery hypertension mainly by inhibiting high levels of PDE-5 (Yamamura et al., 2017) but, is there any interaction between these tyrosine kinase receptors and this PDE-5 inhibitor compound needs further investigation.

A very low immunoreactivity was observed for PDGFR-alpha in 58% of the tumor cases studied. Blood vessels and strap-like cells were mainly reactive to the anti-PDGFR-alpha antibody. Previous studies showed that PDGFA, PDGFC, and their receptor PDGFRA, its subsequent effector molecules MAPK and Akt are overexpressed in primary and metastatic alveolar RMS in humans and are considered as therapeutic targets (Taniguchi et al., 2008). Role of PDGF family is not limited to an alveolar subtype of RMS. In a study including different sub-variants of RMS, overexpression of PDGFC, PDGFD, and PDGFR-alpha in tumor cells and PDGFR-beta in the stromal cells was observed (Ehnman et al., 2013). Expression of this receptor tyrosine kinase was linked with reduced overall survival time in patients with RMS (Armistead et al., 2007). Low immunoreactivity scores for the PDGFR-alpha in the current study may be attributable to a different pathogenesis and heterogeneous cellular population in this chemical carcinogen-induced tumor.

Integrin alpha v expression was noticed in all the tumors except one animal in IP group which showed very low reactivity. However, no statistically significant difference was noticed in the expression of this molecule in different groups. Extensive literature research did not provide any published literature regarding integrin alpha v expression in 3-MCA induced RMS. However, expression of fibronectin which is a ligand for integrin alpha v was observed in 3-MCA induced RMS in mice. The expression of fibronectin was mostly observed around less differentiated cells and was absent in areas of high differentiation (Vogel et al., 1991). *In vitro* studies have shown that increased levels of fibronectin may

delay myotube formation (Podleski et al., 1979). Fibronectin-integrins axis plays an important role in the migration of tumor cells, their invasiveness and distant spread (Akiyama et al., 1995). In the current study, integrin alpha v expression was membranous, cytoplasmic as well as in extracellular/around the cells, and most of the multinucleated cells and strap-like cells were negative for integrin expression. So, an indirect inference may be obtained from the aforementioned studies that high expression of integrin alpha v may also be responsible for poor differentiation of tumors. In human medicine, expression of integrin alpha v has been documented in RMS cells (Cripe et al., 2001; Scherzinger-Laude et al., 2013). Though various heterodimers of integrin alpha v have been implicated in the tumor angiogenesis and metastasis (Kumar, 2003), they are also being used for drug delivery in the tumor by tagging therapeutic molecules with the integrin ligands (Scherzinger-Laude et al., 2013).

Overall, a very weak expression of the E-selectin was noticed in 46% of the tumors. Endothelial cells and polygonal tumor cells along with multinucleated or strap-like cells were immunoreactive. The endothelial cells showed strong staining as compared to tumor cells. Endothelial expression of this endothelial cell adhesion molecule may be attributable to the inflammatory components in the tumor as various inflammatory mediators e.g. tumor necrosis factor alpha, interleukin-1, granulocyte colony-stimulating factor produced by leucocytes are reported to induce *de novo* expression of E-selectin (Barthel et al., 2007) or hypoxic insult in the tumor (Zund et al., 1996). Inflammation is believed to play an important role in the pathogenesis of 3-MCA induced sarcomas (Swann et al., 2008). In the present study, tumor cells especially strap like multinucleated cells also stained positively with the anti-E-selectin antibody. This finding has been previously reported neither in 3-MCA induced RMS nor in the human cases of RMS. Endothelial expression of E-selectin has been associated with tumor invasion and metastasis e.g. in breast cancer (Zen et al., 2008), colon cancer (Maurer et al., 1998), melanomas (Schadendorf et al., 1995) while the epithelial expression of E-selectin has been reported in limited tumors i.e. the prostate cancer and colon cancer (Maurer et al., 1998; Bhaskar et al., 2003). Epithelial expression of E-selectin in prostate cancer is the result of unique and complex interactions of different factors in the tumor microenvironment and was not detected in the xenograft models of prostate cancers

and prostate cancer cell lines (Bhaskar et al., 2003). In humans, head and neck squamous cell carcinoma is associated with the influx of nitric oxide producing myeloid suppressor cells resulting in downregulation of E-selectin and preventing the influx of immunocompetent T cells (Gehad et al., 2012). Tadalafil can suppress nitric oxide production and thus can upregulate E-selectin in the endothelial cells with subsequent recruitment of effective immune cells resulting in tumor growth suppression (Califano et al., 2015). However, as there was no significant difference between tadalafil treated and untreated group regarding the expression of E-selectin, this mechanism of E-selectin expression or upregulation cannot be applied in the current study and further research should be done to define the role of E-selectin expression by tumor cells.

6. CONCLUSION AND RECOMMENDATIONS

The results of the present study indicate that PDGFA and its receptor PDGFR-alpha and integrin alpha v are overexpressed in the canine and feline fibrosarcomas and thus play a significant role in the pathogenesis of these mesenchymal tumors. PDGFA and integrin alpha v expression showed a positive correlation in dog tumors but not in cats. This indicates a complex interaction between different molecular factors in tumor development. Neoplastic cells in some of the canine and feline fibrosarcomas also showed E-selectin expression which has not been reported in both canine and feline fibrosarcomas and has been rarely reported in some of the human cancers.

Induction of RMS by administration of 3-MCA resulted in variable sized tumors and treatment with tadalafil at a dose rate of 10mg/kg of body weight did not show any significant inhibitory effect on tumor development through intraperitoneal route or intratumoral injection. However, the microscopical analysis revealed more differentiated cells in the group treated with tadalafil intraperitoneally. Moreover, PCNA was significantly lower in the intraperitoneally treated group. This shows that tadalafil therapy may have some effect on tumor cell proliferation and differentiation. Contrary to high expression of PDGFA in these mouse RMS, PDGFR-alpha showed quite a low expression. Like in canine and feline fibrosarcomas, cytoplasmic E-selectin expression by tumor cells were also noted in 3-MCA induced RMS.

We recommend that further studies should be carried out to delineate the complex interactions between tyrosine kinases and ECAM in the tumor pathogenesis, especially role of E-selectin expression by tumor cells requires special attention. Moreover, tadalafil administration alone or in combination with a chemotherapeutic agent may be evaluated using different dose rates and duration of time.

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APPENDIX

APPENDIX 1. Approval certificate of ethical committee

RESUME

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