CLINICAL APPLICATION OF ADIPOSE DERIVED MESENCHYMAL STEM CELLS FOR THE MANAGEMENT OF OSTEOARTHRITIS IN DOGS

Thesis

Submitted to the Guru Angad Dev Veterinary and Animal Sciences University in partial fulfillment of the requirements for the degree of

MASTER OF VETERINARY SCIENCE
in
VETERINARY SURGERY AND RADIOLOGY
(Minor Subject: Veterinary Anatomy)

By

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(L-2017-V-91-M)

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LUDHIANA – 141 004

2019

CERTIFICATE I

This is to certify that the thesis entitled **"Clinical application of adipose derived mesenchymal stem cells for the management of osteoarthritis in dogs"** submitted for the degree of **M.V.Sc.**, in the subject of **Veterinary Surgery and Radiology** (Minor subject: **Veterinary Anatomy**) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Vinny Anna Varghese (L-2017-V-91-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

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ACKNOWLEDGEMENTS

First and foremost I am immensely thankful to the ***God Almighty*** *for His endless bounties and opportunities that He provided to me and for letting me through all the time with His grace.*

I wish to thank my family; my father ***Mr. A.P Varghese****, mother* ***Mrs. Nileena Varghese****, sister* ***Nivya*** *and brother* ***Eldhose*** *for their love support and concern which have been my strength all these years.*

I would like to extend my deepest regards and immense indebtedness to my esteemed major advisor ***Dr. Rahul K Udehiya,*** *Assistant Professor, Department of Veterinary Surgery and Radiology, College of Veterinary Sciences, GADVASU for his invaluable guidance during my research work. It was a great privilege to work and study under his guidance.*

I feel privileged to express my gracious thanks to the respected members of my advisory committee ***Dr. N. Umeshwori Devi,*** *Department of Veterinary Surgery and Radiology.* ***Dr. J. Mohindroo,*** *(Dean PGS nominee), Professor, Department of Veterinary Surgery and Radiology and* ***Dr. Anuradha Gupta,*** *Professor, Department of Veterinary Anatomy for their invaluable suggestions and guidance.*

My sincere and whole hearted thanks to the faculty members of my department ***Dr. S. K, Mahajan, Dr. Vanadana Sangwan, Dr. Ashwani Kumar, Dr. Navdeep Singh, Dr. Arun Anand, Dr. Tarunbir Singh, Dr. Pallavi Verma, Dr. Chandan Kumar Singh*** *and* ***Dr. Jasmeet Singh Khosa*** *for rendering help and moral support throughout the study period.*

I express my sincere thanks to ***Dr. Simrat Sagar Singh****, former Dean, Postgraduate Studies, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, for constant encouragement and inspirational guidance throughout the study period.*

I wish to express my heartfull thanks to ***Dr. Manjinder Sharma,*** *Assistant Professor Department of Veterinary Physiology and Biochemistry.* ***Dr.Milindmitra Lonare,*** *Assistant Professor Department of Veterinary Pharmacology and Toxicology for their inspiring guidance, valuable suggestions during the course of investigation.* ***Dr. Neeraj Kashyap,*** *Assistant Professor Department of animal genetics and breeding for helping me in analysis of data of my research.*

A sense of heartful gratitude is felt towards my batchmates ***Ramandeep, Gurkripal, Anjan, Harnoor, Anmol, Tejpal, Raman, Dr. Kuldeep, Harpreet Singh,***

Avantika, Palak, Diksha, Akshita, Harman for their ever willing co-operation and ungrudging assistance wherever and whenever need arose, and also my seniors Dr. Deepti, Dr. Neelam, Dr. Beenish, Dr. Bipasha, Dr. Atul, Dr. Harman, Dr. Kiran, Dr. Jagdeep, Dr. Richa, Dr.Ganga, Dr. Himani, Dr.Daman, Dr. Nishant, Dr. Mahesha, Dr. Priyanka, Dr. Aman, Dr Shruti, Dr Sairam and Dr Deepshika and juniors Vinod, Kavitha, Devinder, Aman, Jasleen, Gurpal, Dr. Ravi, Gurjab, Sehejbir and Sunil for their ever willing co-operation in work and cheerful moments in the department.

I pay my respect to Mrs. Ally Kutty (lab technician), Mr. Harmesh Lal and Jagdish (radiographers), Gurmeet Kaur (Clerk), Pawan Sharma (Storekeeper), Amarjeet, Kishan, Kamarpal, Rajinder, Gurpreet, Mandeep, Jatinder, Vicky, Jaswinder, Raj Kumar, Vishal, Jeevan, Kulwinder and all the other non-teaching staffs of the department for their help and co-operation. The assistance provided by them is duly acknowledged.

A lovely and warm thanks to all my lovely friends namely Arya S Nair, Vishnu Velayuthan, Shina, Ashika, Indhu, Afthab, Swathi, Neelima and Reshmi.

I duly acknowledge the Indian Council of Agricultural Research (ICAR), for the financial aid provided under the "All India network program on diagnostic imaging and management of surgical conditions in animals". I wish to express my thanks to Guru Angad Dev Veterinary and Animal Sciences University for providing required facilities for conducting my research work.

All may not have been mentioned but none is forgotten.

Place: Ludhiana

Date: _____ *(Vinny Anna Varghese)*

Title of the Thesis	**:**	Clinical application of adipose derived mesenchymal stem cells for the management of osteoarthritis in dogs.
Name of the Student	**:**	Vinny Anna Varghese
Admission No.	**:**	L-2017-V-91-M
Major Subject	**:**	Veterinary Surgery and Radiology
Minor Subject	**:**	Veterinary Anatomy
Name and Designation of Major Advisor	**:**	Dr. Rahul K. Udehiya Assistant Professor
Degree to be Awarded	**:**	M.V.Sc.
Year of award of Degree	**:**	2019
Total Pages of Thesis	**:**	72 + VITA
Name of University	**:**	Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana – 141 004, Punjab (India)

ABSTRACT

The study was conducted on 25 clinical cases of dogs diagnosed with osteoarthritis. The study was conducted in two phases. In phase I, Collection, isolation, culture and characterization of canine adipose derived mesenchymal stem cells was done. In phase II, the study was conducted on clinical cases of dog presented with osteoarthritis. Phase II was subdivided to group A control group and group B treated with allogenic canine Adipose Derived Mesenchymal Stem Cells (cADMSCs) treatment group. In group A, Medical management was done in sixteen clinical cases of dogs which were not previously treated with any medication and were treated with traditional conventional treatment per orally. In group B, isolated ADMSCs injected in nine cases of dogs. About 4.5 to 5 million viable ADMSCs cells in DPBS were used for intra-articular injection aseptically using 18G IV cannula under C-arm guidance. Two injections of allogenic canine ADMSCs were given in 15 days time interval. Total of nine joints were injected with canine ADMSCs. In all the animals incidence of osteoarthritis was recorded. The clinical, hemato-biochemical and radiographic observations were recorded in both the groups. However, synovial fluid examination was done only in cADMSCs treated group. All the dogs were observed upto 60 days. Significant improvement in lameness was recorded in group B whereas, no change was recorded hemato-biochemically and radiographically in both the groups. It was concluded that occurrence of osteoarthritis (OA) was found to be high in hip joint of Labrador Retriever as compared to other joints and breeds. Effective restoration of lameness was seen in allogenic cADMSCs treatment as compared to conventional medical treatment. The functional improvement was detected after the second injection and persisted throughout the period.

Keywords: Osteoarthritis, Allogenic Mesenchymal stem cells, Adipose tissue, Dog.

Signature of Major Advisor **Signature of the Student**

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LIST OF ABBREVIATIONS

%	:	Percent
( )	:	Parenthesis
,	:	Comma
.	:	Full stop
/	:	Division slash
-	:	Hyphen
:	:	Colon
;	:	Semicolon
@	:	At the rate
°	:	Degree
’	:	Apostrophe
=	:	Equal
±	:	Plus minus
AP staining	:	Alkaline phosphatase staining
AT-MSCs	:	Adipose Tissue Derived Mesenchymal Stem Cells
B. Wt.	:	Body weight
cADMSCs	:	Canine Adipose Derived Mesenchymal Stem Cells
CCLR	:	Cranial Cruciate Ligament Rupture
CHD	:	Chronic Hip Dysplasia
Cm	:	Centimetre
COX	:	Cyclooxygenase
CT	:	Computed Tomography.
Cumm	:	Cubic per millimetre
DLC	:	Differential Leukocyte Count
DMEM-HG	:	Dulbecco Modified Eagler’s Medium -High Glucose
DPBS	:	Dulbecco Phosphate Buffer Saline
DRF	:	Diameter of rosette of Furstenberg
e.g	:	Example
et al.	:	et alia (and others)
FBS	:	Fetal Bovine Serum
Fig.	:	Figure
gm	:	Gram
gm%	:	Gram Percentage
Hb	:	Haemoglobin
Hrs	:	Hours
i.e.,	:	That is
I/V	:	Intra venous
kg	:	Kilogram

L	:	Lymphocyte
LW	:	Lameness during walk
mg	:	Milligram
ml	:	Millilitre
mm	:	Millimetre
MRI	:	Magnetic Resonance Imaging
MSCs	:	Mesenchymal Stem Cells
N	:	Neutrophil
n	:	Number
NSAIDS	:	Non-steroidal Ant-iinflammatory Drugs
OA	:	Osteoarthritis
OCT	:	Optical Coherence Tomography
P	:	Passage
PDT	:	Population Doubling Time
PP	:	Pain on palpation
TLC	:	Total leukocyte count
U/L	:	Units per litre
WBS	:	Weight bearing during standing
yrs	:	Years

CHAPTER I

INTRODUCTION

Osteoarthritis (OA) is the most common form of arthritis in dogs which affects all type of movable joints. It is a prevalent debilitating joint disorder characterized by erosion of articular cartilage, excessive stiffness, pain, and crepitus (Elders 2000, Noth *et al* 2008). According to United Nation and Australian estimates that one in five dog are affected by OA (Hielm-Bjorkman *et al* 2003, Aragon *et al* 2007, Andreson *et al* 2018). A huge economic pressure imposed in treatment and management of OA leading to stress and decreased quality of life. Osteoarthritis is classified as primary and secondary OA. Primary OA is associated with age, whereas secondary OA is due to developmental diseases and other factors. The degradation of collagen and proteoglycan in OA cartilage leads to a loss in tensile strength and shear properties of cartilage. Although OA manifests as loss of the articular cartilage, it also includes all tissues of the joint, particularly the subchondral bone (Dubey *et al* 2018).

Osteoarthritis mainly affects large breed dogs such as German Shepherd, Labradore Retrievers, Siberian Huskies, Rottweilers as compared to small breed dogs (Bland 2015). Osteoarthritis affect all ages however older dogs are at higher risk. Majority of OA occur in dogs secondary to developmental orthopedic diseases such as canine hip dysplasia (CHD), elbow dysplasia, patellar dislocation, cranial cruciate ligament rupture (CCLR) cases. Other contributing factors can be genetic, age, bodyweight, obesity, gender, exercise and diet (Andreson *et al* 2018). Clinical signs associated with OA are often non-specific and shows activity impairment such as reluctance to exercise, decrease in overall activity, stiffness, lameness, inability to jump, changes in gait such as ‘bunny-hopping’, joint pain on palpation (Budsberg-2004, Bland 2015).

Osteoarthritis is diagnosed with patient’s signalment, history and physical examination, range of motion, stiff-legged gait, deformity of the joints and swelling or pain in the joints. Other diagnostic techniques such as radiography, Computed Tomographys (CT) and Magnetic Resonance Imaging (MRI), Optical Coherence Tomography (OCT) are also helpful for the diagnosis of osteoarthritis (Braun and Gold 2012). Recently, many studies are searching for molecular components of articular cartilage in serum, synovial fluid and urine in an attempt to determine

whether these molecular markers can be used in diagnosis of osteoarthritis (Fox and Cook 2001, Shahid *et al* 2017).

The goal of OA research is to search for new therapeutic strategies that could prevent, reduce or stop the progression of the diseases or alternatively resolve the existing damage to the joint. Large number of animal models has been selected for effective treatment of OA and the therapeutic purpose such as mice, guinea pig, rabbit, dog, sheep, goat and horse (McCoy 2015, Kuyinu *et al* 2016).

Studies suggest that osteoarthritic therapy in dogs with non-steroidal anti-inflammatory drugs not often provide complete pain relief because they rely on a single target receptor or a pathway for their action (Vilar *et al* 2013). Pharmacological interventions are restricted to symptomatic relief with local intra-articular injections of corticosteroids, systemic injection of analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) in addition to these physiotherapy, regular exercise and weight loss (Bijlsma *et al* 2011). Treatments available for OA are limited and most of treatment focuses on symptom management and pain reduction through medication. Surgical management of OA includes reconstructive procedures, joint removal or replacement, removal of aggravating causes like bone or cartilage fragments from the joint (Katz *et al* 2010). Moreover, there is a need of alternative and effective treatment for OA.

Mesenchymal stem cells therapy has been recently added for the management of OA in dogs. It will stimulate regeneration of bones and cartilage that result in considerable improvement in quality of life. In contrast to drug therapies Adipose Derived Mesenchymal Stem Cells (ADMSCs) plays an important function by recruiting endogenous cell to the injured site (Vilar *et al* 2013).

Mesenchymal stem cell (MSCs) can be isolated from various sources such as bone marrow, adipose tissue, synovium, umbilical cord, blood, periosteum, peripheral blood and muscle (Dubey *et al* 2018). MSCs cells have the ability to differentiate into osteoblasts, chondroblasts, adipocytes, tendon, nerve cells and many other tissues. The application of multipotent MSCs in small animal practice are reported to be useful for many disease conditions including joint diseases and have shown to possess chondrogenic differentiation ability (Reich *et al* 2012). They regenerate cartilage both in vivo and in vitro (Dubey *et al* 2018, Markoski 2016).

The current researches have more focused on establishing adult stem cells as therapeutics in cartilage regeneration. The mesenchymal stem cell-based therapy offers various opportunities such as resurfacing whole joint surface, selection of particular mesenchymal stem cells, culturing of stem cells in environmental conditions to develop the desired phenotype, intra-articular stem cell injections and exogenous manipulation of stem cells to regenerate articular cartilage (Dubey *et al* 2018).

Autologous adipose derived mesenchymal stem cells (ADMSCs) are used in veterinary practice for treatment of tendon, ligament injuries and joint diseases in horses on a commercial basis (Smith *et al* 2008, Renzi *et al* 2012). Some studies also shows that intra-articular injection of adipose derived mesenchymal stem cells has excellent healing potential in hip osteoarthritis and improved limb function (Vilar *et al* 2013). Moreover, allogenic MSCs have been applied for many regenerative conditions in small animals and it does not have any immune reaction to the recipient (Black *et al* 2007, Udehiya *et al* 2013, Vilar *et al* 2013, Berglund *et al* 2017).

Only limited systematic studies have been undertaken on the treatment of osteoarthritis in canines by using adipose derived mesenchymal stem cell in dogs in India. There is a need to study application of adipose derived mesenchymal cells for osteoarthritis. So the present study was planned with the following objectives:

1. To study the incidence of osteoarthritis in different joints in dogs.
2. To evaluate the management of osteoarthritis using adipose derived stem cell in dogs.

CHAPTER II

REVIEW OF LITERATURE

Osteoarthritis (OA) is a common disorder in veterinary medicine. Among the domesticated animals, dogs are more affected with osteoarthritis. The detailed review of literature on various aspect of osteoarthritis in dogs has been discussed under the following objective of history, physical and clinical examination, synovial fluid examination, radiographic evaluation, medical treatment, surgical treatment, mesenchymal stem cell therapy, allogenic and autogenic mesenchymal stem cell therapy.

Local delivery of adult mesenchymal stem cells to the injured joints stimulates regeneration of meniscal tissue and retards the progressive destruction causing OA (Murphy *et al* 2003). In human, OA is a common condition limiting the quality of life of the patients, representing a socioeconomic burden (Bijlsma *et al* 2011).

At present stem cell therapy in veterinary patients are not rigorously supervised in any country (Yingling and Nobert 2008). In general the therapeutic role of stem cell in regenerative medicine is to cure or limit a diseases condition (Fortier and Travis, 2011). According to World Health Organization (WHO) Global Burden of Diseases Study (2010) hip and knee OA is the 11^{th} leading cause of disability.

2.1 Signalment

Mele (2007) observed that >50% of arthritis cases are observed in dogs aged between 8-13 years. The musculoskeletal diseases are more common in geriatric patients and nearly 20% of dogs show orthopedic diseases. About 45% of dogs with arthritis are large breed dogs. In puppies obesity and over feeding has been associated with onset of joint diseases particularly hip dysplasia which further lead to OA. Osteoarthritis (OA) is the main cause for chronic pain in dogs, affecting one in five adult dogs.

Anderson *et al* (2018) found males are frequently predisposed to osteoarthritis as compared to females. This could be due to sex hormone or activity differences as well as differences in bodyweight between males and females. Previous studies have reported that neutered dogs are more likely to develop joint diseases. It is suggested that this association is due to the reduction in gonadal hormones which act as

protectors against osteoarthritis or due to the positive association of neutering with weight gain, whereby higher bodyweight is linked with increasing osteoarthritis development.

2.2. Incidence

Arthritis is a commonly occurring chronic illness in human and animal alike. Among all domestic and pet animal species, dogs suffer more often because of excessive running or exercise, injury, and genetic predisposition. In dogs OA is most common than rheumatoid arthritis (Bland 2015). Anderson *et al* (2018) studied on prevalence, duration and risk factors of OA in dogs under primary veterinary care in the UK. A random subset was manually evaluated against a case definition of 455,557 study dogs, 16,437 candidate OA cases were identified; 6104 (37%) were manually checked and 4196 (69% of sample) were confirmed as cases.

2.3. Normal joint

A joint is formed when two bones are brought together and held in place by supporting tissues. Joints may have large ranges of movement, as in the shoulder and hip joints, or have very little movement, as in the joints between the bones in the skull. The primary weight bearing surface in a synovial joint is the articular cartilage. The tissue typically composed of water (approximately 75%), type 2 collagen (approximately 15%), proteoglycans (approximately 10%) and chondrocytes (only 2%) (Pool 2001). Articular cartilage is an avascular connective tissue that lines the surface of the diarthrodial joints, providing a nearly frictionless surface for transmitting mechanical loads over decades of use. Cartilage has little capacity for repair. An injury or diseases of joints are the primary complaint of orthopedic patients (Mow *et al* 1992). The synovial fluid in the synovial joint capsule provides nutrition, lubrication and a cushioning effect for articular cartilage (Pasquini 2007). The blood supply of synovial joint is derived from the arteries through anastomosis around the joint.

Deep to articular cartilage a layer of calcified cartilage which is not very vascular normally. Deep to the calcified cartilage is subcondral bone plate, which is corticalized, not very porous and may not be very vascular and may change its density by remolding and molding. Buttressing the subchondral plate from beneath is subchondral trabecular bone. It is clearly not homogeneous and the distinction

between the subchondral plate and subchondral trabecular bone are often not made clearly, but these two tissues are organized to adapt to mechanical over loads in different ways and have quite different mechanical properties (Martin *et al* 1998). The three different mineralized tissues in the joint- calcified cartilage, subcondral plate and subchondral trabecular bone are different not only mechanically, but also physically (Martin *et al* 1998).

2.4. Physical and clinical examination

A physical examination should be systematic and complete. It is useful to compare the contralateral limb when assessing the range of motion, and also when looking for soft tissue swelling and muscle atrophy (Anderson *et al* 2018). However, since the most common forms of orthopaedic disease (eg, cruciate disease, elbow dysplasia and hip dysplasia) frequently present bilaterally a complete analysis of the gait should be performed. This is done in two ways: The patient can be examined while in the consultation room during the history taking for lameness and this will allows the clinician to monitor the animal lying down and standing up, as well as when walking around the consultation room (Pettitt and German 2015).

Further analysis made by walking the animal away from and back towards the clinician, as pelvic limb lameness is often more obvious when walking away and thoracic limb lameness more obvious when walking back towards the clinician. Osteoarthritis is associated with a variety of clinical and physical signs. These signs include lameness, reluctance to exercise, inactivity stiffness, pain, joint effusion, muscle atrophy, joint thickening and altered range of motion, altered gait, and altered behavior (Bland 2015 and Anderson *et al* 2018).

Assessing thickening and effusions in the hip and shoulder are not possible due to the presence of the overlying muscle masses. Assessment of range of motion made by flexion and extension of affected joints. It is often better to perform these latter tests under sedation as they can be painful for the patient and difficult to interpret in conscious animals. After finishing a complete history and physical examination, the clinician will often have a good idea of potential differential diagnoses (Pettitt and German 2015).

2.5. Diseases Specific-Osteoarthritis

Osteoarthritis is defined as a disorder of joints considering a group of disturbance resulting in progressive destruction of articular hyaline cartilage (Schlueter and Orth 2004). Possible causes: Some dog breeds may have congenital or hereditary conditions that make them more prone to developing arthritis later in life. Trauma to cartilage caused by accidents can damage cartilage, resulting in arthritis later in life and adversely affecting mobility in your dog. Occasionally, infections can lead to the destruction of cartilage and joint tissue (Mele 2007).

In adult specimens, the articular cartilage is devoid of innervations and organization and lymphatic vessels, which result in reduced inflow of blood progenitor cells and a limited reparatory mechanism (Stashak 2006). Thus the repair tissue formed in extensive injuries and superficial defect is composed exclusively of fibrocartilage, which avoids the morphological and functional recovery of the tissue, resulting in progression of osteoarthritis (Coleman *et al* 2010).

Alterations in joint structure, decrease flexibility, severe pain ensues due to lack of hydration and inflammation. Cells within the damaged joints release pro-inflammatory cytokines, which further the inflammatory process. These causes more break down of cartilage collagen type II and proteyoglycans, which result in perpetual destructive cycle and ultimately result in cartilage distraction (Bland 2015).

Osteoarthritis consists of four stages of progression. In stage one minor small lumps of bone called osteophytes may grow lead to inflammation and pain. Stage two is mild in which erosion of bones due to cartilage lesions. Can cause new bone growth osteophyte / bone spur which affect normal joint movement and marked proteoglycan loss of articular cartilage is irreversible and result in joint degeneration. Stage three is moderate the cartilage between bone thins and loses cushion. Symptoms will be severe and inflammation began to occur. The fourth and final stage, in this stage the joint space is dramatically reduced, the cartilage is almost gone and joint mobility is reduced greatly (Renberg 2005).

Pathogenesis- Osteoarthritis results from failure of chondrocytes to maintain homeostasis between synthesis and degradation of extracellular matrix components. It is not known what initiates the imbalance between degeneration and the repair of cartilage. Trauma causing a micro fracture or inflammation causing a slight increase

in enzymatic activity may allow the formation of "wear" particles, which could be then engulfed by resident macrophages. The production of these particles overwhelms the ability of the system to eliminate them and they become mediators of inflammation, stimulating the chondrocyte to release degradative enzymes. Molecules for breakdown of collagen and proteoglycan, also taken up by synovial macrophages, cause release of proinflammatory cytokines like Tumour Necrosis Factor (TNFalpha), Interleukin-1(IL-1), Interleukin-6 (IL-6) (Man and Mologhianu, 2014).

2.4 Synovial fluid

Synovial joints made up of synovial fluid and dense irregular connective tissue, which creates a synovial joint capsule allowing the joint to freely move. The main focus will be on hip joint because these joints are most commonly affiliated with osteoarthritis, especially in canines (Pasquinin 2007).

In the early stages of the disease viscosity is not reduced although, as the hyaluronic acid concentration diminishes over time, the viscosity decreases. Hydroxyapatite crystals are common features in the synovial fluid of human patients with osteoarthritis. Check for hydroxyapatite counts from submitted samples have significance in diagnosing diseases condition in joints. Biomarkers have been investigated as a possible way of diagnosing osteoarthritis and although several potential markers have been identified, none are validated in the dog (Pettitt and German 2015.).

Synovial fluid inflammation plays an important role in the symptom and structural progression of OA.Cytokines and chemokines, are increased in synovial fluid (SF) in OA and promote synovitis (Loeser *et al* 2012). Supporting this, synovitis has been shown to correlate with symptom severity and rate of cartilage degeneration (Roemer *et al* 2011, Conaghan *et al* 2010). Osteoarthritis has multiple causes and risk factors; however, once the cartilage is lost, the joint fails (Eyre *et al* 2006).

Synovial fluid analysis is an under used diagnostic test in clinical practice. With osteoarthritis there are early changes that occur in the synovial fluid. In osteoarthritic joints the volume of fluid may be increased with an increase in cell counts, predominantly monocytes (>88 per cent), are usually low (less than 5×10^9 white blood cells (WBC) and can often be within normal limits ($<2 \times 10^9$ WBC), and the colour remains clear to pale yellow (Pettitt and German 2015).

2.5 Radiographic finding

Braun and Gold (2012) reported that OA has traditionally been diagnosed with radiographic imaging that demonstrate joint space width (JSW) and osteophytes. Despite the development of newer imaging techniques, the radiograph remains the most accessible tool in the evaluation of the OA joint. The knee joint is typically evaluated using the extended-knee radiograph, which is a bilateral anteroposterior image, acquired while the patient is weight-bearing, with both knees in full extension. More recently, flexed-knee radiographs with varying degrees of flexion and x-ray beam angles have been employed to improve intra-articular visualization.

However, radiography provides information on the osseous changes, such as sclerosis and osteophyte formation but provide a limited amount of information about the soft tissues. Radiographic signs of osteoarthritis are relatively non-specific and include: osteophytosis, enthesiophytosis, subchondral sclerosis, effusion, soft tissue swelling, intra-articular mineralization and cysts (Pettitt and German 2015).

Although the presence of osteophytes can be used to diagnose osteoarthritis, there is a tendency for clinicians to focus on these osseous changes even if they do not necessarily correlate well to severity of disease or the clinical situation seen. Elbow dysplasia, for example, produces osteophytes very slowly whereas within three or four weeks of a cranial cruciate ligament rupture there will be radiographic evidence of osteoarthritis. It has also been shown that some breeds produce more osteophytes than others despite having the same degree of hip laxity (Smith *et al* 2001). For this reason, it is important to always evaluate the radiographs in line with the clinical situation in order to appropriately manage the cases.

Subchondral sclerosis is commonly quoted as an indicator of osteoarthritis although its sensitivity on plain radiography is limited, due to the wide variation in exposure factors that can lead to apparent differences in density and detection of subchondral sclerosis was unacceptably low among board-certified radiologists (Innes *et al* 2004). Care should be taken to not over interpret the radiographic finding because, taken in isolation, it is not a reliable indicator of osteoarthritis.

2.6 Medical management

The management of osteoarthritis may involve a combination of medical and surgical approaches. Owners need to be counseled from the outset that osteoarthritis is likely to be a lifelong process with flare-ups that may increase in severity and frequency as the dog ages. The approach to these cases often involves a balancing act between exercise, medical management, weight management (if the dog is overweight) and surgery. Exercise although one study demonstrated that a short period of exercise (1.2 km of trotting) increased the degree of lameness (Beraud *et al* 2010).

In canine patients exercise should be moderated but continued. When advising owners on exercise levels for their dog it is important to advise them to try and avoid exuberant activities such as chasing balls, agility activities or other forms of high-energy exercise. When a flare-up of osteoarthritis has occurred for most patients the best course of action is to reduce the levels of exercise to short frequent lead walks which, if appropriate, are gradually increased over a period of weeks to previous levels of exercise. Medical management Drugs that are used to manage osteoarthritis can be divided into two main categories:

- symptom-modifying
- structure-modifying.

2.6.1 Symptom-modifying

The main drugs that can modify clinical signs are aimed at relieving the pain associated with osteoarthritis. The symptom-modifying drugs used to manage osteoarthritis aim to relieve the pain associated with the condition, and include- NSAIDs, oral opioids (eg, buprenorphine, tramadol), amantadine, and corticosteroi (Hermann *et al* 2018).

It is important for clinicians to abide by the Cascade system when prescribing medication for the management of osteoarthritis and not just go for the more ‘fashionable’ options. Most cases of osteoarthritis, especially in the early stages, can be managed with a balanced approach using licensed medication in combination with other treatment modalities, in particular, weight control. Non-steroidal anti-inflammatory drugs (NSAIDs) these form the backbone of the medical options for the

management of osteoarthritis in dogs (Johnston and Budsberg 1997). NSAIDs work by inhibiting the cyclo-oxygenase (COX) enzyme in order to reduce prostaglandin production. Two main forms of COX exist, namely COX 1 (constitutive) and COX 2 (inducible), although this classification is somewhat simplified and crossover between the functions of the classes exist. A third variety, COX 3, has been identified and may be the target for paracetamol (acetaminophen). COX 3 is found in greatest abundance in the canine brain and paracetamol has a greater ability to cross the blood brain barrier better than other NSAIDs so offers an alternative pathway for pain management. Note, paracetamol is not considered to be a NSAID as it has only weak anti-inflammatory activity (Pettitt and German 2015).

NSAIDs have been developed to be more selective for COX 2 in preference to COX 1 in order to allow analgesia to occur without creating the common side effects (in particular gastrointestinal irritation and ulceration, nausea, diarrhoea and renal papillary necrosis) associated with inhibiting COX 1. A drug that selectively induces COX 2 inhibition at a concentration lower than that needed to inhibit COX 1 is potentially safer, although this is an oversimplification of the actual situation. There is not a clear distinction between the pathways of constitutive and inducible prostaglandins and, in some cases, COX 2 provides a protective effect (eg in inflamed gastrointestinal mucosa). However, drugs (eg, meloxicam and carprofen) that provide analgesia without significant inhibition of COX 1 pathways (so-called COX 2 selective) have been shown to have greater safety profiles (Pettitt and German 2015, Belshaw *et al* 2016).

Tramadol is anecdotally reported to be a popular therapy given by veterinary surgeons although there is little evidence of its efficacy and the drug is not licensed for use in dogs or cats in the UK. It has recently been reclassified as a Schedule 3 controlled drug. Tramadol is metabolised into eight substrates of which only one is active in the dog, and that is only for a short (one to two hours) period. If tramadol is used, careful monitoring of the patient is needed to ensure adequate levels of analgesia are provided (Pettitt and German 2015. Budsberg *et al* 2018).

2.6.2 Structure-modifying drugs

Structure-modifying drugs are less available for dogs due to the strict criteria needed to validate such drugs. The main example includes pentosan polysulfate

(Cartrophen; Biopharm Australia), which is a licensed product made from beech and similar in structure to heparin. Clinical trials of this drug have shown mixed results and two systemic reviews have concluded that only a moderate level of comfort is achieved when using this product for canine osteoarthritis (Aragon *et al* 2007, Sanderson *et al* 2012).

2.7 Surgical treatment

The aim of OA treatment is to alleviate pain and to improve function. The combination of non- pharmacologic with pharmacologic treatment will give the best result. Obesity is one of the most important risk factors for having OA progression, losing weight should be advised by diet or by exercise. The first line of pharmacologic OA treatment is acetaminophen to treat mild and intermediate symptoms. If symptoms are getting worse, non- steroidal anti-inflammatory drugs can be used. In case of refractory pain after non-pharmacologic and pharmacologic treatment, Total Joint Replacement (TJR) can be considered (Yusuf 2016). Numerous clinical practice guidelines specific non-pharmacological strategies as the core management approach for OA (Murphy *et al* 2016).

Intra-articular administration of corticosteroids may be considered especially for flare of knee pain, accompanied by effusion. Failing conservative pharmacological options, surgical treatment may be considered, while alternative or complementary therapies e.g, acupuncture, have also been associated with benefits in OA. Among the different treatment modalities available for the management for OA, viscosupplmentaion with hyaluronic acid (HA) is widely accepted in clinical practice (Balazs and Denlinger 1993).

2.8 Mesenchymal Stem Cells

Stem cells are non differentiated cells that have the ability of prolification, regeneration, conversion to differentiated cells and tissue production. Regeneration means that, cells have the ability of asymmetric division which one of the resulting cells remains as stem cell while other cell, which is called daughter cells, becomes one cell of germ layer. Stem cells may remain inactive for a long time till they enter cell division again. Mesenchymal stem cells are found in many adult tissues and represent an alternative stem cell category for regenerative medicine and cell therapy purpose (Mimeault and Batra 2008). MSC possess a robust self-renewal potential,

show a constant growth and a known proliferation kinetic; furthermore, MSC are able to differentiate *in vitro* and forms numerous specialized cell types (Zuk *et al* 2002). An adult mesenchymal progenitor cell population was identified in human adipose tissue and lipoaspirate (Zuk *et al* 2002, Lee *et al* 2004).

Mesenchymal stem cells (MSCs) are considered as promising option for the treatment of OA. The increased incidence of OA and an ageing population, coupled with insufficient therapeutic choice, has led to focus on the potential stem cells as a novel strategy for the cartilage repair. MSCs are multipotent progenitor cells with capability of self-renewal, high plasticity, immunosuppressive and anti-inflammatory action and the possibility to differentiate into selected lineages including chondrocytes (Manferdini *et al* 2013). One of the fields for MSCs use is regenerative medicine in the treatment of bone defects. In various animal models, MSCs have been used to repair segmental bone defects of critical size (Kon *et al* 2000, Petite *et al* 2000). The cell therapy approach targeted to osteoblast formation from MSC was first investigated on murine model. In addition there are also observations indicating the usefulness of MSC transplantation in myocardium regeneration after myocardial infarction (Toma *et al* 2002, Wang *et al* 2000, Pittenger and Martin 2004, Mathur and Martin 2004).

Currently, several experimental strategies have investigated whether mesenchymal stem cells (MSCs) can be used for regeneration and maintenance of articular cartilage in OA (Vilar *et al* 2013). Black *et al* (2007) studied effect of adipose derived mesenchymal and regenerative cells on lameness in dogs with chronic osteoarthritis of coxofemoral joints. Significant improvement in lameness scores parameters mainly lameness, pain and range of motion compared with control dogs. MSCs have been used successfully in studies for the repair of intervertebral disc regeneration in rat models (Skovrlj *et al* 2004).

Otto and Wright (2011) examined the ability of MSCs to modulate liver, kidney and intestine repair and to update their opposing qualities of being less immunogenic. However, such observations have not been replicated in the clinic. Recent studies showing the clinical safety of MSC in several pathologies were discussed. The possible opposing power of MSC need careful understanding and control if their clinical potential is to be realized with long-term safety for patients.

Kirby *et al* (2015) suggested that cell therapies can help to heal cutaneous wounds when used appropriately but there is risk in clinical use such as the evidence for standardized method of cell characterization and delivery, as well as randomized clinical trial. Wounds are a growing clinical burden on healthcare system and with an ageing population as well as increasing incidence of obesity and diabetes, this problem is set to increase. Cell therapies may be the solution. A range of cell based approach has begun to cross the rift from bench to bedside and the supporting data suggests that the appropriate administration of stem cells can accelerate wound healing.

In heamatopoietic disorders the severity of graft versus host diseases (GVHD) can be minimized with the transplantation of MSCs as demonstrated in preclinical studies as reported by Yanez *et al* (2006). Clinical trials done in human patients using MSC for treatment of severe, steroid resistant GVHD have shown very promising results by Le Blanc *et aI* (2004) and Ringden *et al* (2006). Application of MSCs in patients suffering from leukemia resulted in hematopoietic stem cell (HSC) engraftment, rapid platelet recovery and low incidence of GVHD by Lazarus *et al* (2005) and Lee *et al* (2002).

2.8.1 Autologenic mesenchymal stem cells therapy.

Spinal cord injury (SCI) can cause irreversible damage to neural tissues. However, there is currently no effective treatment for SCI. The therapeutic potential of adipose-derived mesenchymal stem cells (ADMSCs) has been emerged with effects and safety of the intrathecal transplantation of autologous ADMSCs in patients with SCI (Hur *et al* 2016). Autologus MSCs derived from adipose tissue coated in hydroxyapatite scaffold was found successful in treating the SCI (Michel *et al* 2015).

Studies were made to evaluate the effect of autologous adipose-derived mesenchymal stem cell therapy in the treatment of acromio-clavicular joint having osteoarthritis. Case report is to evaluate the efficacy of mesenchymal stem cell (MSC) therapy in the treatment of small joint osteoarthritis (OA). Acromio-clavicular (AC) joint OA is an under-diagnosed and yet frequent source of shoulder pain. MSCs have shown evidence of benefit in the treatment of knee OA. This is the first report to describe the use of MSC therapy in OA of the upper limb (Freitag *et al* 2019). Brittberg and his colleagues first injected chondrocyte suspension (autologous

chondrocyte transplantation,) to cure human joint cartilage defects in 1994. MSCs have been used to repair joint and cartilage defects in various animal models using various carrier devices (Wakitani *et al* 1994, Caplan *et al* 1997). Chronic inflammatory diseases are common in cats and autologous mesenchymal stem cells (MSC) are injected intraperitoneal in cats a promising therapeutic approach for management of inflammatory diseases. Serious adverse effects were not observed in any MSC injected cats. Intraperitoneal MSC injection was well tolerated with only mild, self- limiting adverse effects being observed in two cats (Parys *et al* 2016).

2.8.2 Allogenic mesenchymal stem cell therapy.

Kriston-Pal *et al* (2017) study was to evaluate the long-term effects of administering MSCs dispersed in an HA solution on regeneration of osteoarthritic elbow joints in dogs. Allogeneic adipose tissue derive mesechymal stem cells (AT-MSCs) were isolated from surgical waste obtained from the spaying of female mixed-breed dogs and were characterized by marker analysis and differentiation. The cells were suspended in HA and injected into joints of dogs suffering from elbow dysplasia with OA. The treatment efficacy was assessed using questionnaires completed by the dog owners based on changes in the dogs' lameness. Transplantation of MSCs resulted in highly significant long-term improvement (over a 1-year period) in the condition of the involved animals. Allogenic transplantation of canine MSCs was proved useful in a critical sized segmental defect to regenerate new bone by Arinzeh *et al* (2003).

Teshima *et al* (2017) study was to investigate the effects of allogenic AT-MSCs on acute liver injury by carbon tetrachloride in dogs and to compare the therapeutic effects of AT-MSCs transplanted via the peripheral vein (PV) or splenic vein (SV). After transplantation of AT-MSCs through the PV or SV, serum liver enzymes were decreased significantly and SV injection was more effective compared with PV injection. By comparing the number of engrafted AT-MSCs in the liver, SV injection was significantly more effective than PV injection in acute hepatic injury in dogs.The hypothesis that allogenic adipose-derived mesenchymal stem cells (ADMSCs) alleviated brain death (BD)-induced remote organ damage and events of post heart-transplant acute rejection. In conclusion, ADMSCs suppressed BD-caused remote organ damage and heart-transplant rejection (Yip *et al* 2017).

2.8.3 Adipose derived mesenchymal stem cells and Bone marrow derived mesenchymal stem cells

Adipose tissue is a rich and very convenient source of cell for regenerative medicine therapeutic approach. However, a characterization of the population of adipose-derived stromal stem cells (ASCs) with the greatest therapeutic potential remains unclear (Bourin *et al* 2013).

Black *et al* (2007) studied case series on dogs with OA. Concentrated ADSCs were made from adipose tissue obtained-abdominal area, inguinal, or thoracic wall regions and 1 dose of injection given follow up taken up to 180 days 40% improvement in lameness, range of motion, and pain on manipulation over time compared with baseline values. Abdulazeez (2015) discussed the resource and progress made in the field of stem cell research for diabetic treatment.

Numerous studies (Rebelatto *et al* 2008) have demonstrated that human mesenchymal stem cells derived from adipose tissue (A-MSC) express classic MSC markers as CD90, CD44, CD73, CD105, CD117 and CD106 they possess the same capacity of bone marrow-derived stem cells (BMSC) to differentiate into osteogenic, adipogenic and chondrogenic lineages. Moreover, less than 1% of genes are differentially expressed between A-MSC and BMSC; this data support the notion that A-MSC and BMSC are originated from a common precursor (Bianco *et al* 2008).

Koh *et al* (2013) studies 18 patients with knee OA. The study was to evaluate the clinical and imaging results of patients who received intra-articular injections of autologous mesenchymal stem cells for the treatment of knee osteoarthritis. The results were encouraging and show that intra-articular injection of infrapatellar fat pad-derived mesenchymal stem cells is effective for reducing pain and improving knee function in patients being treated for knee osteoarthritis. Direct regeneration of joint surface was considered as basic features of MSCs but recent and increasing body of research is beginning to suggest that the primary benefit of these cells is derived from their pancreatic activity (Barry and Murphy, 2013). MSCs express alkaline phosphates enzyme activity, phenotypes markers (CD105 and CD166), collagen type I and collagen type II genes and can differentiate into chondrogenic, osteogenic and adipogenic lineage in vitro (Gugjoo *et al* 2015)

Tharasanit *et al* (2011) isolated, identified, characterized and examined the differentiation capability of canine MSCs. Two cell lines were examined for the expression of CD34, CD44 and CD90, using flow cytometry. The result showed that these fibroblast like MSCs expressed MSCs markers (CD44 and CD90), while fewer than 5% of these MSCs were tested positive to hemopoietic stem cell marker (CD34).

There are few criteria defined by International Society for Cellular Therapy for characteristics of mesenchymal stem cells (MSCs) (Dominici *et al* 2016):

- MSCs must be plastic- adherent when maintained in standard culture condition.
- MSC population must express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b,CD79 alpha or CD19 and Human Leukocyte Antigen class II (only 2% positive or less) surface molecules.
- MSC must differentiate into osteoblasts, chondroblasts and adipocytes, when they are cultured under standard differentiating conditions.

Punwar and Khan (2001) studied the current treatment strategies for articular cartilage, described use of mesenchymal stem cells for articular cartilage repair along with the result of clinical studies and described the future direction that these strategies are likely to take. Current treatment options including the use of procedures that stimulates repair through the stimulation of subchondral bone marrow and result in the formation of fibro-cartilage.

Guercio *et al* (2012) comparative study was done on dogs with OA and their possible application in the cellular therapy. To assess whether the cellular therapy can replace drug therapy, the clinical effect of single intra-articular injection(i) ADSCs + PRP and(ii) ADSCs + HA. Adipose derived cells taken and expanded, from abdominal area, subcutaneous, visceral, and inguinal fat. Follow up was taken up to 1 month. Both groups showed functional improvements in their disability, lameness on trotting, and pain on manipulation of the joints. Wakitani and his colleagues injected BM- MSCs in twelve patients inside the knee, on the medial femoral condyle in 1994. They observed that the patients treated with MSCs showed better arthroscopic and histological grading scores as compared to control patients without receiving MSCs. These clinical observations clearly prove the acceptability, safety and efficacy of

MSC therapy for repair and regeneration of cartilage by reducing inflammatory reactions within the joint.

Das *et al* (2012) conducted study to reveal the efficacy of bone marrow derived mesenchymal stem cells (BM-MSCs) based therapy in healing of chronic non- healing and ulcerative wound in bovine species. Results showed that the evaluated value on zero day and after healing (18 days). Showed significant difference in relation to collagen content, tensile strength and physical characteristic of wound like extent of wound, size of wound, type exudates and photography. But clinical, heamatological and biochemical data shows no significant difference. They concluded that BM-MSCs were the main pioneers to bring the chronic ulcerative wound towards healing. The procedure is simple, safe and effective in bringing out healing without showing any adverse effect on host.

CHAPTER III

MATERIALS AND METHODS

The study was conducted on 25 clinical cases of dogs diagnosed with osteoarthritis (OA) which were presented to the Department of Veterinary Surgery and Radiology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, during the period from April 2018- March 2019. Only those dogs were selected which were diagnosed with OA. The study was conducted in two phases.

Phase 1: Collection, isolation, culture, subculturing, characterization of adipose derived mesenchymal stem cells was done under this section.

3.1 Chemicals for mesenchymal stem cell culture

All the chemical and media used for culture of adipose derived mesenchymal stem cells (ADMSCs) were procured from the Sigma Chemicals Co, St. Louis Missour, US which were cell culture grade and endotoxin tested. The buffers and reagents were prepared in autoclaved Milli-Q water and filtered through membrane filters (0.22 µm) purchased from Tarsons Products Pvt.Ltd. (Kolkata, India). The fetal bovine serum (FBS)(Cat. No.092916754) was purchased from MP Biomedicals (US) and this was aliquoted in 15ml centrifuge tube and store at -20°C until use.

3.2 Antibiotics for characterization of ADMSCs in dog

Primary antibody CD44 Monoclonal, (Cat. No.MA1-19277) and secondary antibody (Goat anti-rat IgG, Cat. No. A-21247) used for characterization of ADMSCs were procured from Thermo Fisher Scientific (Invitrogen).

3.3 Plastic ware and glassware

All the plastic wares used for the culture viz., culture dishes, multiwall plates, centrifuge tube and culture flask etc., were purchased from Corning. All the glasswares used for culture were washed, packed and autoclaved at 121°C and 15psi for 15min. All plastic wares and glasswares were UV treated in laminar air flow for 10 min before use.

3.4 Growth medium and reagents

3.4.1 The growth medium 50ml for canine mesenchymal stem cell culture was prepared as per given composition.

Stock medium (DMEM-HG)	42.45ml
Fetal Bovine Serum (5%)	7.5ml
Gentamicin (50µg/ml)	0.05ml

The pH of media was adjusted to 7.4. The growth media was stored at 4°C after checking the sterility by incubating 2ml of growth medium at 37°C for 48 hours in CO_2 incubator.

3.4.2 Phosphate buffer saline (PBS)

Sodium Chloride (NaCl)	8.0g
Pottasium Chloride (KCl)	0.20g
Disodiumhydrogen orthophosphate (Na_2HPO_4)	1.15g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.02g

Above components were mixed in small quantity of Milli-Q water, pH was adjusted to 7.4 with the help of NaOH and HCl. Final volume was made upto 1000ml and then sterilized by autoclaving.

3.4.3 RBC lysis buffer

RBC lysis buffer(100 ml) was prepared as below

Ammonium chloride (NH_4Cl)	0.826gm
Sodium bicarbonate ($NaHCO_3$)	0.1gm
EDTA	0.004gm

Above components were mixed in small quantity of triple distilled water, pH was adjusted to 7.3 with the help of NaOH and HCl. Finally the volume was made upto 100 ml and then sterilized by autoclaving and stored at 4° C till use.

3.4.4 Alkaline phosphatase staining

Tris HCl	25mM
Sodium chloride (NaCl)	150mM
Magnesium chloride ($MgCl_2$)	4Mm
Sodium Naphthyl Phosphate	0.4 mg/ ml
Fat red	1mg/ml

3.5 Collection and transportation of adipose tissue

The subcutaneous adipose tissue, during abdominal surgeries were collected (approximately 5-10gm) from dogs which were brought for elective surgeries (Fig. 1). This adipose tissue was completely devoid of any contamination and pathological lesions. The adipose tissue was taken aseptically in a tube containing DPBS and Gentamicin to laboratory within half an hour after collection at ambient temperature for further processing (Fig. 2).

3.6 Isolation, culturing and subculturing

Subcutaneous adipose tissue was washed properly 3-4 times with Dulbecco Phosphate Buffer Saline (DPBS) in laminar air flow and followed by digestion with collagenase type 1 (Cat No.17100-017, from GIBCO). This adipose tissue was placed onto a magnetic stirrer at 37 °C for half an hour. Digested sample was filtered using cell strainers and centrifuged at 2200 rpm for 15 minutes at room temperature. Stromal vascular fraction (SVF) was then suspended (Fig. 3) in RBC lysis buffer for 10 minutes. Cells were cleared of cell lysis buffer by washing with DPBS thrice followed by resuspension in stock Dulbecco modified Eagler's medium (DMEM) and washed at 800 rpm for 10 minutes. Finally the SVF was resuspended in culture medium that consist of high- glucose DMEM (Cat. No. D6429) with L-glutamine (2 mM) 15% of FBS and gentamycin solution (10µg/ml, Cat. No.G1272). ADMSCs were cultured in 25 cm^2 flask at 37°C in humidified atmosphere with 5% CO_2 in CO_2 incubator (Fig. 4).

The culturing flask kept undisturbed for 48-72 hours in an incubator at 37°C and was observed under phase contrast microscope (Nikon Eclipse Ti, Japan) to check contamination and attachment of cADMSCs. The growth medium was replaced at every 2-3 days with new growth medium and regularly observed the growth of ADMSCs. The subculturing was done at 70- 80% confluence by trypsinization i.e. cells were detached by enzymatic digestion in 0.25% (w/v) trypsin –EDTA solution (Cat. No. T4049). In brief, the growth media was discarded and the confluent cells were washed twice with PBS. Thereafter, 0.5ml of trypsin –EDTA solution was added to cover the surface of cells and flask was kept at 37°C for 1 minute for the cell detachment. Growth medium containing FBS was added to the flask for inhibiting the trypsin activity after rounding of cells as observed under phase contrast inverted

microscope. Pipetting was done to detach the cells from the surface of tissue culture flask and to make a uniform suspension. Trypsinized cells were reseeded at cell density of $2x10^6$ in new 75 cm² flask. Characterization of stem cells and population double time (PDT) was done after third passage.

3.7 Population Doubling Time (PDT)

Population doubling time was done in 4^{th} passaged cells. Approx. $3x10^5$ - cells/ml/ well were plated in 6 well plates. The time was noted during plating of ADMSCs. The cells were then harvested with trypsin-EDTA after 72 hours of plating and total number of cells in each culture well was counted using a haemocytometer at noted time. Population doubling time was calculated using the equation:

PDT= Culture time (CT)/ Cell doubling (CD)

Where

CD= log (NH/NI)/ log2

NH (harvested cell number).

NI (initial cell number).

3.8 Characterization of ADMSCs

3.8.1 Alkaline phosphatase staining

Third passaged cells were subjected to alkaline phosphatase (AP) staining for characterization as per procedure given below:

1. Medium was removed from the culture and the cells were washed properly in PBS for three times.
2. Cells were fixed in 4% paraformaldehyde prepared in PBS for 3 minutes.
3. After fixation, the cells were washed properly with PBS.
4. MSCs were incubated with Alkaline Phosphatase staining for 2 hour at 37°C.
5. AP positive stained cells were observed in bright field as well as fluorescence under inverted microscope.

3.8.2 Immunohistochemistry

Monoclonal antibody CD44 were used to characterize the ADMSCs by immunohistochemistry 60-70% confluent cells was subjected to immunohisto-chemistry as described below:

Fig. 1: Photograph showing site for collection of adipose tissue.

Fig. 2: photograph showing collected adipose tissue in a sterile centrifuge tube containing DPBS and gentamycin.

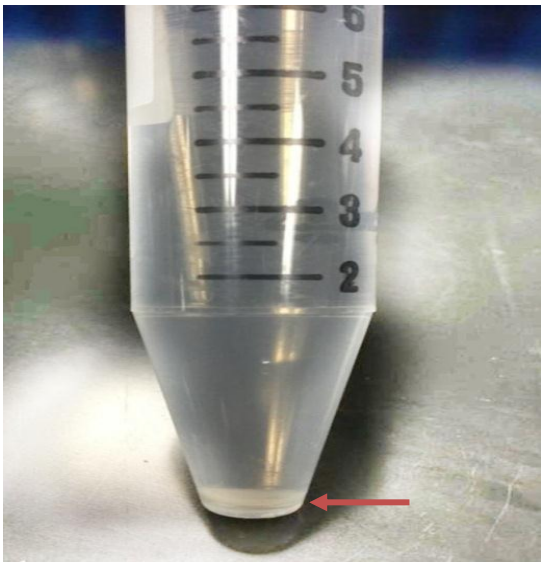


Fig. 3: Photograph showing SVF at bottom of centrifuge tube (arrow).

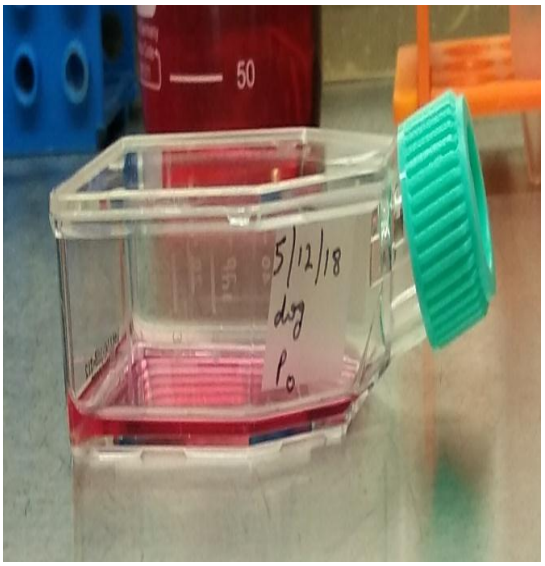


Fig. 4: Photograph showing culture of canine ADMSCs in tissue culture flask on day zero.

1. After removing the medium, the cells were washed thoroughly for three times in PBS.
2. Cells were fixed in 4% formalin solution prepared in PBS for 10min at 37°C and rinsed properly in PBS for three times.
3. After fixation cells were covered with antigen retrieval solution 0.1M sodium citrate for 30 minutes at 37°C
4. Non-specific binding were blocked by incubating the cells with goat serum (1:10 dilution in PBS) for 30 min at 37°C.
5. Cells were then properly rinsed in PBS for three times.
6. MSCs were then incubated with primary antibody (1:100 dilution in PBS) for 1 hr at 37°C and washed properly with PBS for three times. Followed by incubation with secondary antibody (1:200 dilutions in PBS) conjugated with Flurescein Isothiocyanate (FITC) for 1hr at 37°C.
7. Washing was done properly with PBS to remove unbind secondary antibodies.
8. Cells were covered with Hoechst staining (1µg/ ml) for nuclear stain and kept in incubator for 5 minutes. After incubation cells were washed and covered with DPBS.
9. The stained ADMSCs were observed under fluorescent microscope with filters of respective wave length.

3.9 Viability test

Trypan blue exclusion viability test was used to measure the proportion of viable cells. Viable cells exclude the dye, whereas dead cells take the dye. After staining with Trypan blue, the cells should be counted immediately since viable cells also take up the dye after a few minutes of exposure of dye. To perform the test, 10µl of the cell suspension was transferred to an eppendorf tube and an equal volume of 0.1% Trypan blue (1:1 ratio, dilution factor 2) was added to the cells suspension and mix well. After about five minutes, hemocytometer chamber was charged with a small volume of cell suspension to count the unstained cells in the four corners square (WBC square) using a microscope. The number of viable cells per ml was given by the formula:

$$\text{Number of viable cells per ml} = \frac{\text{Total number of cells}}{4} \times \text{Dilution factor} \times 10^4$$

3.10 Cell senescence detection

Adipose stem cells used upto 15th passage for the treatment of OA for the present study and at every passage cells were detected for senescence using cell senescence detection kit (Cat. No. CCK063 from Himedia) as per direction of manufacture.

3.10.1 Preparation

3.10.1.1 Fixing solution

1. Diluted the 10X fixing solution CCK063 (E) with sterile cell culture grade water in 1:9 ratio to obtain 1X fixing solution.
2. Stored 1X fixing solution at -20°C after use

3.10.1.2 1X Dulbecco's Phosphate Buffered Saline (DPBS)

1. Diluted Dulbecco's Phosphate Buffered Saline CCK06 3(F) with sterile cell culture grade water in 9:1 ratio to obtain 1X Dulbecco's Phosphate Buffered Saline.
2. Stored at room temperature after use.

3.10.1.3 X-gas solution

1. Thawed X-gas solution CCK063(D) at room temperature
2. Warmed the thawed solution at 37°C water bath for 1 hour. Warming of X-gas solution avoids formation of aggregates that could interfere with the visualization of stained cells.

3.10.1.4 Staining mixture

1. Thawed staining solution A 10X CCK063 (A) at room temperature
2. Prepared staining mixture in polypropylene tube as given below

Component	**Volume For 2 ml**
Staining solution A 10X CCK063(A)	200µl
Staining solution B 10X CCK063(B)	25µl
Staining solution C 10X CCK063(C)	25µl
X-gal solution	50µl

10X CCK063(D)

Cell culture grade water 1700µl

3. Filtered the staining mixture through 0.22µ syringe to ensure that the solution is free of any aggregates.

3.10.2 Staining procedure

1. Aspirated the growth media form cells.
2. Added 1ml of 1X DPBS to each of the dish and swirl gently to remove trace of medium.
3. Discarded DPBS by aspiration and repeated the washing step once again.
4. Added 1ml of 1X fixing solution to the dish and incubated the plate at room temperature for 8-10 minutes. Staining mixture was prepared freshly during fixation time.
5. Discarded fixing solution after incubation by aspiration.
6. Added 1ml of 1X DPBS and swirl gently to removed trace of fixing solution.
7. Repeated washing twice.
8. Added 1ml of staining mixture to the dish and incubated the plate at 37°C without CO_2 for overnight (as SA-beta-gal activity is pH dependent).
9. Observed the cells under microscope after incubation time.
10. Cells stained blue showed scenescence / aging.
11. Healthy cells were remained unstained and appeared red after counter staining with Neutral Red Dye (0.33 gm%) for 5 min and washed with DPBS.

Phase II: In this section, the study was conducted on clinical cases and the following observations were recorded.

3.11 Signalment

When the animals were presented to the clinic detailed signalment including age, sex, breed and weight were recorded in all animals.

3.12 History

History of the littermates (pedigree), genetic history if available, type of feed given, duration of lameness, response to any treatment, any history of trauma or other

inciting cause, behavioral change, response to amount of exercise performed, previous blood work and surgical history was recorded.

3.13 Physical examination

Thorough physical examination was done in all the animals.

1. Range of motion with associated pain was recorded.
2. Palpation of the affected part was done and the nature of gait abnormality was determined in all animals
3. Dog was observed from distance while sitting, standing and walking and recorded as vedioes.

Each limb was palpated while the dog was being placed in lateral recumbence from toe to hip or shoulder joint for evidence of any pain or any abnormality.

3.14 Special examination for hip and stifle joint

The ortolani maneuver for the pelvic joint was recorded. The hip was positioned in a neutral position with the femur parallel to the table and firm pressure was applied along the long axis of the femur, which causes subluxation of the femoral head when there was increased hip joint laxity. The cranial drawer test was performed to diagnose concurrent cranial cruciate ligament rupture (CCLR). The index finger of one hand was placed on the proximal patellar region, while the thumb of the same hand was placed caudal to the lateral febella. The index finger of the opposite hand was placed on the cranial aspect of tibial crest, and the thumb placed on the caudal aspect of fibular crust. With the femur stabilized, the tibia was pushed cranially and then pulled caudally. This was repeated several times and was performed gently but quickly detect if there was any moment (1-10mm) of the tibia in relation to femur.

3.15 Lameness Score

Clinical and physical signs including lameness, pain and weight bearing during standing were recorded as under. Evaluation of lameness during walk was done for animals in both the groups on basis of lameness score system given by Black et al., (2007) with minor modification as follow.

1. **Pain on palpation (PP)**

   Pain on palpation was recorded on a scale of 1-3 as;

   1- No pain on palpation.

2-Mild pain slightly withdrawal of limb.

3- Sever pain immediate limb withdrawal.

2. **Weight bearing during standing (WBS)**

Weight bearing during was recorded on a scale of 1-3 as;-

1-Equal weight bearing on all forelimbs.

2-Partial weight bearing while standing.

3-Non-weight bearing while standing

3. **Lameness during walk (LW)**

Lameness during walk was recorded on a scale of 1-4 as;-

1-Not detectable; walk normally.

2- Intermittent slightly lame when walking.

3- Persistent severely lame when walking.

4- Persistent non-weight bearing

On follow up after intra-articular injection with ADMSCs therapy and conventional medical treatment the animals were evaluated at interval of 15^{tt}, 30^{th} and 60^{th} days.

Owner's feedback was taken regarding post treatment assessment of quality of life of patient. In terms of;

- Poor- No improvement in pain and day to day activity.
- Satisfactory - Mild improvement in pain and day to day activity.
- Good –Appreciable improvement in pain and day to day activity.
- Very good- Marked improvement in pain and day to day activity.
- Excellent – No pain seen and animal able to perform normal activities.

3.16 Radiographic evaluation

3.16.1 Radiographic Positioning

For pelvic joint the dogs were positioned according to the directions suggested by American Veterinary Medical Association (AVMA) Panel. The dogs were placed in dorsal recumbency and both the hind limbs were pulled caudally until the stifle and hock were fully extended. The limbs were then adducted until the femurs were

parallel to each other. Both femurs were rotated medially until the patella was centered dorsally. The dogs were symmetrically positioned to avoid any rotation of the pelvis.

For stifle joint medio-lateral view was used to assess the position of patella, presence of joint effusion (cranial displacement of infra-patellar fat pad and increased radio-opacity of the joint space) and any evidence of OA. The anterioposterior (AP) radiograph of stifle joint was allowed an adequate evaluation of medial and lateral side to evaluate the osteoarthritis change. The radiographic changes were evaluated according to primary osteoarthritis include non-uniform joint space loss, cyst formation, osteophyte formation and subchondral sclerosis. As disease progress subluxation may occur.

3.16.2 Imaging Technique

Repeated radiography was done before and after treatment to assess the efficacy of the treatment adopted. Suitable radiograph views were used to take radiograph for evaluation of the affected joint. The radiography was evaluated on 15^{th}, 30^{th} and 60^{th} day's intervals after the treatment. The radiographic criteria of joint OA severities used in this study were according Takahashi Scoring system (1998)-

Grade 0 (normal) - Not affected

Grade I (mild) - Doubtful narrowing of joint space and possible osteophytic lipping (Fig 5).

Grade II (moderate) - Definite osteophytes and possible narrowing of joint space (Fig 6).

Grade III (severe) - Moderate multiple osteophytes, definite narrowing of joint space, some sclerosis and possible deformity of bone contour (Fig 7).

Grade IV (very severe) - Large osteophytes, marked narrowing of joint space sclerosis and definite deformity of bone contour.

3.17 Grouping

Phase II was subdivided to group A control group and group B treatment group. Group A (n=16) normal conventional medical treatment and group B (n=9) treated with allogenic canine ADMSCs

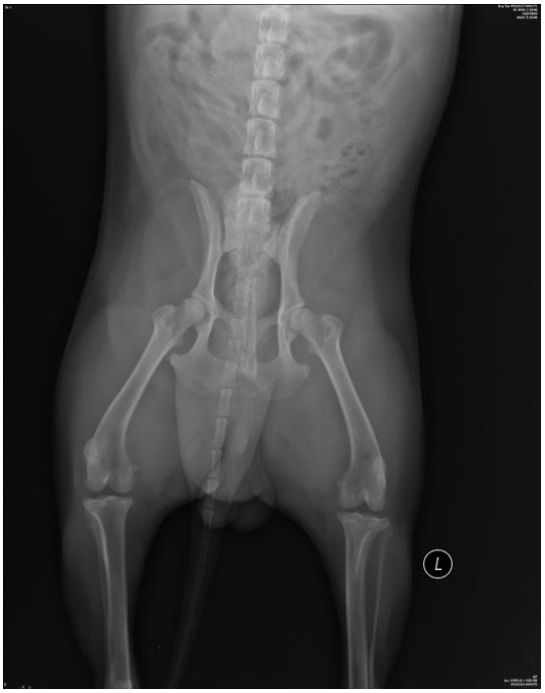


Fig.5: Radiograph showing bilateral mild periosteal reaction with subluxation in ventro-dorsal view of pelvis.

Fig.6: Radiograph showing moderate periosteal reaction on left hip joint with in ventro-dorsal view of pelvis.

Fig.7: Radiograph showing bilateral severe periosteal reaction with subluxation in ventro-dorsal view of pelvis.

3.18 Hematological parameter

The blood sample of (2ml) were collected aseptically from cephalic/ saphenous vein in vials containing Ethylenediaminetetraacetic acid (EDTA) @ 2mg/ml the samples of blood were collected on 0 day, 15^{th}, 30^{th} and 60^{th} day were subjected to following estimation:

1. **Haemoglobin (Hb g%):** Hemoglobin was determined by acid haematin method using Sahil's haemocytometer.
2. **Total leukocyte count (TLC per µL):** Total leukocyte count was evaluated using Neubar's counting chamber method Jain (1986).
3. **Diffrential leukocytes count (DLC%):** leukocyte count was determined as per method described by Jain (1986).

3.19 Serum Biochemical Examination

The blood sample (5ml) in each animal was collected aseptically from cephalic/ saphenous vein in a vial without coagulant and allow to clot, after clotting these were centrifuged to separate serum which was stored at-20°C for further use for the estimation of calcium (g/dL), phosphorus (g/dL) and alkaline phosphatase (U/L).

3.20 Pre-treatment management

Treatment with allogenic canine ADMSCs was done in 9 dogs on hip joint, elbow joint and stifle joint. It was scheduled for the earliest possible date in all cases. Owner was advised to withhold food and water for 12 hours prior to surgery. On the day of surgery an IV cannula was placed in cephalic vein and 0.9% of saline solution was administered at a rate of 10ml/kg/hour throughout the treatment until the dog were extubated. Pre-anesthetic medication of butorphenol @ 0.2mg/ kg body weight (Butodol-2; Neon Laboratories Ltd., India), acepromazine (Ilium- Acepril-10; Troy Laboratories LTD., Australia) @ 0.05mg/ kg body weight administered intramuscularly. Hair clipping of the joints were done with a wide area around the site. Induction of anesthesia was done with propofol @ 4mg/ kg (1%) (Neorof; Neon Laboratories Ltd., India) given intravenously. Maintenance of anesthesia was done with 1-2% isoflurane (Isoflurane USP; Raman and Weil Pvt. Ltd., India) the surgical area was scrubbed using savlon and then painted with povidone iodine solution before start of surgery.

3.21 Synovial fluid examination

Synovial fluid was aspirated aseptically under C-arm guided from the affected joints. The samples were collected only from those joints in which the allogenic canine ADMSCs were injected. The samples were taken before the treatment and 15 days after the treatment and was evaluated for inflammatory cells.

3.22 Medical Management

Group A: Medical management was done in 16 clinical cases of dogs and the standard treatment protocol followed:

1 Tablet petjoint* were given as 1 gm/day for 20kg body weight. Tablet contain glucosamine hydrochloride 500mg, chondroitin sulphate 400mg, magnesium sulphate 5mg, vitamin C 12.5mg.

2 Tablet Carodyl[#] 100mg @ 2-4.4mg/kg body weight (non-steroidal anti-inflammatory drug) contains Carprofen with selective COX2 inhibitor.

3 Calcirol sachet[$] sachet 60,000 IU/gm @ used vitamin D3 (Cholicalciferol oral).

The medical treatment given were evaluated by clinically, physically lameness scoring and radiographic methods during the subsequent follow up period.

Group 1A- Hip joint OA (n=12).

Group 2A- Stifle joint OA (n=3).

Group 3A- Elbow joint OA (n=1).

3.23 Treatment of Osteoarthritis with viable ADMSCs

Group B: Approximately 4-5 million viable allogenic canine ADMSCs suspended in PBS was used for intra-articular injection aseptically using 18G IV cannula under C-arm guided (Fig.8). Two intra-articular injections were given in nine dogs with 15days time interval. A total of nine joints were injected with allogenic canine ADMSCs. The intra-articular injection was given in hip joint, stifle joint and elbow joint with OA lesion.

Group 1B-Hip joint OA (n=6).

Group 2B- Stifle joint OA (n=2).

Group 3B- Elbow joint OA (n=1).

Fig. 8:Photograph showing intra-articular injection of canine ADMSCs in hip joint by using 18G IV cannula.

Fig. 9: Photographs showing needle placement at right hip joint in lateral view under C-arm guidance (arrow).

Fig. 10: Photograph showing intra-articular injection of canine ADMSCs in stifle joint by using 18G needle.

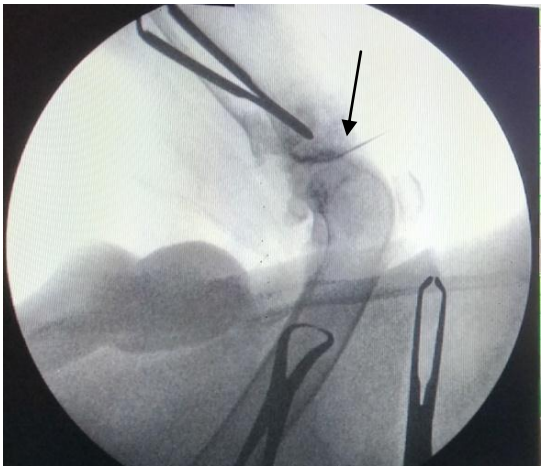


Fig. 11: Photographs showing needle placement at right stifle joint in lateral view under C-arm guidance (arrow).

Fig. 12: Photograph showing collection of synovial fluid from stifle joint.

Fig. 13: Photograph showing intra-articular injection of canine ADMSCs in elbow joint by using 24G needle.

For hip joint lateral approach for intra-articular injection was used. The greater trochanter of femur, tuber ischii, and wing of illium were the landmark for insertion of the needle. Palpated the greater trochanter then slightly abducted and rotated the femur traction was applied to opening the joint. The needle was positioned medially proximal to the greater trochanter and inserted in a slightly distal and ventral direction. The synovial fluids was collected first then were injected in the hip joint with prepared ADMSCs under C-arm guided (Fig.9).

For stifle joint intra-articular injection of ADMSCs in dog placed in lateral recumbency (Fig.10). The landmarks for injection were the distal tip of the patella and tibial tuberosity. The half way spot between these two palpate the patella tendon and the needle was inserted perpendicular to skin once through the skin angle the needle slightly, ie under the patellar tendon towards the inter-condylar fossa of the femur. The needle should be inserted until it hits the bone then adjust slightly until to get synovial fluid under C-arm guided (Fig. 11). Synovial fluid was collected from stifle joint before injection of ADMSCs (Fig. 12).

For elbow joint- Flex the elbow to opening up the joint space between the humerus and the ulnar. Inserted the needle medially, just lateral and parallel to the olecranon, then directed the needle downwards between the lateral chondyl and the ancorneal process. The synovial fluids was collected first then were injected in the hip joint with prepared ADMSCs under C-arm guided (Fig.13).

On follow up after intra-articular injection with ADMSCs therapy the animals were evaluated at interval of 15^{th}, 30th and 60^{th} days. Functional outcome was based on the weight bearing and gait during the postoperative period. Stifle, hip and elbow joint movement were assessed clinically for any abnormalities. Complications encountered were recorded.

3.24 Statistical Analysis

The data was analyzed using SAS (Statistical Analysis System) software version 9.3. The hemato-biochemical parameters and lameness score were compared for different days between stem cell therapy and normal medical treatment using t-test. The synovial fluid at day zero and 15^{th} compared using pared t-test.

CHAPTER IV

RESULT AND DISCUSSION

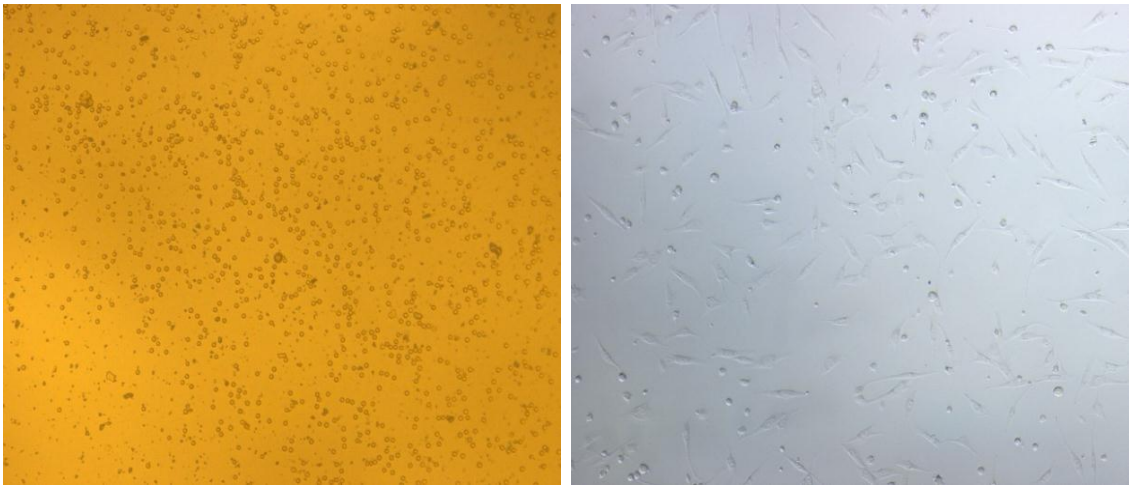
The study included twenty five canine osteoarthritis cases which were brought to the Department of Veterinary Surgery and Radiology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, during the period from April 2018- March 2019. Diagnosis was done in these dogs based on history, signalment, physical examination, radiography and laboratory information's. The study was conducted in two phase.

Phase I- Culture, subculturing and characterization of adipose derived mesenchymal stem cells was done under this section.

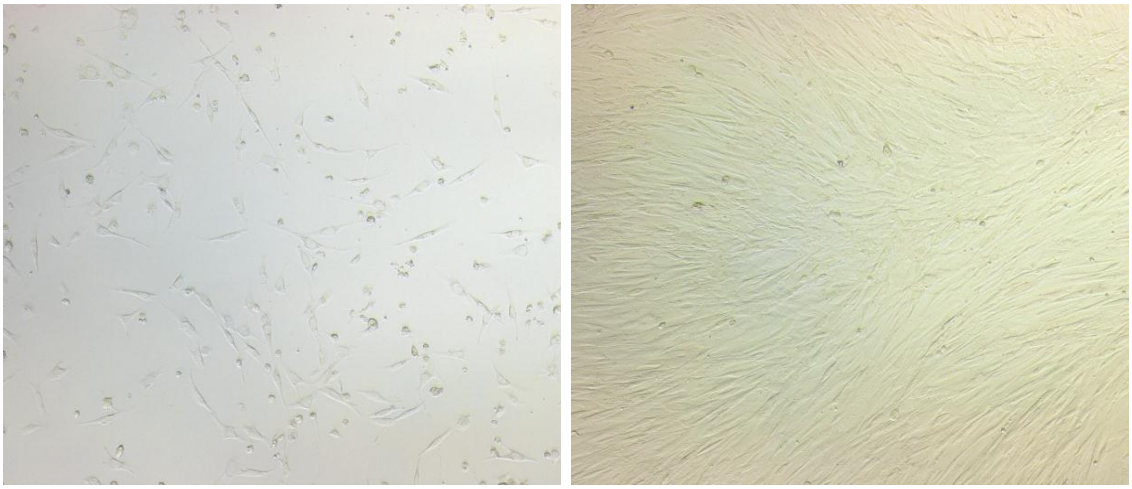
4.1 Culture and morphology of canine mesenchymal stem cells.

Mononuclear cells were isolated from adipose tissue sample under proper aseptic condition. Mesenchymal stem cell was isolated on the basis of their plastic adherence property under standard culture condition. During primary culture, adhered cell attached to the cell culture flask showed round, non- fibroblastic, morphology during initial days of culture (Fig 14A). Mesenchymal stem cells attached to cell culture flask after 72-96 hours, consisting of round and spindle shaped cells (Fig14B). In corroboration to present study Vieira *et al* (2010) reported that spindle shaped fibroblast-like cells isolated from canine adipose tissue could be sufficient for a therapeutic purpose. Similar feature related to morphological characteristics of mesenchymal stem cell was reported by Naskar (2013) and Kersic *et al* (2017) in canine, Devi *et al* (2017) in buffallo and Zuk *et al* (2001) in human.

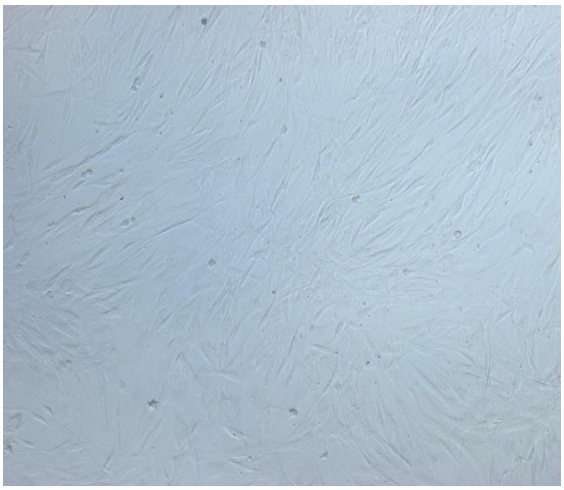
Initially very little number of cells was adhered to plastic surface but subsequently cell starts to grow and divide gradually. Upto five days after initial seeding, mesenchymal stem cells proliferate slowly (Fig14C). As the growth continues ADMSCs attains 70-80% confluence at seven to ten days (Fig14D) and thereafter cells subcutured to get more purified cell population (Fig14E). In corroboration to present study mesenchymal stem cells were isolated on the basis of their plastic adherence and fibroblastic morphology under standard culture condition. AD-MSCs attained 80% confluence at 5 to 7 days (Yaneselli *et al* 2018). Similar



(A) **(B)**



(C) **(D)**



(E)

Fig. 14: Development of the dog mesenchymal stem cells at different time and passages. A: Day 0; B: Day 3; C: Day 5; D: Confluent monolayer; E: Confluent monolayer at 3rd passage (Original magnification- A: X20 ; B, C, D, E: X10)

findings were obtained in canine MSCs harvested after six to seven days 70% confluent by Marx *et al* (2014), Clark *et al* (2016) and Bahamondes *et al* (2017).

The *in vitro* culture conditions required for growth and maintenance of canine ADMSCs were assessed. In present study, the cells were well maintained in culture medium that contains of high-glucose DMEM containing L-glutamine with 15% FBS. The cells were cultured at 37°C in humidified atmosphere with 5% CO_2. The medium changed every 3-4 days. Similar culture media was used by Sasaki *et al* (2018), for culturing the ADMSCs. Devireddy *et al* (2019) used 10% to 20% of FBS culturing stem cells. FBS is very essential component of the media containing a high content of growth factors as well as nutrition and physiochemical compounds required for cell maintains and growth (Jung *et al* 2012). Therefore the cell progression is largely depending upon the presence of FBS in culture medium (Eslaminejad and Nadri 2009).

4.2 Population doubling time

Population doubling time (PDT) was calculated in 4th passaged cells which were found to be 32.33±1.19 hours (mean±SE) in this study. In contrast to present study Zhan *et al* (2019) calculated population doubling time at 3rd passage was 15.8±0.318h in canine ADMSCs. However, Devi *et al* (2017) studied PDT at 15% serum concentrations was 38.63±1.70 hours before cryopreservation in buffalo mesenchymal cells. Gade *et al* (2012) also found similar results of PDT (36.66 hrs) at 15% serum concentrations. Buffalo bone marrow derived MSCs have similar range of population doubling time as compared to MSCs from other origins like ovine (McCarty *et al* 2009), caprine (Nair *et al* 2009), canine (Laura *et al* 2008) and human (Bruder *et al* 1997). However, higher PDT (55.79±0.12 hrs) was found by Shivakumar *et al* (2015) in humans.

4.3 Characterization of mesenchymal stem cells

4.3.1 Alkaline phosphatase staining

Alkaline phosphatase (AP) staining activity was detected in 3rd passage cells of canine adipose derived mesenchymal stem cells that appear red after staining under bright field microscope (Fig.15A) and given red fluorescence under florescent microscope (Fig.15B). Nucleus was stained using Hoechst stain which appears blue.

In corroberation to present study, Tang *et al* (2006) studied higher expression of AP in passage 3 canine MSCs and found the canine ADMSCs have an active proliferative ability in passage culture. However, O' Connor *et al* (2008) reported presence of AP activity in canine adipose derived MSCs considered as an embryonic marker because of increase activity of this enzyme in embryonic stem cells.

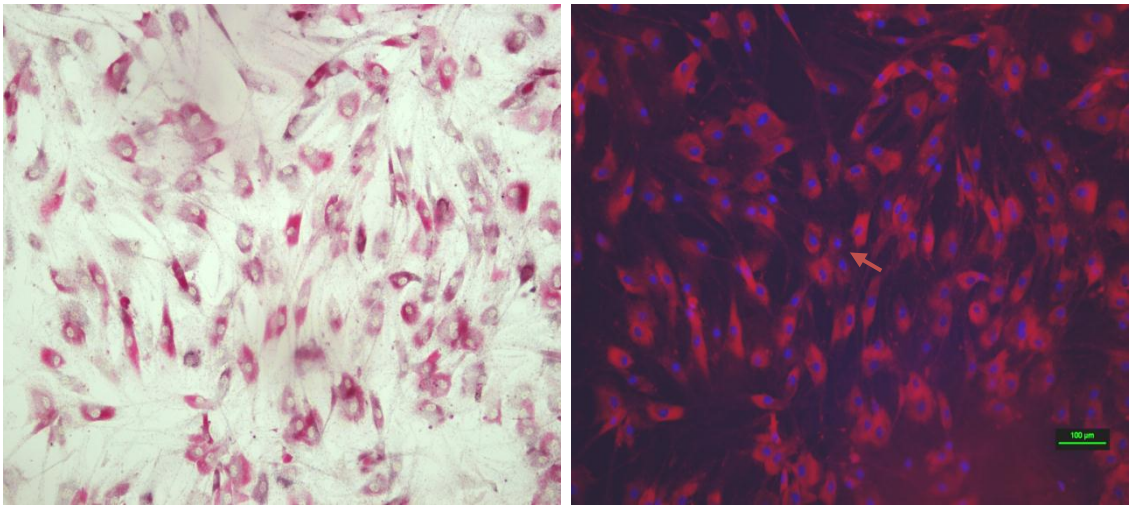
4.3.2 Immunohistochemistry

Monoclonal antibody CD44 specific to MSCs was used to characterize the canine MSCs by immunohistochemistry. It was demonstrated that the adipose tissue derived MSCs showed positive expression of cell surface marker CD44 as revealed by green colored immunofluorescence all over the cell surface under florescent microscope. Nucleous is stained by Hoechst staining an appeared blue in colour . More than 95% of cell population expressed CD44 under florescent microscope (Fig.16).

Kresic *et al* (2017) observed canine ADMSCs express low level of autofluroscency in Passage 1-Passage 2 and it starts to increase in Passage 3. Under the present culture conditions, more than 95% of the AD-MSCs expressed CD44, which is similar to results presented by Yaneselli *et al* (2018), Lee *et al* (2014). Although Martinello *et al* (2011) described a lower CD44 expression level (88%) for Visceral -AD-MSCs. Similar results were reported by Russell *et al* (2016) in canine and Baglioni *et al* (2009) in human.

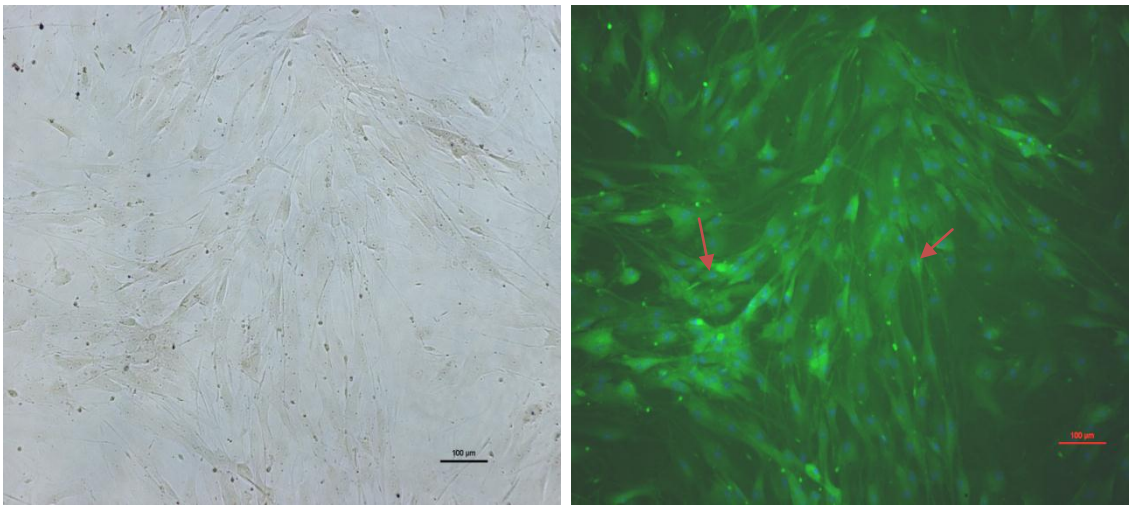
4.4 Cell senescence test

In the present study, ADMSc used upto 15^{th} passage for clinical cases of OA and every passage cells were tested for senescence qualitatively so as to inject the healthy cells in clinical cases. Senescence cells appeared blue in colour under bright microscopy indicative of increased beta- galactosidase activity in aged cells and healthy cells appeared red after counter staining with neutral red (Fig.17). This finding helped for us to use healthy cells in OA cases upto 15^{th} passage. In contrast to present study Kresic *et al* (2017) observed senescent appearance in P4 in seeded cells and P7 represents the upper cultivation limit. With each passage (8-15) higher number of cells showed marked SA-beta- Gal activity. Whereas, highest population of senescent cells at P5 (70%) by Bertolo *et al* (2015).



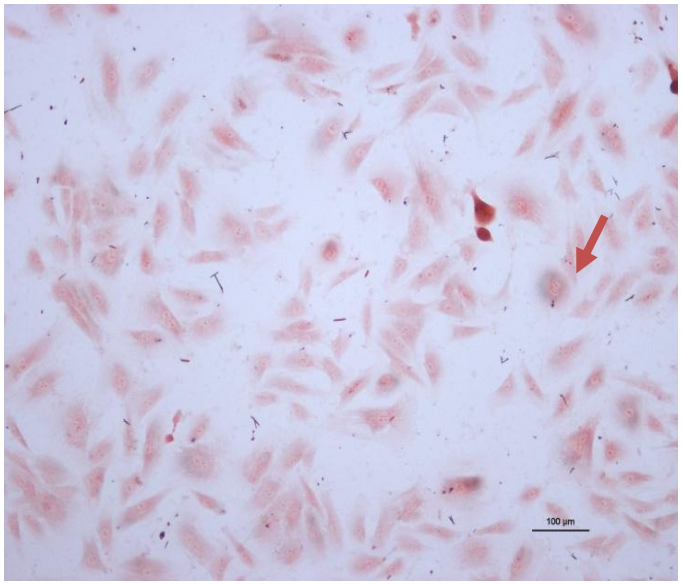
(A) **(B)**

Fig. 15: Alkaline phosohatase staining in mesenchymal stem cells A. under bright field microscopy, B.under flourescent microscope. Nuclear staining done with Hoechst stain (Nucleous yellow arrow)(Original magnification: X10).

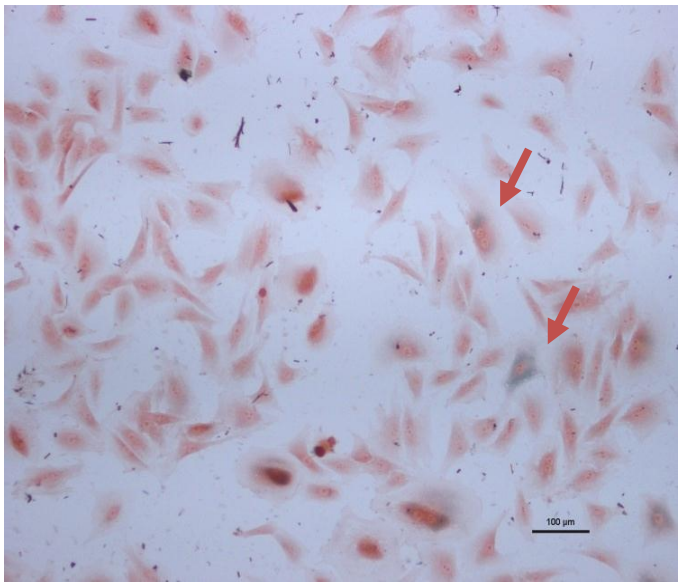


(A) **(B)**

Fig. 16: Immunofluorescent staining of dog adipose derived mesenchymal stem cells for localization of CD44 (A) under bright field (B) under fluorescence. Nuclear staining done with Hoechst stain (yellow arrow) (Original magnification: X10).



(A)



(B)

Fig 17. Cell senescence done in dog adipose derived mesenchymal stem cells. A: At 8 passage; B: At 15 passage . Senecsent cells appeared blue in colour (yellow arrow). Healthy cells are counter stained with neutral red (Original magnification: X10)

Early senescence could be due to high seeding density and high percentage of FBS (20%) in basal media (Kresic *et al* 2017). However, we used 15% FBS in culture media and seeded 2 million cells in 75cm² flask which might probably the reason for better growth of cells and very less no of senescent cells. The canine adipose derived mesenchymal stem cell (cADMSCs) can be maintained in vitro for extended period with stable population doubling and low level of senescence. The increasing numbers of senescent stem cell, which accumulate with sequential passages, may cause a reduction in treatment effects during *in vivo* applications and can cause malignant transformation of cells through interactions with adjacent cells (Krtolica *et al* 2001, Lee *et al* 2014).

Phase II: In this section, the study was conducted on clinical cases and the following results were recorded.

4.5 Incidence of osteoarthritis

The most common osteoarthritis seen in dogs were hip joint osteoarthritis (72%) followed by stifle joint osteoarthritis (16%) followed by elbow joint osteoarthritis (8%). The incidence of each OA is detailed in Table 1. The least common arthritis was elbow arthritis (Fig. 18).

Table 1: Incidence of OA

Osteoarthritis	Number of dogs	Percentage (%)
Hip joint	18	72%
Stifle joint	5	20%
Elbow joint	2	8%

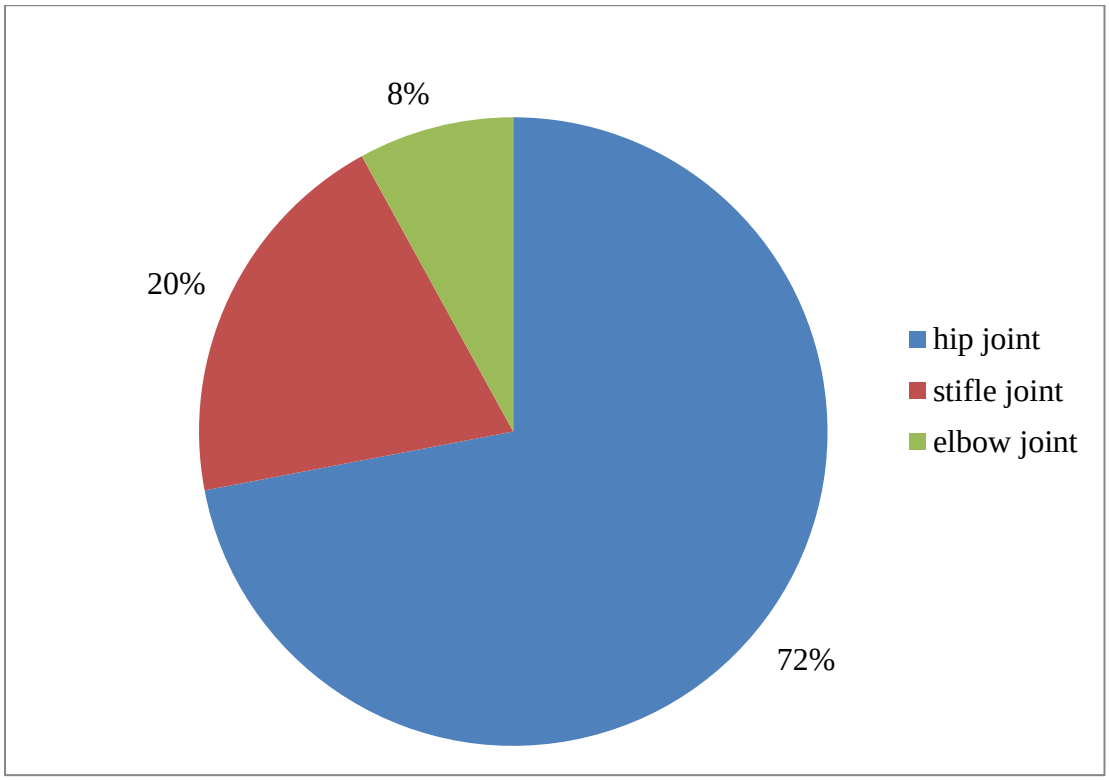


Fig. 18. Graphical representation of osteoarthritic incidence in dog

In corroboration of present study Anderson *et al* (1999) reported osteoarthritis may affect all the joints but hip, stifle and elbow joints were at more risk. Mele (2007), Rialland *et al* (2012) and Millis and Levine (2013) reported similar findings, that dogs were affected with osteoarthritis included hip, tarsus, carpus, stifle, shoulder, elbow in descending order. Moreover, Smith *et al* (2006) reported higher incidence of hip osteoarthritis in Labrador retrievers and attributed to excessive body weight. While Wahl *et al* (2008) reported higher incidence of hip osteoarthritis in German shepherd dog and attributed to hereditary origin. However, Lamani *et al* (2019) reported that highest occurrence of osteoarthritis in hip joint followed by elbow joint OA and then followed by hock, carpal joint, tarsal joint, shoulder, stifle and spine joints. In contrast to present study Denny and Gibbs (1980) and Leighton (1981) reported highest incidences in stifle joint compared to the elbow joint.

4.6 Prevalence in breeds

Prevalence of mostly affected breed were calculated and recorded. Large breed dogs were affected more as compared to small breed (Fig.19). Prevalence of OA in different breeds were recorded as Labrador retriever (64%), Rottweiler (12%), German Shepherd (8%), Pug (8%), Bullmastiff (4%) and Pomeranian (4%) (Table.2).

Table 2: **Prevalence breeds distribution**

Breeds	Number of dogs	Percentage (%)
Labrador Retriever	16	64%
Rottweiler	3	12%
German shepherd	2	8%
Pug	2	8%
Bullmastiff	1	4%
Pomeranian	1	4%

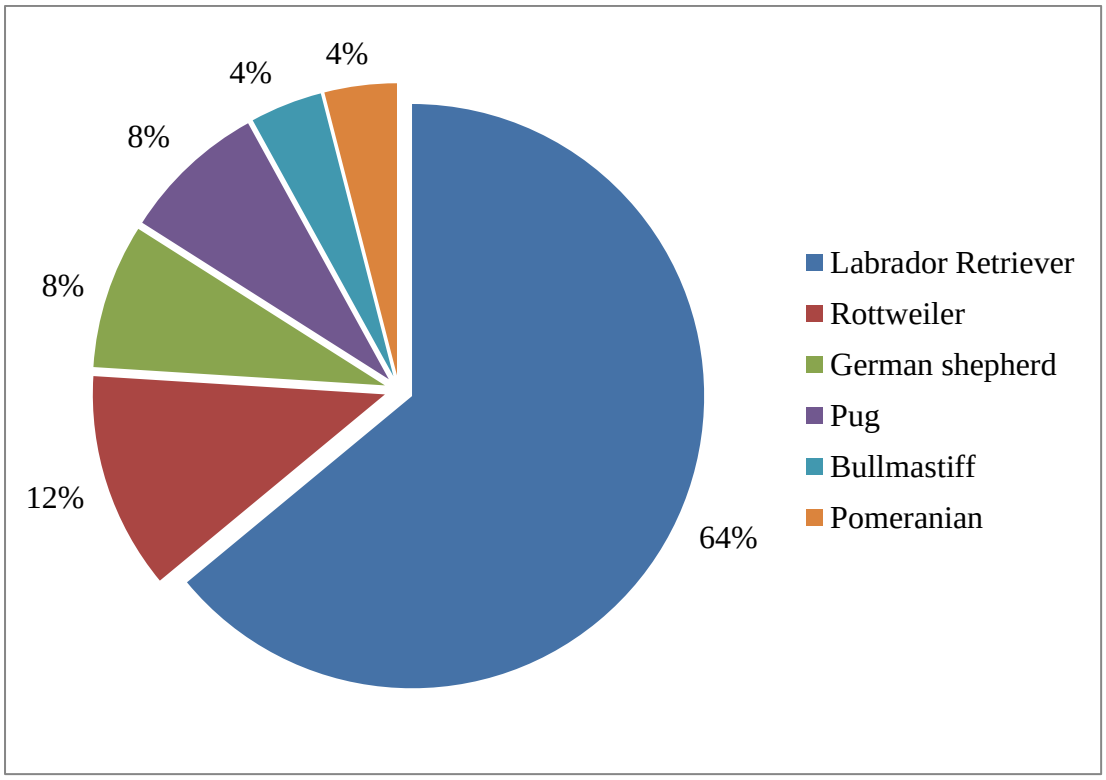


Fig. 19. Graphical representation of prevalence in breed distribution.

In corroborated of present study Mele (2007) described that breeds, such as Labrador Retriever and the German Shepherd dogs were mostly affected with osteoarthritis and concluded that the large breed dogs affect more with OA in comparison with median and small breed dogs. Anderson *et al* (2018) and Bland (2015) reported similar findings that large breeds were prone to osteoarthritis including Golden Retriever, Labrador Retriever, Rottweiler and German Shepherd in descending order.

4.7 Age distribution of dogs with osteoarthritis

A diagnosis of OA was made in dogs with different age groups. Six (24%) dogs were less than 4 years old. Nine (36%) were between four and eight years of age. Ten (40%) dogs were more than 8 years of age (Fig.20). The age distribution of dog with OA is detailed in Table 3.

Table 3: Age distribution of dogs with osteoarthritis

Age	Number of dogs	Percentage %
>5	6	24%
7-8	9	36%
< 8	10	40%

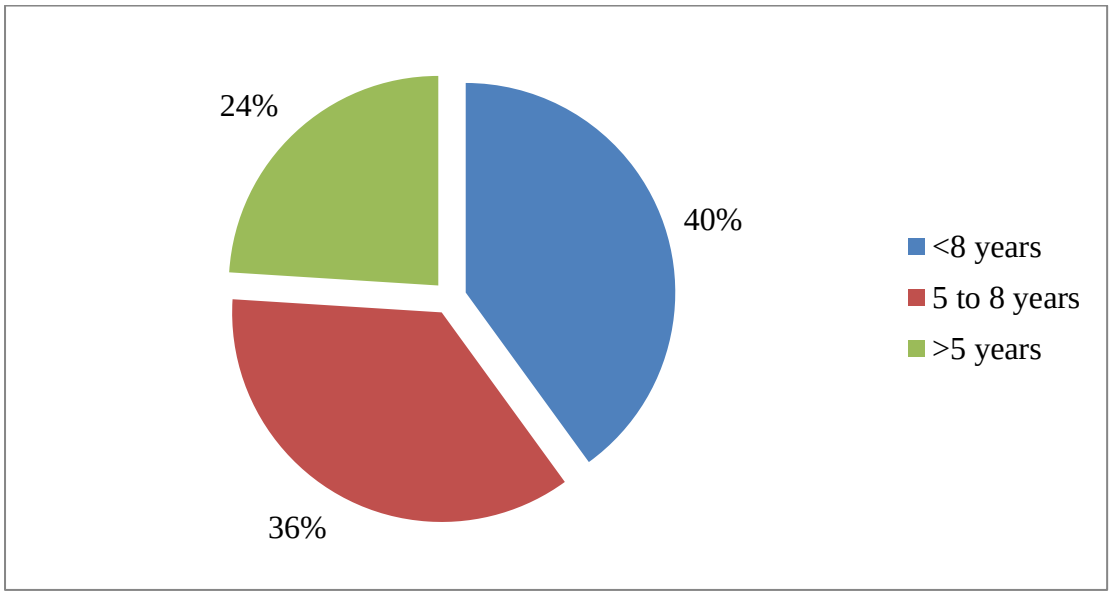


Fig. 20. Graphical representation of age distribution of dogs with osteoarthritis.

In support of present study Mele (2007) recorded that dog aged between 8-13 years were affected more due to musculoskeletal diseases in older dog. Johnston (1997), Pettitt and German (2015), Anderson *et al* (2018) reported similar findings based on radiographic and clinical data. The dogs older than eight years were more affected than the younger dogs. In contrast to present study Boothe *et al* (1996) recorded those dogs of age groups 7-9 years were found to be most affected. In corroborated to present study Whitehair *et al* (1993) and Duval *et al* (1999) reported the highest incidence of osteoathritis in 7 to 10 years of age. Restricted feeding has been shown to increase longevity of an animal which in turn could lead to age related

degeneration and development of OA at an older age (Lawler *et al* 2008, Runge *et al* 2008).

4.8 Body weight distribution of dog with OA

Animals with OA (n=25) were weighed. Out of 25 dogs, 16 (64%) dogs were having weight < 35kg. 6 (24%) dogs were having body weight range from 25-35kg and three (12%) dogs were having body weight of > 25 (Fig. 21). The body weight distribution of dog with OA is detailed in Table 4.

Table 4: Body weight distribution of dog with OA

Body weight	Number of dogs	Percentage %
>25	3	12%
25-35	6	24%
< 35	16	64%

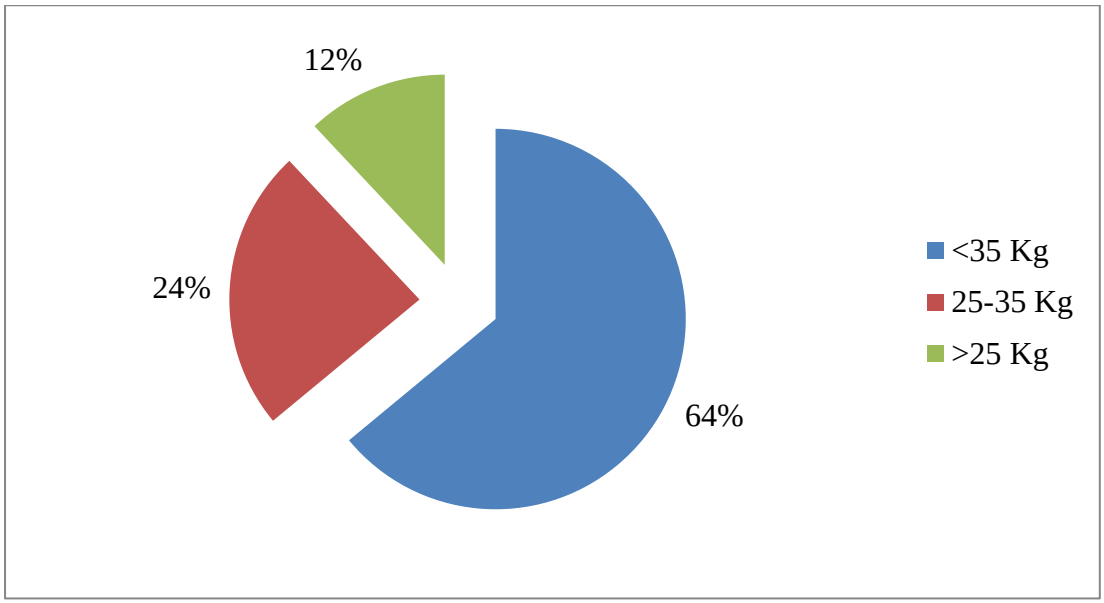


Fig. 21. Graphical representation of body weight of dogs with osteoarthritis.

In corroborated to present study the work of Kealy and colleagues (2000) reported that obesity may be a risk factor for the development of OA in the dog and this relationship is most convincing for hip OA secondary to dysplasia (Smith *et al* 2006). Moreover, Hadhammar and Krook (1974) reported diet have a great influence on the onset of OA, since overfeeding growing puppies causes a more rapid growth, which increases both bone length and body weight. McLaughlin and Roush (2002)

and Smith *et al* (2001) reported similar findings that growing large breed puppies are often affected with developmental orthopaedic conditions like hip dysplasia, which can lead to osteoarthritis. However, Marshall *et al* (2010) results indicated that body weight reduction causes a significant decrease in lameness obese dogs with OA. Sanderson (2012) reported in dogs, there is a positive association between obesity and OA, obesity with chronic inflammation, which plays a major role in the development of OA.

4.9 Gender distribution

Out of 25 dogs 19 (76%) were males while 6 (24%) were females. Out of 18 cases of hip joint osteoarthritis, 14 (77.7%) were males and 4(22.2%) were females. Whereas in stifle joint osteoarthritis (n=5), 4(80%) were males and 1 (20%) was female; In elbow joint osteoarthritis (n=2), one was (50%) was male and one was (50%) female (Fig. 22). Overall percentage showed higher incidence of OA in males. The gender distribution of dog with OA is detailed in Table 5.

Table 5: Gender distribution

Osteoarthritis	Number	Males		Females	
		No.	%	No.	%
Hip joint	18	14	77.7%	4	22.2%
Stifle joint	5	4	80%	1	20%
Elbow joint	2	1	50%	1	50%
Total	**25**	**19**	**76%**	**6**	**24%**

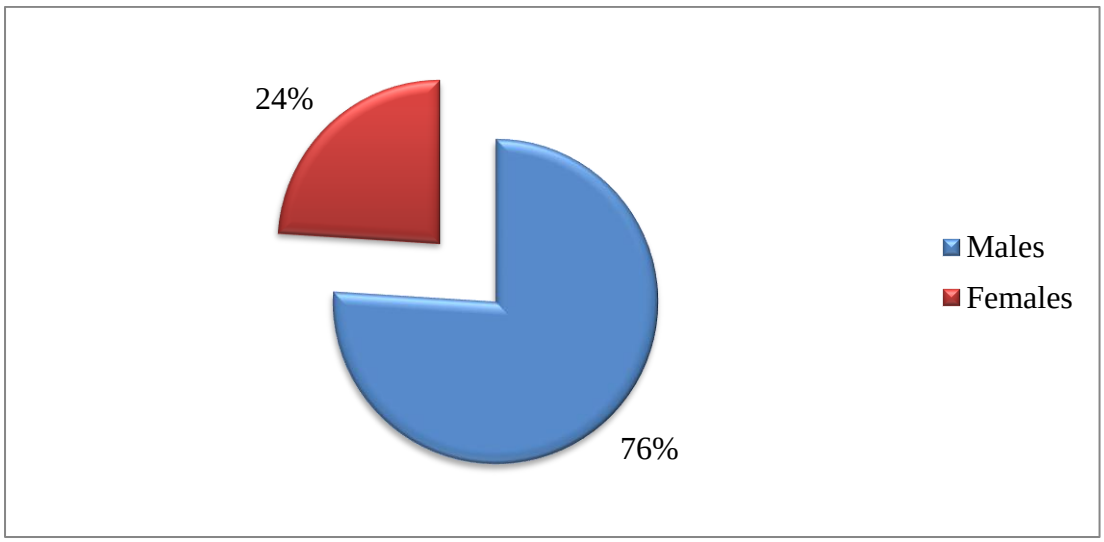


Fig. 22. Graphical representation of gender distribution in dogs with osteoarthritis.

In general, OA often associated with primary disorders, which may be more prevalent in males as compared to females. The fragmentation of the coronoid process was more in males than females due to their higher growth rate, in males compared to females by Mele (2007). Similar findings reported by Lamani *et al* (2019), Bendele (2001) and Henrotin *et al* (2005). Many research suggested that male dogs have greater odds of developing osteoarthritis than females due to sex hormone differences as well as differences in weight between males and females (Hays *et al* 2007).

4.10 Clinical observation

4.10.1 History and clinical observations

Group A: In control group, clinical signs were observed for osteoarthritis with progressive lameness in 16 dogs. Three dogs were treated by local veterinarian one or two week prior presentation. No relevant history of trauma was reported in any of these cases. Non- weight bearing lameness with occasional dragging was the complaint in all cases. Medical treatment was given in this group.

Group 1A: Twelve cases of hip joint oseoarthritis were presented. The clinical signs were recorded as difficulty to rise, reluctant to climb up stairs, pain, bunny- hopping gait and exercise intolerance in majority of dogs with large body weight. In corroboration to present study Pettitt and German (2015) investigated the most common manifestation of osteoarthritis was inactivity stiffness and lameness seems to resolve during exercise but then worsens after periods of rest. Other common presentations included change in the animal's ability to climb the stairs or to jump.

Group 2A: Three cases of stifle OA were presented and the clinical signs were intermittent non- weight bearing, pain on palpation, muscle atrophy were present. Dogs with OA are usually presented with a complaint of lameness or gait abnormalities. Leach *et al* (1997) recorded similar finding that, the animal was not able to jump with as much vigor as previously, difficulty in climbing a flight of stairs that previously were easily negotiable.

Group 3A: Right elbow joint OA (n=1) case was presented with lameness. There was no relevant history of trauma. Predominant presenting complaint was progressive lameness, pain, skipping gait, limb weakness. In corroboration to present study Hercock *et al* (2009) done clinical examination on thoracic limb lameness and one or

more clinical signs of elbow OA such as pain on manipulation of the elbow joint, enlargement of the elbow joint, reduced range of motion and crepitus of the elbow joint.

Group B: ADMSCs treatment group, clinical signs were observed for osteoarthritis with progressive lameness in nine dogs. Non- weight bearing lameness with occasional dragging, reluctance to exercise, stiffness were the complaint. No history of trauma was reported.

Group 1B: Pelvic limb lameness from 1-2 weeks was observed. Hip joint OA caused severe lameness in one case. Poor weight bearing with pain and limb weakness was present. Fry and Clark (1992) observed pelvic limb OA general behaviors often attributed to lameness, gait abnormalities and hind limb muscle atrophy in older dogs. Hind limb lameness can be due to CHD joint changes, including pelvic, metabolic bone disease, ligament rupture, patellar luxation, and spine disorders.

Group 2B: Two cases of right stifle joint OA was presented with lameness. The dog in this group was showing hind limb lameness and stiffness, after rest followed by exercise, muscle atrophy. Clicking noise was also heard while palpation. Piermattei *et al* (2006) recorded joint crepitus associated with irregular joint surface that results from the loss of cartilage detected with manipulation of stifle.

Group 3B: Only one case of elbow joint OA was presented with reduced range of motion and pain of elbow, moderate weight bearing was noticed, showed difficulty in getting up from a resting position.

Clinical and physical signs associated with osteoarthritis showed lameness, reluctance to move, inactivity stiffness commonly associated with inflammation and can be attributed to edema of peri-articular tissue or joint effusions. Other signs include edema in tissue which can alter range of motion, pain, muscle atrophy, joint thickening, altered gait and behavior by Bland (2015), Piermattei *et al* (2006) and Anderson *et al* (2018).

4.10.2 Special clinical examination

Ortolani's maneuver gave a positive result in all dogs with severe and moderate hip joint OA. Bunny-hopping gait and pain on manipulation of hip flexion and extension were tolerated by most of the dogs, however showed pain in severe

cases of stifle joint osteoarthritis. Clicking noise was heard while doing cranial drawer test. The ortolani's test is a subjective evaluation of coxofemoral joint laxity by Schachner and Lopez (2015). The test is also used as a canine hip dysplasia (CHD) screening test (Chalman and Butler 1985). Lack of an Ortolani sign does not necessarily mean that the hip is normal. Joint changes associated with dysplasia, like thickening of the joint capsule and joint tissue, may interfere with the displacement required for a positive sign.

Definitive diagnosis of rupture of the cranial cruciate ligament rupture (CCL) demands an assessment of stifle joint stability by means of the cranial "drawer" test, best done on the laterally recumbent animal. Painful response in a dog with a CCL injury, often detected with the stifle flexed (Harasen 2002, Piermattei and Flo 1997, Moore and Read 1996).

4.11 Orthopedic evaluation of lameness

On day 0, there were no significant difference in scores of lameness during standing, walking and pain while palpation, between animals of group A and group B (Fig.23). After the 1st treatment by ADMSCs no significant difference between 0 day and 15 day scores were recorded in both the groups. The improvement in clinical scoring was observed to be significant on day 30 in animals of group B after second dose of injection. This improvement remained significant ($P \leq 0.05$) in treatment group B up to 60 days of follow-up (Fig. 24) from day 0 in terms of pain on palpation (Fig. 25), weight bearing during standing (Fig. 26), lameness during walking (Fig. 27 & Table. 6). No significant improvement was seen in animal of group A in terms of lameness and pain scoring throughout the follow-up period.

Out of 16 animals treated by traditional conservative method in group A owner's feedback on quality of life post treatment showed poor (n=8) to satisfactory improvement (n=5). Only few animals (n=3) showed good quality of life post treatment in this group. Among animals of group B which were treated by allogenic canine ADMSCs the owner feedback showed good (n=4) to very good (n=3) quality of life post treatment. Interestingly two animals showed excellent quality of life and did not show any pain upto 60 days post treatment the follow up of this animals extended upto 3months and animal remained pain free till last follow-up.

Table 6: Orthopedic evaluation of lameness

Parameters	Treatment	0day	15day	30day	60 day
Lameness during walking (LW)	Group A	2.25±0.26	2.18±0.26	1.87±0.23	2.00±0.24
	Group B	2.33±0.23	1.77±0.14	1.55±0.24	1.44±0.24
Pain on palpation (PP)	Group A	1.93±0.14	1.93±0.14	1.75±0.11*	1.62±0.12*
	Group B	1.77±0.14	1.77±0.14	1.33±0.66*	1.22±0.14*
Weight bearing during standing (WBS)	Group A	2.18±0.13*	2.12±0.12	2.00±0.12*	1.93±0.77*
	Group B	1.66±0.16*	1.77±0.22	1.33±0.23*	1.22±0.66*

*Represent significant value at $P \leq 0.05$.

No significant difference from baseline value (day 0) in lameness and pain score upto day 15 in animals of both the groups suggested that a single dose of allogenic canine ADMSCs and conservative treatment failed to provide immediate relief to the animals. However, significant improvements in pain and lameness scores in animals belong to group B during the post treatment period upto 60^{th} day suggested that allogenic canine ADMSCs provide greater degree of relief from pain and improve the quality of life of the patient. This is similar to finding of Black *et al* (2007), Naskar (2013), Cuervo *et al* (2014). The improvement in pain and lameness score could be attributed in new cartilage formation as reported by Nathan *et al* (2003). Chopp *et al* (2000) and Caplan and Dennis (2006) also reported clinical benefits in regenerative medicine. The significant improvement in pain and lameness scores in the present study might be due to the stimulatory effect of cytokines and growth factors on the recovering cartilaginous tissue in a paracrine manner (Fang *et al* 2007, Gimble *et al* 2007, Marx *et al* 2014).

Fig. 23: Photograph showing lameness of left hind limb with dragging of limb (on 0 day).

Fig. 24: Photograph showing proper weight bearing on left hind limb after injection of ADMSCs (on 60 days).

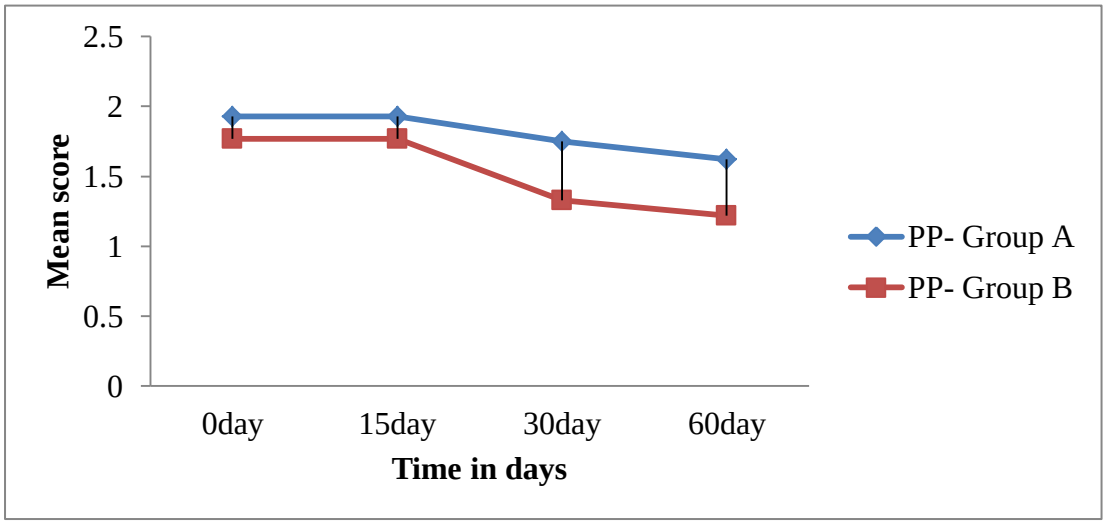


Fig. 25: Line diagram showing effect of treatment on orthopedic examination score in dogs based on pain on palpation (PP).

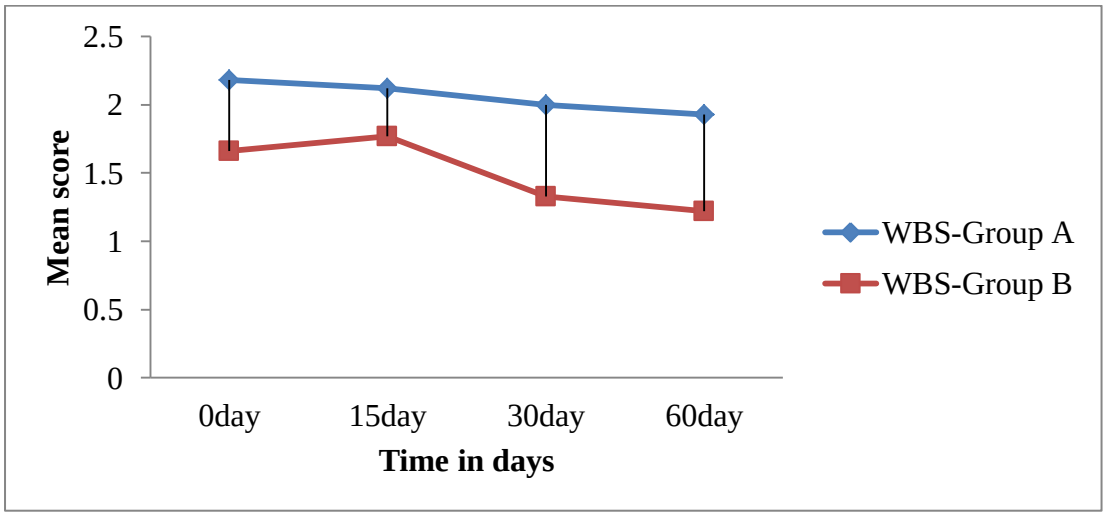


Fig. 26: Line diagram showing effect of treatment on orthopedic examination score in dogs based on weight bearing during standing (WBS).

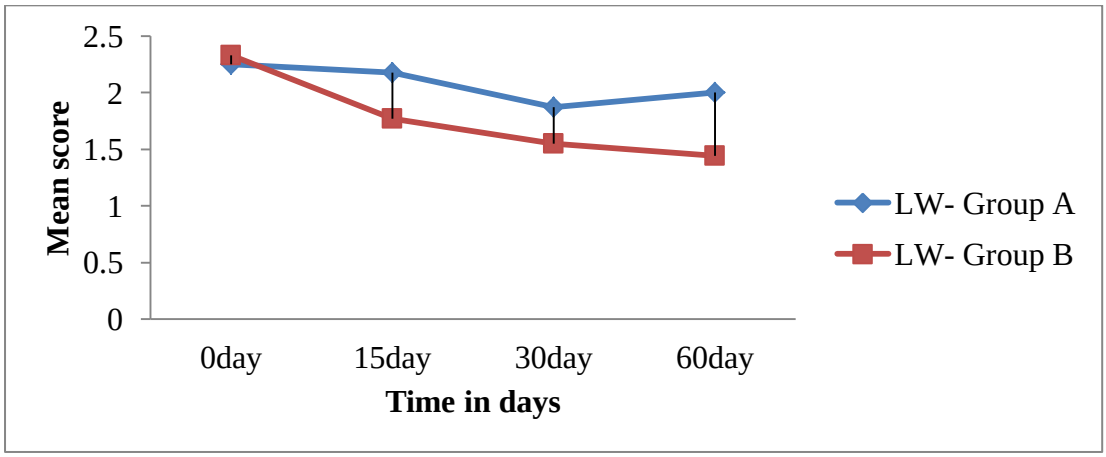


Fig. 27: Line diagram showing effect of treatment on orthopedic examination score in dogs based on lameness during walking (LW).

4.12 Radiographic evaluation of osteoarthritis

Group A: Day zero, 15th, 30th and 60th the radiographic evaluation was done in group A which was treated with normal medication (control group). No radiographic changes were evident after medication on selected intervals. Similar finding were reported by Carapeba *et al* (2016), all the radiographic score at 60th day were the same as the score at baseline (day zero).

Group 1A: Ventro-dorsal (VD) view of the hip was found to be the most helpful to access the diagnosis of osteoarthritis in present study. Lust *et al* (1985) had remarked that standard ventro-dorsal radiographs reveal abnormalities of hip undetectable in other projections. Osteoarthritic changes include periosteal reaction; reduction in joint space and a thin layer developed beneath the cartilage in the hip joints were evident in all cases. In VD pelvic incongruency of the hip joint seen with mild osteoarthritic changes less than 50% femoral head in the acetabulum seen along with broadening of femoral neck in majority of cases. Osteophytes seen on left acetabulum in one case. Bilateral hip dysplasia seen in majority of the cases with severs osteoarthritic changes. Worth *et al* (2012) reported that dysplasia typically occurs in the hip or elbow as a result of developmental malformation causing joint instability. This commonly leads to pain and lameness and often to laxity of the joint, causing further degeneration due to trauma. Morgan (1992 &1969) described characteristic radiographic features of OA include sclerosis of subchondral bone, subchondral bone cysts, osteophyte formation, subluxation, narrowing of the joint space, and intra-articular or periarticular calcification. No radiographic changes were evident after medication on selected intervals.

Group 2A: Medio-lateral view of stifle was helpful in assessing stifle joint arthritis. One of the case showed periosteal reaction involving femoral condyle, tibial plateau and increased tissue density in joint space. Periosteal reaction and lytic changes cold be seen at the level of femoral condyl and ventral border of patella. The infra patellar fat pad seen in medio-lateral view.

Periosteal reaction and a bone chip seen close to femoral condyle. In one case the periosteal reaction involving patella and patella appears to be superimposed over femoral condyles. The case was diagnosed with osteochondritis dissecans (OCD) with osteoarthritis. Piermattei *et al* (2006) reported increased joint space; free ossicles may

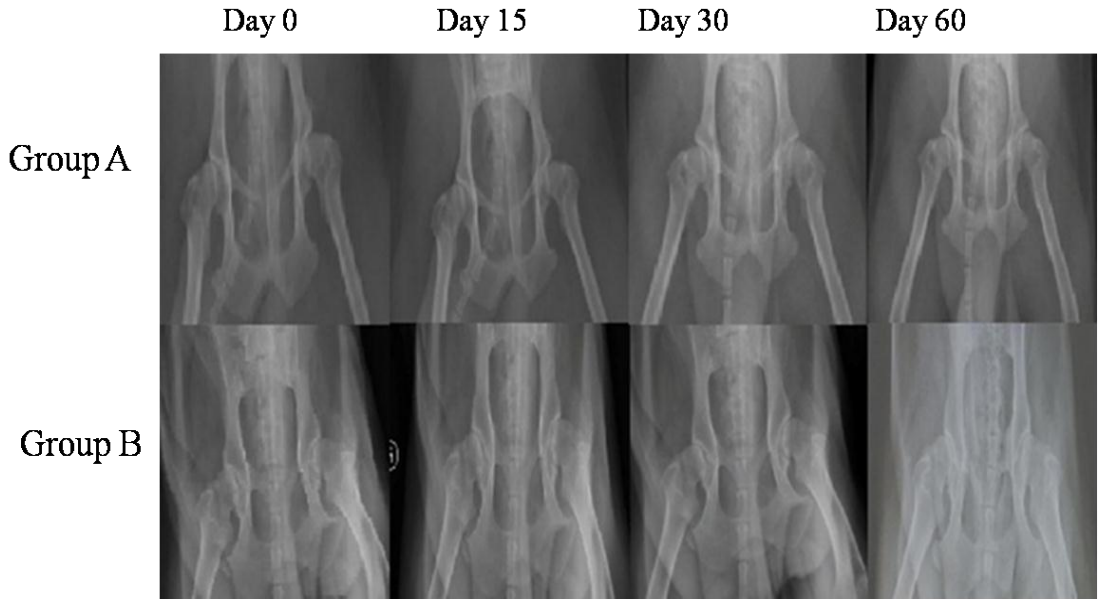


Fig. 28: Ventro-dorsal radiograph of Hip joint of group A (Upper row) and group B (Lower row) on days 0, 15, 30 and 60.

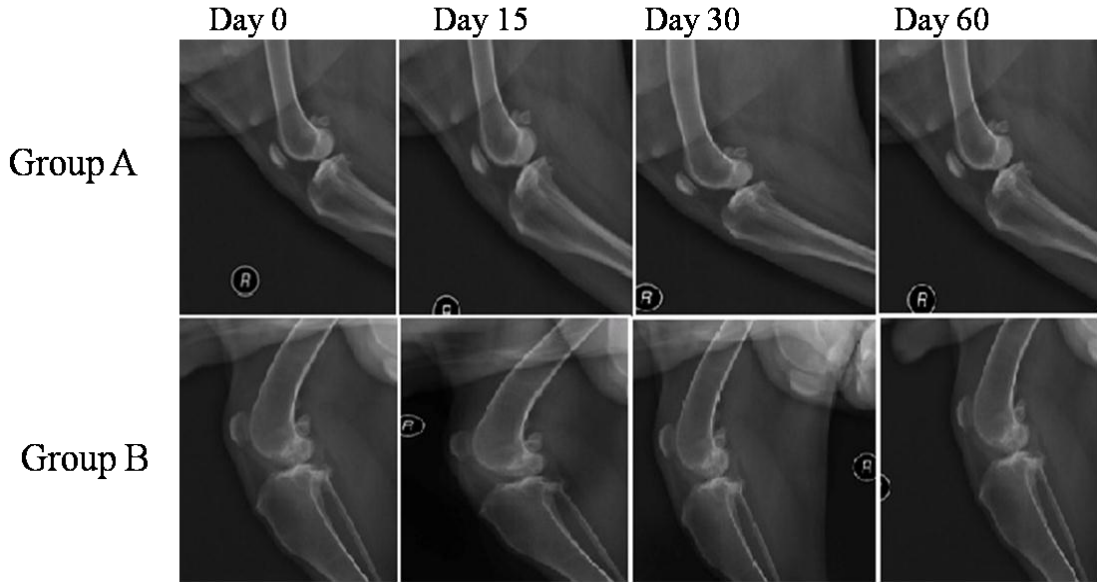


Fig. 29: Ventro-dorsal radiograph of stifle joint of group A (Upper row) and group B (Lower row) on days 0, 15, 30 and 60.

be seen occasionally. Some fragments may be extruded and trapped under the ligament. No radiographic changes were recorded after medication on selected intervals.

Group 3A: Medio-lateral view of elbow was helpful in assessing moderate elbow joint osteoarthritis. Moderate radiographic changes are seen with periosteal reactions in all the cases. Similar findings were reported by Hercock *et al* (2009). No radiographic changes were recorded after medication on selected intervals.

Group B: Day zero, 15^{th}, 30^{th} and 60^{th} the radiographic evaluation was done for the groups B treated with ADMSCs (treatment group). No radiographic changes were evident after medication on selected intervals. Similar finding were reported by Cuervo *et al* (2014), all the radiographic score at 60^{th} day were the same as the score at baseline (day zero).

Group 1B: Ventro-dorsal (VD) view of the hip taken to access the diagnosis of osteoarthritis. Osteoarthritic changes were mild, moderate and sever with reduced joint space, thinning of the joint capsule, periosteal reaction. When subluxation occurs, the articular cartilage is worn and roughened on the dorsal surface of the femoral head which contact with the acetabular rim.

Group 2B: A mediolateral view of stifle was found to be helpful to access cranial cruciate ligament rupture with mild osteoarthritic reaction in one of the case. Osteophytic reaction was seen at the caudal aspect of tibia. Increased fat pad area was noticed (DeLuke *et al* 2012). Moreover, Anderson *et al* (2018) reported that conditions such as hip dysplasia, patellar luxation, osteochondritis dissecans, fragmented coronoid process and cruciate ligament disease can all be considered a risk factors for developing OA in dogs. The cranial cruciate ligament rupture was repaired with togged pin method and ADMSCs cell were implanted.

Group 3B: Sever periosteal reaction with reduced joint space on right elbow joint. Alam *et al* (2011) reported similar finding that the chronic and progressive OA results in degradation within the joint, accompanied by changes within the bone and formation of osteophytes.

Radiographic signs of osteoarthritis are relatively non-specific and include osteophytosis, effusions, soft tissue swelling, intra-articular mineralization, having some degree of hip laxity (Pettitt and German 2015). However, Poole (1994) recorded

loss of joint cartilage, a narrowing of the joint space, sclerosis of subchondral bone, and the production of joint osteophytes. Radiography was inconclusive to assess the treatment out come in both groups during various time intervals. One reason to explain these findings could be related to technical limitations of radiography in detecting and evaluating the progression of osteoarthritis. These results do not necessarily rule out changes at a cartilage structure level and advanced techniques like magnetic resonance imaging (MRI) may be helpful in evaluating the changes at cartilage level. Invasive technique like biopsy can also be used subjected to owners consent but may have ethical issues (Roemer *et al* 2011, Cuevro *et al* 2014).

Table 7: Radiographic changes in OA

Radiographic changes	No. of cases	A		B	
		No.	%	No.	%
Mild arthritic change	6	3	50%	3	50%
Moderate arthritic change	12	9	75%	3	25%
Severe arthritic change	7	4	57.1%	3	42.8%

In group A, out of 16 animals three animals showed mild osteoarthritic changes and nine animals showed moderate osteoarthritic changes while four animals showed severe osteoarthritic changes on day zero. In group B, 9 animals showed mild osteoarthritic changes (n=3), moderate osteoarthritic changes (n=3) and severe osteoarthritic changes (n=3) on day zero. Follow-up radiograph were taken in days 15, 30, 60 revealed no improvement in radiographic features from day zero in any animal irrespective of groups (Table 7 and Fig. 28 & 29). The radiographic progression of osteoarthritis of the stifle joint, secondary to cranial cruciate ligament deficiency. In the index stifle joints all features were characterized by significant change over time, but osteophytosis had the greatest degree of change. In addition, 40% of contra lateral joints showed progressive osteophytosis (Innes *et al* 2004).

Although there was no significant improvement in follow-up radiograph of cases treatment with allogenic canine ADMSCs but the owner satisfaction and improvement of quality of life in this patient may be an important consideration for selecting ADMSCs as treatment of choice for canine osteoarthritis.

4.13 Hematological parameter

The mean ± SE values of various hematological parameters in animals of different group are presented in Table 8.

Table 8: Hematological results of dogs with OA

Parameters	Treatment	0day	15day	30day	60 day
Hemoglobin Concentration (Hb)g/dl	Group A	13.22±0.49	13.22±0.47	13.41±0.50	13.46±0.48
	Group B	13.13±0.42	13.33±0.44	13.41±0.41	13.46±0.41
Total leukocyte count (TLC)	Group A	14767±277.4	14631.6±322.4	14946.0±338	14554.6±318.4
	Group B	14980.9±306	15093±360.3	14997±413.4	14979.3±332.9
Neutrophils %	Group A	75.8± 3.22	75.83±2.68	73.93±2.47	76.36±2.54
	Group B	81.1±3.60	80.68±3.19	80.33±3.60	80.33±3.74
Lymphocytes %	Group A	18.07±2.46	17.83±2.42	18.86±2.27	19.10±2.24
	Group B	18.62±2.16	19.6±2.19	19.73±2.09	20.11±2.34

The TLC and neutrophils count were slightly elevated in all the animals throughout the study period. An increase in the number of TLC (Fig. 30) and neutrophils (Fig. 31) value may be suggestive of an inflammatory response. No significant change in various hematological parameter was recorded from the baseline (day 0) values in animals of both the groups (Fig. 32 & 33). A complete blood count can effectively tracks a dog's response to illness. Osteoarthritis is characterized by mild inflammation (Piermattei *et al* 2006). Similarly Alam *et al* (2006) recorded that changes in the complete blood count (CBC) and Chemistry Screening (CS) parameters can be impressive but cannot be the sole determinants of osteoarthritis in dogs. However, Alam *et al* (2006) and Pettitt and German (2015) observed the hematological parameter is not routinely taken in OA cases unless warranted by history and clinical signs.

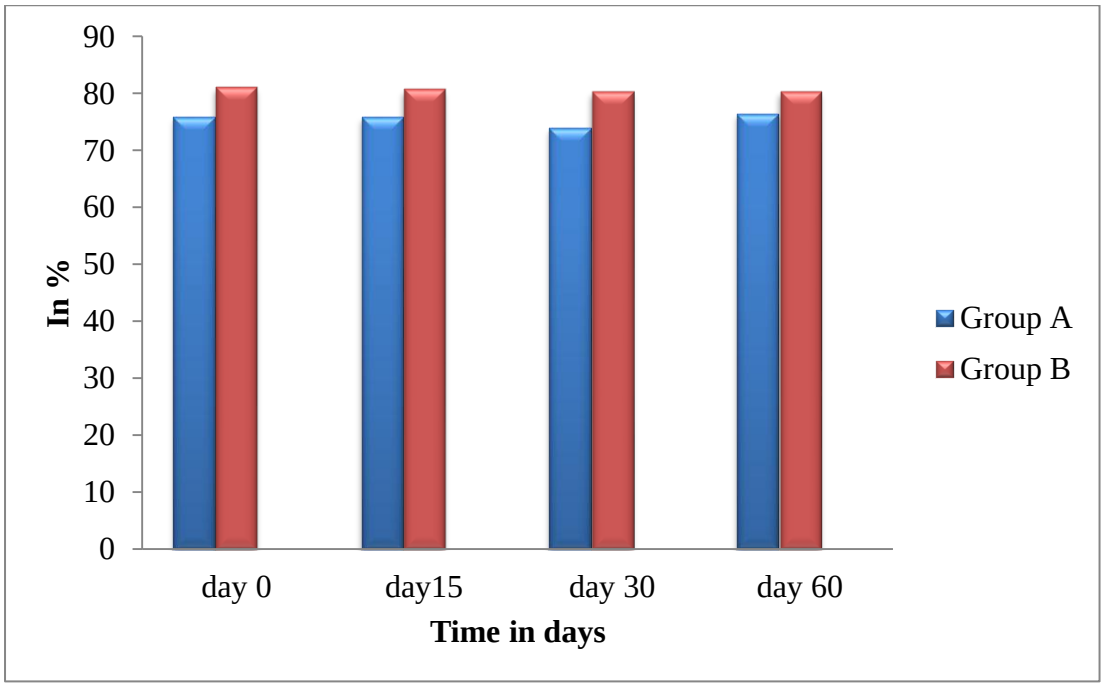


Fig. 30. Graphical representation of Total Leucocytes count (µ/dl) in dogs with osteoarthritis in both the groups.

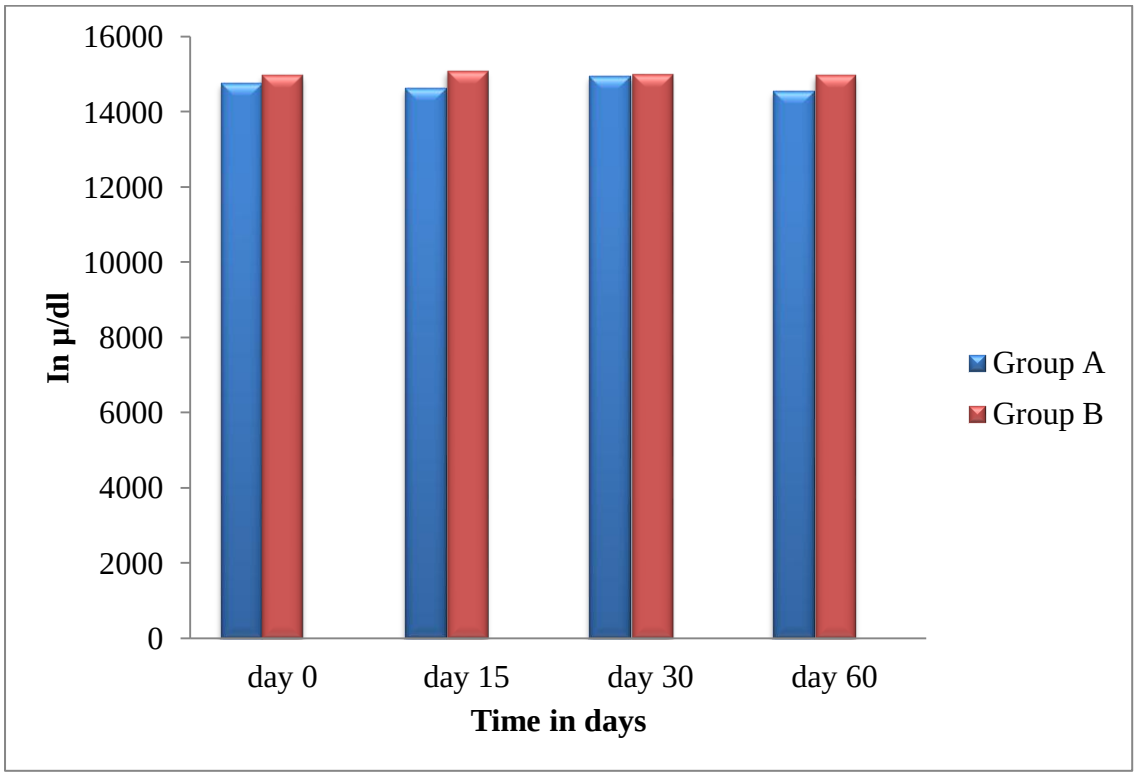


Fig. 31. Graphical representation of neutrophils (%) in dogs with osteoarthritis in both the groups.

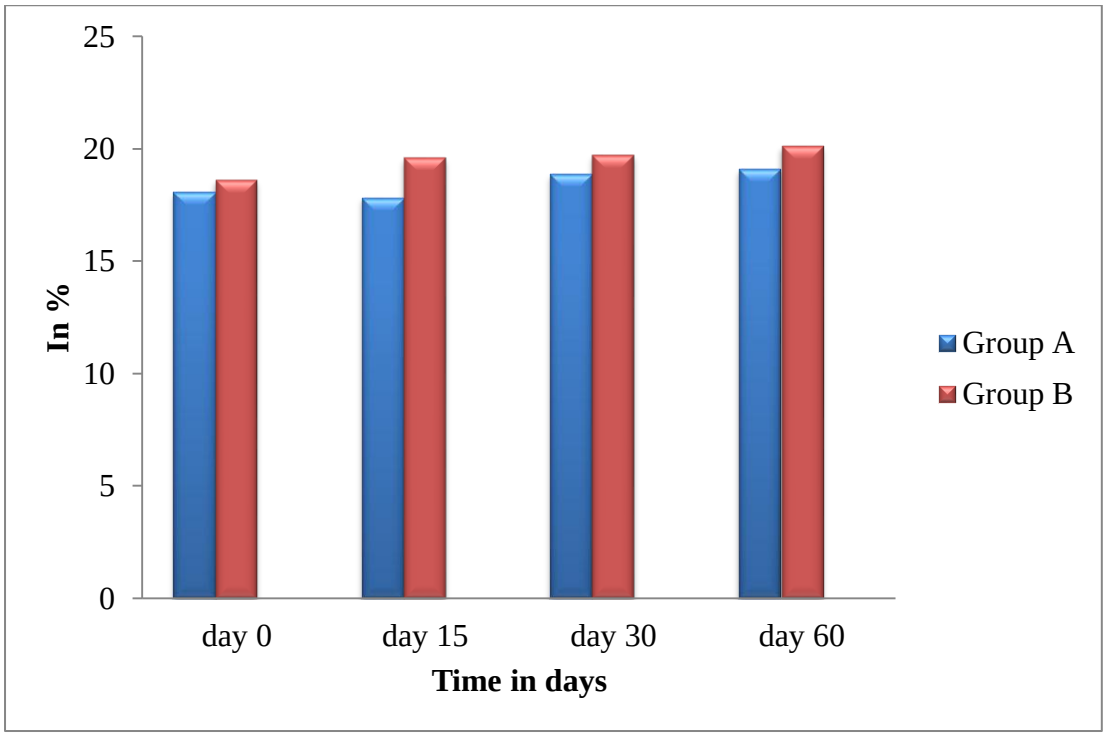


Fig. 32. Graphical representation of lymphocytes (%) in dogs with osteoarthritis in both the groups.

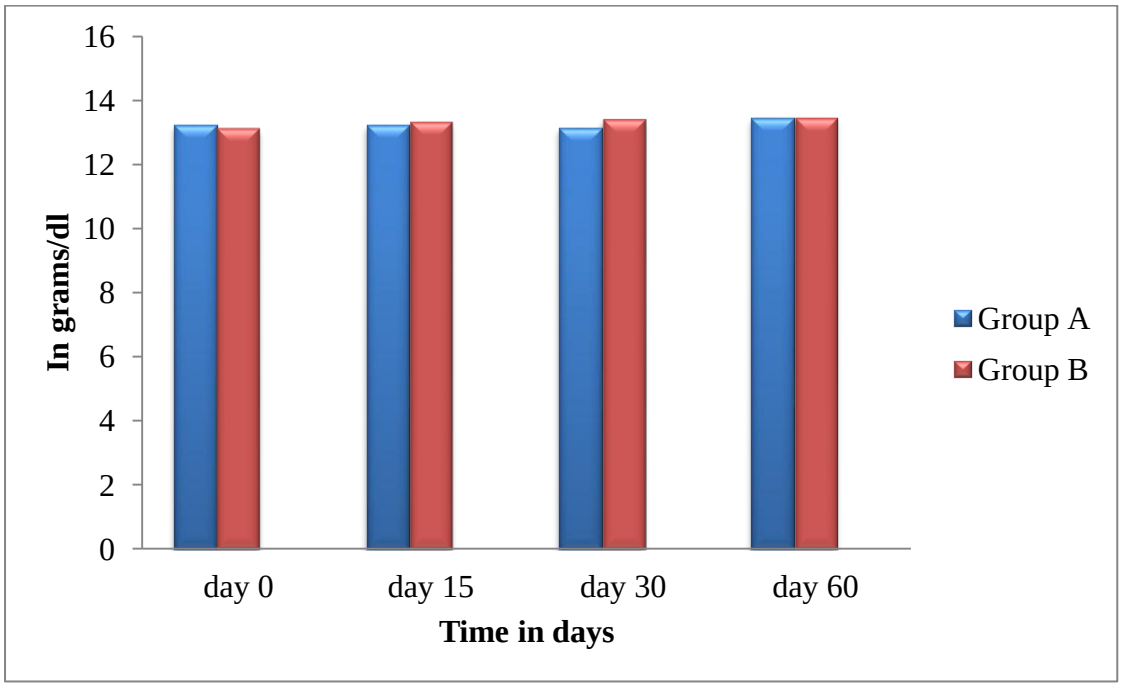


Fig. 33. Graphical representation of hemoglobin (g/dl) in dogs with osteoarthritis in both the group.

4.14 Serum biochemistry

The mean ± SE values of various serum biochemistry parameters in animals of different group are presented in Table 9.

Table 9: Serum biochemistry values

Parameters	Treatment	0day	15day	30day	60 day
Calcium mg/dl	Group A	9.00±0.28	9.01±0.34	9.05±0.31	8.89±0.24
	Group B	8.73±0.67	8.24±0.85	9.12±0.55	9.03±0.54
Phosphorus mg/dl	Group A	3.63±0.16	3.85±0.18	4.02±0.17	3.96±0.15
	Group B	3.62±0.27	3.83±0.29	3.96±0.28	3.88±0.21
Alkaline phosphates IU/L	Group A	48.65±1.75	48.56±1.69	47.83±1.51	47.65±1.56
	Group B	49.15±3.21	52.33±2.79	47.91±2.50	48.96±2.22

The serum biochemical findings were not statistically significant as the mean value were within normal reference range in both the group (Fig. 34-36). In contrast to present study activation of osteoclasts and subchondral bone remodeling is a major histologic feature of early-stage osteoarthritis (OA), which can be accompanied by an increase of calcium (Ca) and phosphate (P) level in the subchondral milieu (Jung *et al* 2018).

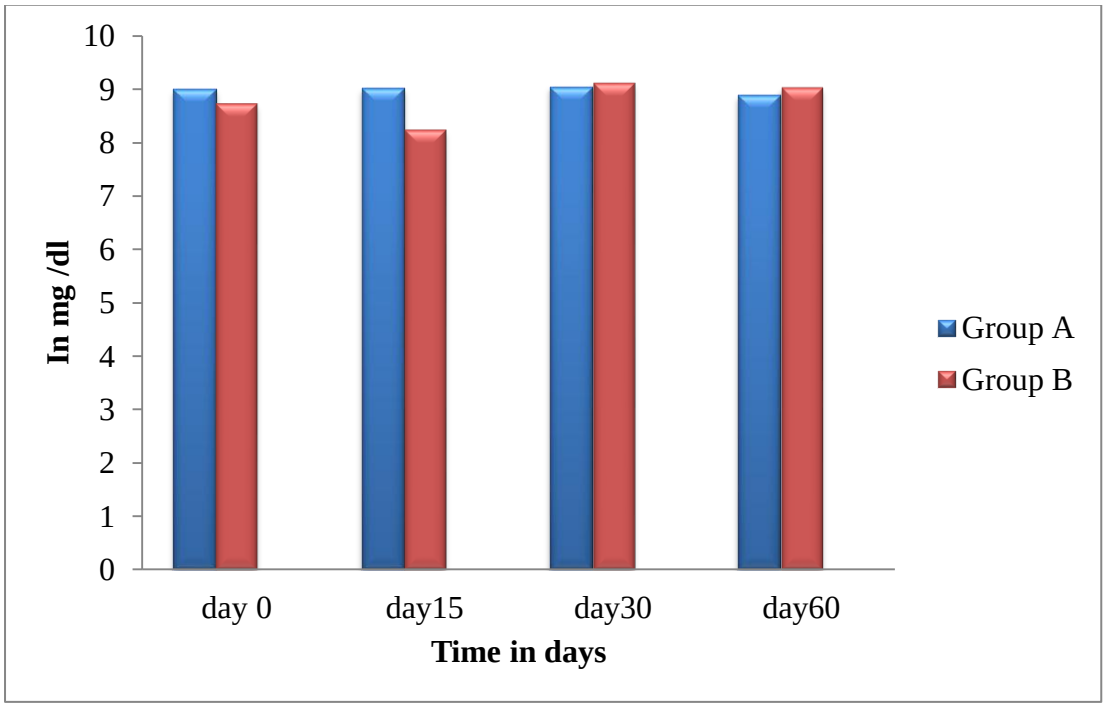


Fig. 34. Graphic representation of serum calcium values (mg /dl) in both the groups.

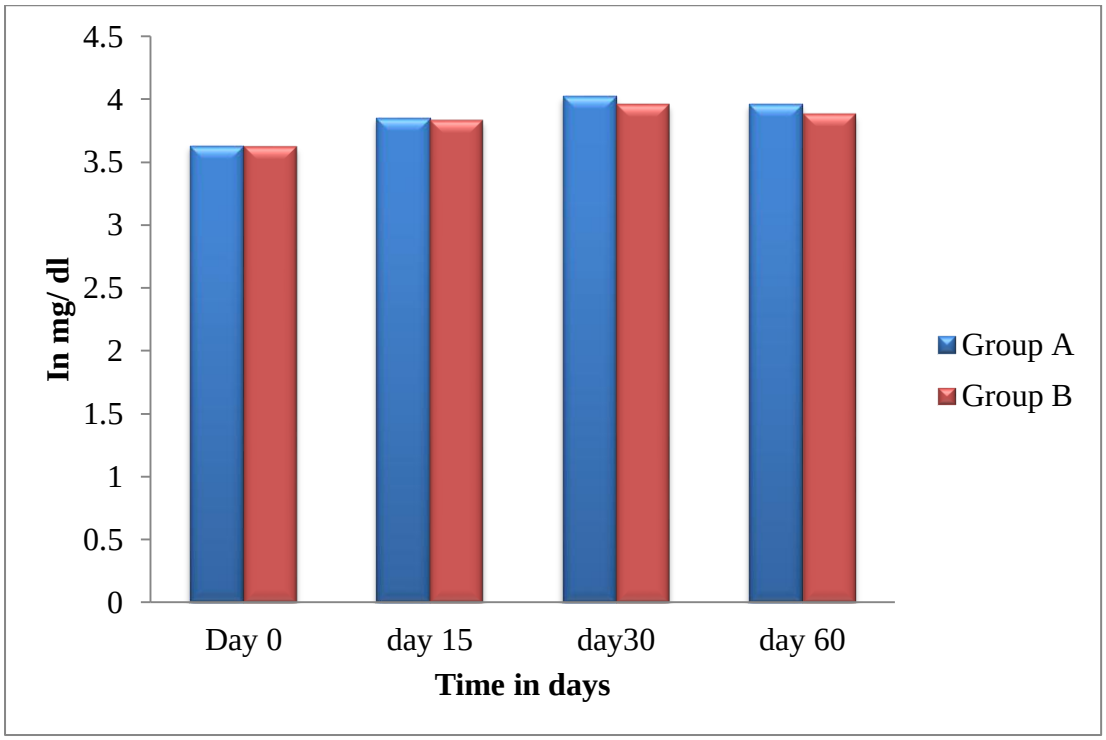


Fig. 35. Graphical representation of serum phosphorus values (mg/dl) in both the groups.

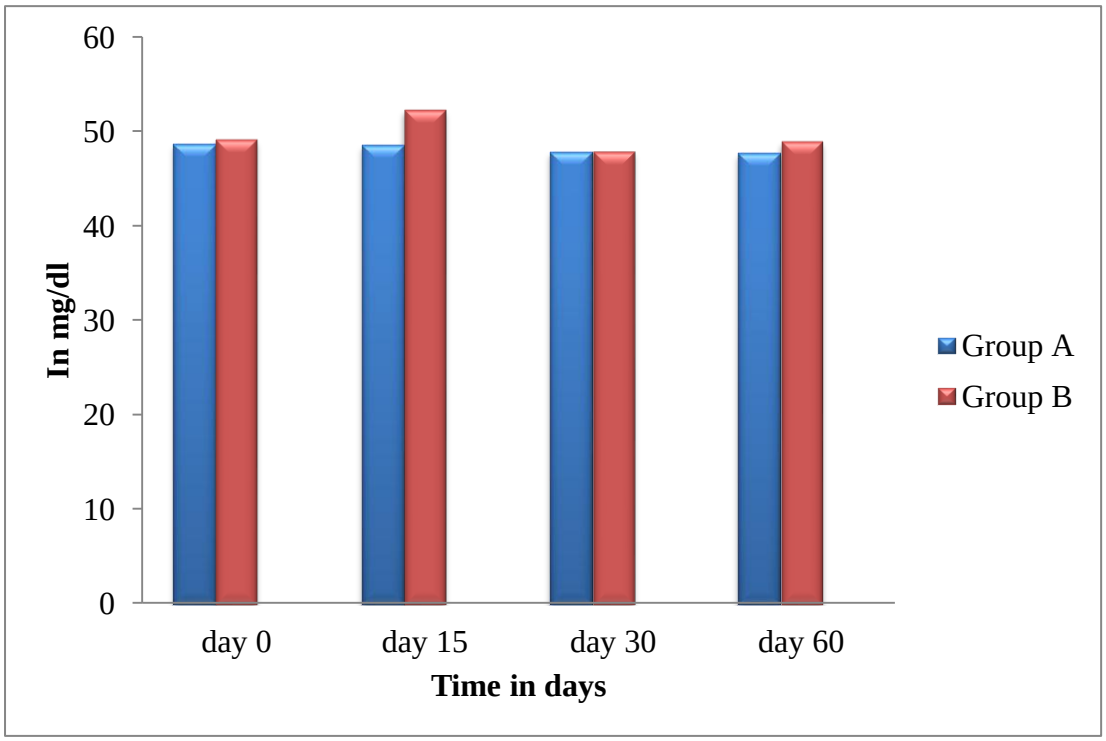


Fig. 36. Graphical representation of serum Alkaline Phosphates (IU/L) in both the groups.

4.15 Synovial fluid evaluation

The mean ± SE values synovial fluids in different animals are presented in Table 10. The colour of synovial fluid varied from creamy (n=4) to red tinged (n=5) on 1st day of collection before injection of ADMSCs. The neutrophils and lymphocytes count were 50.33±5.54 and 49.66±5.54respectively. On 15th day the colour of synovial fluid improved and became clearer as compared to day 0. The neutrophils and lymphocytes count were 49.33±5.25 and 56.11±5.25respectively. The neutrophils counts were decreased after ADMSCs treatment. However, there reduction was not significant (P>0.05). The count could be decreased due to anti-inflammatory activity of ADMSCs (Abumaree *et al* 2012).

Table 10: Synovial fluid values

Neutrophils		**Lymphocytes**	
Day 1	**Day 15**	**Day 1**	**Day 15**
50.33±5.54	49.33±5.25	49.66±5.54	56.11±5.25

In contrast present study Kiefer *et al* (2015) suggested that neutrophils and lymphocyte count may not be sensitive indicator of joint diseases in dogs. The volume of synovial fluid was found to be increased in dogs with joint disorders when compared to the synovial fluid volume in normal dogs. The synovial fluid from inflammatory joints shows variable discoloration. The normal fluid is clear to colorless. The red tinged colour could be due to hemorrhage during collection. In normal synovial fluid, neutrophils count ranges between 0-12 %. Similar observations were treated by Anirudh and Ranganath (2015). Similarly Brar et al (2000) reported neutrophils nearly 10% in normal synovial fluid. The percentage increase in septic arthritis (70-95%) and non-erosive arthritis (50-90%). In this study neutrophils values were around 50% so there was no septic arthritis present in any of the cases.

CHAPTER V

SUMMARY AND CONCLUSION

The present study was conducted on 25 clinical cases of dogs diagnosed with osteoarthritis (OA) which were presented to the Department of Veterinary Surgery and Radiology, College of Veterinary Sciences, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, during the period from April 2018- March 2019. Only those dogs were selected which were diagnosed with OA. The study was conducted in two phases.

In phase 1 collection, isolation, culture, subculturing, characterization of adipose derived mesenchymal stem cells was done under this section. The subcutaneous adipose tissue was collected aseptically (approximatily 5-10gm) from dogs brought for elective surgeries. The collected adipose tissue was then taken aseptically in a tube containing DPBS and Gentamicin to the laboratory, within half an hour after collection at ambient temperature, for further processing. Cells were successfully isolated by digesting with collagenase enzyme and then filtering the sample using cell strainers and centrifuged at 2200 rpm for 15 minutes at room temperature. The cells were cultured in medium that consists of high- glucose DMEM with L-glutamine (2 mM) 15% of FBS and gentamycin solution. Characterization was done in third passaged cells using Alkaline Phosphates staining and immunohisto-chemistry (CD44) and expanded for further use in clinical cases.

In phase II, the study was conducted on clinical cases of dog presented with osteoarthritis. The following observations were recorded as signalment, history, clinical signs, radiographic, hemato-biochemical analysis and synovial fluid examination. Phase II was subdivided to group A control group and group B treatment group. In group A, Medical management was done in sixteen clinical cases of dogs which were not previously treated with any medication and the standard treatment protocol followed:-Tablet petjoint 1 gm/day for 20kg, Tablet Carprofen 100mg, vitamin D3 granules 60,000 IU/gm as orally throughout the period. Group B, isolated ADMSCs were placed in sterile 1 ml syringe. About 4.5 to 5 million ADMSCs viable cells in DPBS, was used for intra-articular injection aseptically using 18G IV cannula under C-arm guidance. Two injections of allogenic canine ADMSCs were given in 15 days time interval. Total of nine joints were injected with allogenic canine ADMSCs.

The intra-articular injection was given in hip joint (6), stifle joint (2) and elbow joint (1) with OA lesion. On follow up after intra-articular injection with ADMSCs therapy and conventional medical treatment the animals were evaluated at interval of 15^{tt}, 30^{th} and 60^{th} days. The entire examination findings were recorded in 0 day, 15^{th}, 30^{th} and 60^{th} day after treatment. Lameness score was recorded with some modification according to Black *et al* (2007) on basis of three parameters lameness on walk (LW), Pain on palpation (PP) and Weight bearing while standing (WBS)

Repeated radiography was done before and after treatment to assess the efficacy of the treatment adopted. The radiography was done on 15^{th}, 30^{th} and 60^{th} days intervals after the treatment. The radiographic criteria of joint OA severities used in this study were according to the classification by FAO classification for hip joint as mild, moderate, severe.

The blood samples (5ml) were collected aseptically from cephalic/ saphenous vein. The samples of blood were collected on 0 day, 15^{th}, 30^{th} and 60^{th} day were subjected to following estimation: Haemoglobin (Hb g%),Total leukocyte count (TLC/ µL), Diffrential leukocytes count (DLC%) calcium (g/dL), phosphorus (g/dL), and alkaline phosphatase (U/L). Synovial fluid evaluation was done only in ADMSCs treatment group for inflammatory cells, mainly for neutrophils, lymphocytes and for colour. The samples were taken before the treatment and 15 days after the treatment with allogenic canine ADMSc. The data was analyzed using SAS (Statistical Analysis System) software version 9.3.

In phase 1 results, during primary culture, adhered cell attached to the cell culture flask showed round, non- fibroblastic, morphology during initial days of culture. Mesenchymal stem cells attached to cell culture flask after 72-96 hours, consisting of round and spindle shaped cells. As the growth continues ADMSCs attains 70-80% confluence at seven to ten days. Mesenchymal stem cell was subculture upto third passage. Alkaline phosphatase staining activity was detected in 3^{rd} passage cells of canine adipose derived mesenchymal stem cells that appear red after staining. Monoclonal antibody CD44 specific to MSCs was used to characterize the canine MSCs by immunohistochemistry. More than 95% of cell population expressed CD44 marker revealed by green colour immunofluorescence under florescent microscope.

In phase II results, the most common OA seen in dogs were hip joint osteoarthritis (72%) followed by stifle joint osteoarthritis (16%) followed by elbow joint osteoarthritis (8%). Prevalence of OA in different breeds was recorded as Labrador retriever (64%), Rottweiler (12%), German Shepherd (8%), Pug (8%), Bullmastiff (4%) and Pomeranian (4%). This study supported that older dogs above 8 years of age (40%) more affected than younger dogs. Out of 25 dogs, 16 (64%) dogs were having weight < 35kg and 76% of the dogs were males while 24% were female dogs.

At day 0, there were no significant difference found in between group A and group B in all the lameness scores. After the 1st treatment by ADMSCs on 15th day veterinary orthopedic examination score was done but no significant difference noticed. The improvement in clinical scoring was observed significant in group B after second dose of injection on day 30. This improvement was significant (P> 0.05) in treatment group B up to 60 days from 0 day in terms of pain on palpation, weight bearing on standing, lameness on walking. No significant improvement was seen in group A in terms of score at all intervals.

No radiographic change was evident after medication in both the groups. All the radiographic changes at 60th day were the same as the changes at baseline (day zero). The hemato-biochemical finding was not statistically significant as the mean value were within normal reference range for hemoglobin, TLC and Lymphocytes calcium, phosphorus, alkaline phosphate in both the groups throughout the experimental period. An increase in the number of neutrophils value was recorded in all the groups.

The neutrophils counts were decreased on 15th days after the 1st treatment of ADMSCs. The colour of synovial fluid on day 0 was red tinged to straw colour; however on day 15 the colour was clear and watery.

Conclusions:

1. The occurrence of OA was found to be high in hip joint of Labrador Retriever as compared to other joints and breeds.
2. Large breeds, heavy body weight and old age were predisposing factor for OA in dogs.

3. Alteration in physical character of synovial fluid and high neutrophils count in synovial fluid were good indicators of dogs with OA.
4. Radiography, hematology and biochemistry were not reliable in assessing the treatment outcome in dogs with OA.
5. C-arm guided intra-articular injection of allogenic viable canine ADMCs was effective in management of OA in dogs in terms of improved weight bearing, pain score and quality of life.

REFERENCES

Abdulazeeb S S. 2015. Diabetes treatment- a repair review of the current and future scope of stem cell research. *Saudi Pharmacological Journal* **23**(4): 333-40.

Abumaree M, Al Jumah M, Pace R A, Kalionis B. 2012. Immunosuppressive properties of mesenchymal stem cells. *Stem Cell Reviews* **8**(2): 375-92.

Alam M R, Lee H B, Kim M S and Kim N S. 2011. Surgical model of osteoarthritis secondary to medial patellar luxation in dogs. *Veterinarni Medicina* **56**(3): 123-30.

Alam M R, Lee H R, Park S Y, Lee Y H, Kim I S, Kang H S and Kim N S. 2006. Changes in the haematobiochemical parameters in experimental stifle osteoarthritis in dogs. *Pakistan Journal of Biological Sciences* **9**(15): 2869-74.

Anderson K L, O' Neil D G, Brodbelt D C, Church B D, Meson L R, Sargan D, Summer J F, Zulch H and Collins L M. 2018. Prevalence, duration and risk factors for appendicular osteoarthritis in a UK dog population under primary veterinary care. *Scientific Reports* **8**: 5641.

Anderson M A, Slater M R and Hammad T A (1999) Results of a survey of small animal practitioners on the perceived efficacy and safety of an oral neutraceutical. *Preventive Veterinary Medicine* **38**(1): 65-73.

Anirudh A and Ranganath L. 2015. Synovial fluid analysis in dogs with elbow, hip and stifle joint disorders. *International Journal of Applied and Pure Science and Agriculture* **1**(7): 15-18.

Aragon C L, Hofmeister E H, Budsberg S C. 2007. Systematic review of clinical trials of treatments for osteoarthritis in dogs. *Journal of the American Veterinary Medicine Association* **230**(4): 514-21.

Arinzeh TL, Peter SJ, Archambault MP, van den Bos C, Gordon S, Kraus K, Smith A, Kadiyala S. 2003. Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. *Journal of Bone and Joint Surgery American* **85**(10): 1927-35.

Baglioni S, Francalanci M, Squecco R, Lombardi A, Cantini G, Angeli R, GelminiS, Guasti D, Benvenuti S, Annunziato F, Bani D, Liotta F, Francini F, Perigli G, Serio M and Luconi M. 2009. Characterization of human adult stem-cell populations isolated from visceral and subcutaneous adipose tissue. *FASEB Journal* **23**(10): 3494-505.

Bahamondes F, Flores E, Cattaneo G, Bruna F,Conget P.2017. Omental adipose tissue is a more suitable source of canine mesenchymal stem cells. BMC *Veterinary Research* **13**(1): 166.

Balazs E A and Denlinger J L. 1993. Viscosupplementation: a new concept in the treatment of osteoarthritis. *Journal of Rheumatology Supplement* **39**: 3-9.

Barry F and Murphy M. 2013. Mesenchymal stem cell in joint diseases and repair. *Nature Reviews Rheumatology* **9**(10): 584-94.

Belshaw Z, Asher L and Dean R S. 2016. The attitudes of owners and veterinary professionals in the United Kingdom to the risk of adverse events associated with using non-steroidal anti-inflammatory drugs (NSAIDs) to treat dogs with osteoarthritis. *Preventive Veterinary Medicine* **131**: 121-26.

Bendele A. 2001. Animal models of rheumatoid arthritis. *Journal of Musculoskelet and Neuronal Interaction* **1**(4): 377-85.

Beraud M, Kolb A, Monteil V, D'Alayer J, Norel F. 2010.A proteomic analysis reveals differential regulation of the σ(S)-dependent yciGFE(katN) locus by YncC and H-NS in Salmonella and *Escherichia coli* K-12. *Molecular and Cellular Proteomics* **9**(12): 2601-16.

Berglud a k, Fortier L A, Antczak D F and Schnedl L V. 2017.Immunoprivileged no more: measuring the immunogenicity of allogenic adult mesenchymal stem cells. *Stem Cell Research and Therapy*. **8**: 288.

Bertolo A, Steffen F, Malonzo-Marty C and Stoyanov J. 2015. Canine mesenchymal stem cell potential and importance of dog breed : implication for cell based therapy. *Cell Transplant* **24**(10): 1969-80.

Bianco P, Robey P G and Simmons P J. 2008. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* **2**(4): 313-39.

Bijlsma J W, Berenbaum F and Lafeber F P. 2011. Osteoarthritis: An update with relevance for clinical practice. *Lancet* **377** (9783): 2115-26.

Black L L, Gaynor J, Gahring D, Adams C, Aron D, Harman S, Gingerich D A and Harman R. 2007. Effect of adipose derived mesenchymal and regenerative cells on lameness in dogs with chronic osteoarthritis of the coxofemoral joints: A randomized, double blinded, multicenter controlled trial. *Veterinary Therapeutics* **8**(4): 272-84.

Bland S. 2015. Canine osteoarthritis and treatment: A review. *Veterinary Science Development* **5**(2): 84-89.

Boothe D L, Gelfand A, Turkell R, Camp G, Ross G and Willer R. 1996. Round table discussion. Degenerative joint disease in dogs, part 1. *Canine Practice* **21**: 6-11.

Bourin P, Bunnell B A, Casteilla L, Dominici M, Katz A J, March K L, Redl H, Rubin J P, Yoshimura K, and Gimble J M. 2013. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: A joint statement of the international federation for adipose therapeutics (IFATS) and science and the international society for cellular therapy (ISCT). *Crtotherapy* 15(6): 641-48.

Brar R S, Sandhu H S and Singh A. 2000. Veterinary clinical diagnosis by laboratory method. 1st edition. *Kalyani Publications*, pp. 363.

Braun H J and Gold G E. 2012. Diagnosis of osteoarthritis: Imaging. *Bone* **51**(2): 278-88.

Brittberg M. 1994. Autologous chondrocyte implantation for cartilage repair. *Sports Medical Journal* : 40-47.

Bruder S P, Jaiswal N and Haynesworth S E. 1997. Growth kinetics, self renewal and osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following dryopreservation. *Journal of Cell Biochemistry* **64**(2): 278-94.

Budsberg S C, Torres B T, Kleine S A, Sandberg G S, Berjeski A K. 2018. Lack of effectiveness of tramadol hydrochloride for the treatment of pain and joint dysfunction in dogs with chronic osteoarthritis. *Journal of the American Veterinary Medical Association* **252**(4): 427-32.

Budsberg S C. 2004. Canine osteoarthritis: understanding the etiology, clinical presentation and diagnosis. Department of Small Animal Medicine and Surgery, College of Veterinary Medicine. The University of Georgia, Athens, GA 30602.

Caplan A I and Dennis J E. 2006. Mesenchymal stem cells as trophic mediators. *Journal of Cell Biochemistry* **98** (5): 1076-84.

Carapeba G O, Cavaleti P, Nicacio G M, Brinholi R B, Giuffrida R and Cassu R N. 2016. Intra-articular hyaluronic acid compared to traditional conservative treatment in dogs with osteoarthritis associated with hip dysplasia. *Evidence-Based Complement Alternative Medicine* **2016**(2076921): 1-10.

Chalman J A and Butler H C. 1985. Coxofemoral joint laxity and the Ortolani sign. *Journal of the American Animal Hospital Association* **21**: 671-76.

Chopp M, Zhang X H, Li Y Wang L, Chen J, Lu D, Lu M and Rosenblum M. 2000. Spinal cord injury in rat: Treatment with bone marrow stromal cell trans-plantation. *Neuroreporlogy* **11**(13): 3001-05.

Clark K C, Kol A, Shahbenderian S, Granick J L, Walker N J and Borjesson D L. 2016. Canine And Equine Mesenchymal Stem Cells Grown In Serum Free Media Have Altered Immunophenotype. *Stem Cell Review and Report* **12**(2): 245-56.

Coleman C M, Curtin C, Barry F P, O'Flatharta C and Murphy J M., 2010. Mesenchymal stem cells for osteoarthritis; remedy or accomplice?. *Human Gene Therapy* **21**: 1239-50.

Conaghan P G, D Agostino M A, Le Bar M, Baron G, Schmidely N, Wakefield R, Ravaud P, Grassi W, Martin-Mola E and So A. 2010. Clinical and ultrsonographic predictors of joint replacement for knee arthritis result from a large, 3-year perspective EULAR study. *Annals of the Rheumatic Diseases* **69**: 644-47.

Cuervo B, Rubio M, Sopena J, Dominguez JM, Vilar J, Morales M, Cugat R, Carrillo JM. 2014. Hip osteoarthritis in dogs: a randomized study using mesenchymal stem cells from adipose tissue and plasma rich in growth factors. *International Journal of Molecular Science* **15**(8): 13437-60.

Das J, Nath I, Das R K, Routray P and Behera S S. 2012. Autologus stem cell therapy to treat chronic ulcer in heifer – A case study. *Veterinary World* **5**(12): 771-74.

DeLuke A M, Allen D A, Wilson E R and Lineberger J A. 2012. Comparison of radiographic osteoarthritis scores in dogs less than 24 months or greater than 24 months following tibial plateau leveling osteotomy. *Canadian Veterinary Journal* **53**(10): 1095-99.

Denny H R and Gibbs C .1980. Osteochondritis dissecans of the canine stifle joint. *Journal of Small Animal Practice* **21** (6): 317-322.

Devi P, Sharma M, Singh D V, Lonare M K and Udehiya R. 2017. Viability and expression pattern of cryopreserved mesenchymal stem cells derived from buffalo bone marrow. *Ruminant Science* **6**(1): 7-12.

Devireddy L R, Myers M, Screven R, Liu Z and Boxer L. 2019. A serum- free medium formulation efficiently supports isolation and propagation of canine adipose- derived mesenchymal stem cell/ stormal cells. *PLoS ONE* **14**(2): e0210250.

Dominici M, Nichols K M, Levine A D, Rasko J E, Forte M, O'Donnell L, Koh M B, Bollard C M, Weiss D J. 2016. Science, ethics and communication remain essential for the success of cell- based therapies. *Brain Circlation* **2**(3): 146-51.

Dubey N K, Mishra V K, Dubey R, Syed-Abdul S, Wang J R., Wang P D, Deng W P. 2018. Combating osteoarthritis through stem cell therapies by rejuvenating cartilage: A review. *Stem Cells International* **2018**: 5421019.

Duval J M, Budsberg S C, Flo G L and Sammarco J L. 1999. Breed, sex, and body weight as risk factors for rupture of the cranial cruciate ligament in young dogs. *Journal of the American Veterinary Medical Association* **215**(6): 811-84.

Elders M J. 2000. The increasing impact of arthritis on public health. *Journal of Rheumatology* **60**: 6-8.

Eslaminejad M B and Nadri S. 2009. Murine Mesenchymal stem cell isolated and expanded in low and high density culture system: surface antigen expression and osteogenic culture mineralization. *In Vitro Cellular and Developmental Biology - Animal* **45**(8): 451-59.

Eyre D R, Weis M A and Wu J J. 2006. Articular cartilage collagen: An irreplecable framework?. *European Cells and Materials* **12**: 57-63.

Fang B, Song Y, Lin Q, Zhang Y, Cao Y, Zhao R C and Ma Y. 2007. Human adipose tissue- derived mesenchymal stromal cells as salvage therapy for treatment of

severe refractory acute graft-vs.-host disease in two children. *Pediatric Transplantation* **11**(7): 814-17.

Fortier L A and Travis L A. 2011. Stem Cell in Veterinary Medicine. *Stem Cell Research Therapy* **2**(1): 9.

Fox D B and Cook J L. 2001. Synovial fluid markers of osteoarthritis in dogs. *Journal of American Veterinary Medical* **219**(6): 756-61.

Freitag J, Wickham J, Shah K and Tenen A. 2019. Effect of autologous adipose-derived mesenchymal stem cell therapy in the treatment of acromioclavicular jointosteoarthritis. *BMJ Case Report* **12**(2): 15-18.

Fry T R and Clark D M. 1992. Canine hip dysplasia: clinical signs and physical diagnosis. *Veterinary Clinics of North America: Small Animal Practice* **22**(3): 551-58.

Gade N E, Pratheesh M D, Nath A, Dubey P K, Amarpal, Sharma B, Saikumar G and Sharma G T. 2012. Molecular and cellular characterization of buffalo bone marrow-derived mesenchymal stem cells. *Reproduction in Domestic Animals* **48**(3): 10-15.

Gimble J, Katz A and Unnell B A. 2007. Adipose-derived stem cells for regenerative medicine. *Circular Research* **100**(9): 1249-60.

Guercio A, Di Marco P, Casella S, Canella V, Russotto I, Purpari G, Di Bella S and Piccione G. 2012. Production of canine mesenchymal stem cell from adipose tissue and their application in dogs with chronic arthritis of humeroradial joint. *Cell Biology International* **36**(2): 189-94.

Gugjoo M B, Amarpal K P, Aithal H P, Ansari M M, Pawed A M and Sharma G T. 2015. Isolation, culture and characterization of New Zealand white rabbit mesenchymal stem cells derived from bone marrow. *Asian Journal of Animal and Veterinary Advances* **10**(10): 537-48.

Hadhammar A and Krook L. 1974. Over nutrition and skeletal disease; an experimental study in growing Great Dane dogs. *Cornell Vet* **64**(5): 1-59.

Harasen G. 2002. Diagnosing rupture of the cranial cruciate ligament**.** *Canadian Veterinary Journal* **43**(6): 475-76.

Hays L, Zhang Z, Mateescu R G, Lust G, Burton-Wurster N I and Todhunter R J. 2007. Quantitative genetics of secondary hip joint osteoarthritis in a labrador retriever-greyhound pedigree. *American Journal of Veterinary Research* **68**(1): 35-41.

Henrotin Y, Sanchez C and Balligand M. 2005. Pharmaceutical and nutraceutical management of canine osteoarthritis: present and future perspectives. *Veterinary Journal* **170**(1): 113-23.

Hercock C A, Pinchbeck A, Giejda P D, Clegg J F and Innes. 2009. Validation of a client-based clinical metrology instrument for the evaluation of canine elbow osteoarthritis. *Journal of Small Animal Practice* **50**(6): 266-27.

Hermann W, Lambova S and Muller-Ladner U. 2018. Current treatment options for osteoarthritis. *Current Rheumatol Review* **14**(2): 108-116.

Hielm-Bjorkman A K, Kuusela E, Liman A, Markkola A, Saarto E, Huttunen P, Leppaluoto J, Tulamo R M, Raekallio M. 2003. Evaluation of methods for assessment of pain associated with chronic osteoarthritis in dogs. *Journal of American Veterinary Medical Association* **222**(11): 1552-58.

Hur JW, Cho TH, Park DH, Lee JB, Park JY and Chung YG. 2016. Intrathecal transplantation of autologous adipose-derived mesenchymal stem cells for treating spinal cord injury: A human trial. *The Journal Spinal Cord Medicine* **39**(6): 655-64.

Innes J F, Costello M, Barr F J, Rudorf H and Barr A R. 2004. Radiographic progression of osteoarthritis of the canine stifle joint: A prospective study. *Veterinary Radiology and Ultrasound* **45**(2): 143-48.

Johnston S A. 1997. Osteoarthritis. Joint anatomy, physiology, and pathobiology. *Veterinary Clinics of North America-Small Animal Practice* **27**(4): 699-723.

Johnston S A and Budsberg S C. 1997. Non-sterodial anti- inflammatory drugs and corticosteroids for the management of canine osteoarthritis. *Veterinary clinic north america*: *small animal practice* **27**(4): 841-862.

Jung S, Panchalingam K M, Rosenberg L and Behie L A. 2012. *Ex vivo* expansion of human mesenchymal stem cells in definite serum free media. *Stem Cell International* **2012**: 1-21.

Jung Y K, Han M S, Park H R, Lee E J, Jang J A, Kim G W, Lee S Y, Moon D W and Han S. 2018. Calcium-phosphate complex increased during subchondral bone remodeling affects earlystage osteoarthritis. *Scientific Reports* **8**: 487.

Katz J N, Earp B E and Gomoll A H. 2010. Surgical management of osteoarthritis. *Arthritis Care Res (Hoboken)* **62**(9): 1220-28.

Kealy R D, Lawler D F, Ballam J M, Lust G, Biery D N, Smith G K and Mantz S L. 2000. Evaluation of the effect of limited food consumption on radiographic evidence of osteoarthritis in dogs. *Journal Of The American Veterinary Medical Association* **217**(11): 1678-80.

Kiefer K M, O' Brien T D, Elizabeth G Pluhar, Conzemius M. 2015. Canine adiposed derived stromal cell viability following exposure to synovial fluid from osteoarthritic joints. *Veterinary Record Open* **2**(1): e000063.

Koh Y G, Jo S B, Kwon O R, Suh D S, Lee S W, Park S H, Choi Y J. 2013. Mesenchymal stem cell injections improve symptoms of knee osteoarthritis. *Arthroscopy* **29**(4): 748-55.

Kon E, Muraglia A, Corsi A, Bianco P, Marcacci M, Martin I, Boyde A, Ruspantini I, Chistolini P, Rocca M, Giardino R, Cancedda R and Quarto R. 2000. Autologous bone marrow stromal cells loaded onto porous hydroxyapatite ceramic accelerate bone repair in critical-size defects of sheep long bones. *Journal of Biomedical Materials Research* **49**(3): 328-37.

Kresic N, Simic I, Lojkic I And Bedekovic T .2017. Canine adipose derived mesenchymal stem cell transcriptome composition alteration: a step towards standardizing therapeutic. *Stem Cell International* **2017**: 4176292.

Kriby G T , Mills S J , Cowin A J and Smith L E. 2015. Stem cell for cutaneous wound healing. *BioMed Research International* **2015**: 285869.

Kriston-Pal E, Czibula A, Gyuris Z, Balka G, Seregi A, Sukosd F, Süth M, Kiss-Toth E, Haracska L, Uher F and Monostori E. 2017 Characterization and therapeutic application of canine adipose mesenchymal stem cells to treat elbow osteoarthritis. *Canadian Journal of Veterinary Research* **81**(1): 73-78.

Krtolica A, Parrinello S, Lockett S, Desprez P Y and Campisi J. 2001. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proceeding of the National Academy of Science of the U S A* **98**(21): 15-18.

Kuyinu E L, Narayan G, Nair L S and Laurenci C T. 2016. Animal model of osteoarthritis: classification, update and measurements of outcomes. *Journal of Orthopedic Surgery and Research* **11**: 19-45.

Lamani S D, Ranganath L, Nagaraja B N, Satyanarayana M L, Jamuna K V and Kamran A. 2019. Incidence of osteoarthritis in dogs – a clinical study of 64 patients therapy. *International Journal of Science, Environment and Technology* **8**(3): 559-64.

Laura C, Groza I, Oana L, Pall E, Tean C P, Catana P and Cenariu M. 2008. Canine mesenchymal stem cells isolation from bone marrow aspirates. *Bulletin of University of Agricultural Sciences and Veterinary Medicine* **65**: 96-101.

Lawler D F, Larson B T, Ballam J M, Smith G K, Biery D N, Evans R H, Greeley E H, Segre M, Stowe H D and Kealy R D. 2008. Diet restriction and ageing in the dog: Major observations over two decades. *British Journal of Nutrition* **99**(4): 793-805.

Lazarus H M, Koc O N, Devine S M, Curtin P, Maziarz R T, Holland H K, Shpall E J, McCarthy P, Atkinson K, Cooper B W, Gerson S L, Laughlin M J, Loberiza F R Jr, Moseley A B and Bacigalupo A. 2005. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biology Blood and Marrow Transplant* **11**(5): 389-98.

Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M and Ringden O. 2004. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lance*t **363**(9419): 1439-41.

Leach D, Sumner-Smith G and Dagg A J. 1977. Diagnosis of lameness in dogs: A preliminary study. *Canadian Veterinary Journal* **18**(3): 58-63.

Lee R H, Kim B, Choi I, Kim H, Choi H S, Suh K, Bae Y C, Jung J S. 2004. Characterization and expression analysis of mesenchymal stem cells from human bone marrow and adipose tissue. Cellular Physiology Biochemistry **14**(4-6): 311-24.

Lee S J, Klein J P, Barrett A J, Ringden O, Antin J H, Cahn J Y, Carabasi M H, Gale R P, Giralt S, Hale G A, Ilhan O, McCarthy P L, Socie G, Verdonck L F, Weisdorf D J and Horowitz M M. 2002. Severity of chronic graft-versus-host disease: association with treatment-related mortality and relapse. *Blood*. **100**(2): 406-14.

Lee, K. S., Kang, H. W., Lee, H. T., Kim, H. J., Kim, C. L., Song, J. Y., Lee, K. W., Cha, S. H., 2014. Sequential sub-passage decreases the differentiation potential of canine adipose-derived mesenchymal stem cells. *Research in Veterinary Sciences* **96**: 267–75.

Leighton R L. 1981. Surgical treatment of osteochondritis dessicans of the canine stifle. *Journal of Veterinary Medicine Small Animal Clinics* **76**(12): 1733-36.

Loeser R F, Goldring S R, Scanzello C R and Goldring M B. 2012. Osteoarthritis: a diseases of the joint as an organ. *Arthritis Rhematology* **64**(6): 1697-707.

Lust G, Rendano U T and Summers B A. 1985. Canine hip dysplasia concepts and diagnosis. *Journal of American Veterinary Medical Assosiation* **187**: 638-40.

Man G S And Mologhianu G. 2014. Osteoarthritis pathogenesis - A complex process that involves the entire joint. *Journal of Medicine Life* **7**(1): 37-41.

Manferdini C, Maumus M, Gabus E, Piacentini A, Filardo G, Peyrafitte J A, Jorgensen C, Bourin P, Fleury- Cappellesso S, Facchini A, Noel D and Lisignoli G. 2013. Adipose derived mesenchymal stem cells exert anti-inflammatory effects on chondrocytes and synoviocytes from osteoarthritis patients through prostaglandin E2. *Arthritis Rhematology* **65**(5): 1271-81.

Markoski M M. 2016. Advances in the use of stem cells in veterinary medicine: from base research to clinical practice. *Scientifica(Cairo)* **2016**: 4516920.

Marshall W G, Hazewinkel H A W, Mullen D, Meyer G D, Baert K and Stuart C. 2010. The effect of weight loss on lameness in obese dogs with osteoarthritis. *Veterinary Research and Communications* **34**(3): 241-53.

Martin R B, Burr D B and Sharky N A. 1998. Skeletal Tissue Mechanics. *Sciendific Research.* New York. Springer.

Martinello T, Bronzini I, Maccatrozzo L, Mollo A, Sampaolesi M, Mascarello F, Decaminada M and Patruno M. 2011. Canine adipose derived mesenchymal stem cells do not lose stem features after a long term crypreservation. *Research in Veterinary Science* **91**(1): 18-24.

Marx C, Silveira M D, Selbach I, DaSilva A S, Braga L M, Camassola M and Nardi N B. 2014. Acupoint injection of autologous stromal vascular fraction and allogeneic adipose-derived stem cells to treat hip dysplasia in dogs. *Stem Cells International* **2014**: 391274

Mathur A and Martin J F. 2004. Stem cells and repair of the heart. *Lance*t **364**(9429): 183-92.

McCarty RC, Gronthos S, Zannettino AC, Foster BK and Xian CJ. 2009. Characterisation and development potential of ovine bone marrow derived mesenchymal stem cells. *Journal of Cell Physiology* **219**: 324-33.

McCoy A M. 2015. Animal models of osteoarthritis: Comparison and key consideration. *Veterinary Pathology* **52**(5): 803-18.

Mclaughlin R M and Roush J K. 2002. Medical therapy for patients with osteoarthritis. *Veterinary Medicine.* **97**.

Mele E. 2007. Epidemiology of osteoarthritis. *Veterinary Focus* **17**(3): 15-18.

Michel J, Penna M, Kochen J and Cheung H. 2015. Recent Advances in Hydroxyapatite Scaffolds Containing Mesenchymal Stem Cells. *Stem Cells International* **2015**: 305217.

Millis D L and Levine D.2013. Canine Rehabilitation and Physical Therapy.2nd Edition. *Veterinary Science and Veterinary Medicine* : 1-784.

Mimeault M and Batra S K. 2008. Recent progress on tissue-resident adult stem cell biology and their therapeutic implications. *Stem Cell Reviews and reports* **4**(1): 27-49.

Moore K W and Read R A. 1996. Rupture of the cranial cruciate ligament in dogs. Part II. Diagnosis and management. *Compendium on Continuing Education for the Practising Veterinarian* **18**(4): 381-391

Morgan J P. 1969. Radiological pathology and diagnosis of degenerative joint disease in the stifle joint of the dog. *Journal of Small Animal Practice* **10**(9): 541-44.

Morgan J P. 1992. Radiology in Veterinary Orthopedics, pp 185-186. Philadelphia, Lea & Febiger, 1972.

Mow V C, Ratcliffe A, Poole A R. 1992. Cartilage diarthrodial joints as paradigms for hierarchical materials and structurs. *Biomaterials* **13**: 67-97.

Murphy J M, Fink D J, Hunziker E B, Barry F P. 2003. Stem cell therapy in caprine model of osteoarthritis. *Arthritis Rheumatology* **48**(12): 3464-74.

Murphy S L, Robinson- Lane S G, Niemiec S L S. 2016. Knee and hip osteoarthritic management: A review of current and emerging non-pharmacological approaches. *Current Treatment Option in Rheumatology* **2**(4): 296-311.

Nair M B, Varma H K, Menon K V, Shenoy S J and John A. 2009. Reconstruction of goat femur segmental defects using triphasic ceramic-coated hydroxyapatite in combination with autologous cells and platelet rich plasma. *Acta Biomaterialia* **5**: 1742-755.

Naskar T. 2013. 'Studies on isolation, characterization of adipose tissue derived stem cells and its application in canine osteoarthritis patients'. M.V.Sc. Thesis, Kolkata University, India.

Nathan S, Das De S, Thambyah A, 2003. Cell based therapy in repair of chondral defect**:** A novel use of adipose tissue. *Tissue Engineering* **9**: 733-744.

Noth N, Steinert A F, Tuan R S. 2008. Technology insight: Adult mesenchymal stem cells for osteoarthritis therapy. *Nature Clinical Practice Rheumatology* **4**(7): 371-80.

O'Connor M D, Kardel M D, Iosfina I, Youssef D, Lu M, Li M M, Vercauteren S, Nagy A and Eaves C J. 2008. Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human embryonic stem cells. *Stem Cells* **26**: 1109-16.

Otto W R and Wright N A. 2011. Mesenchymal stem cell for experiment to clinic. *Fibrogenesis and Tissue Repair* **4**: 20.

Parys M, Nelson N, Koehl K, Miller R, Kaneene J B, Kruger J M and Yuzbasiyan-Gurkan V. 2016. Safety of Intraperitoneal Injection of Adipose Tissue-Derived Autologous Mesenchymal Stem Cells in Cats. *Journal of Veterinary Internal Medicine* **30**(1): 157-63.

Pasquinin C, Spurgeon T and Pasquinin S. 2007. *Anatomy of Domestic Animal Systemic and Regional Approach*. 11th Edn. pilot point. Sudz.

Petite H, Viateau V, Bensaïd W, Meunier A, De Pollak C, Bourguignon M, Oudina K, Sedel L and Guillemin G. 2000. Tissue-Engineered Bone Regeneration. *Nature Biotechnology* **18**(9): 959-63.

Pettitt R A, and German A J. 2015. Investigation and management of canine osteoarthritis. *In Practice* **37**: 1-8.

Piermattei D L and Flo G L. 1997. *Handbook of Small Animal Orthopedics and Fracture Repair*, 3rd Edn. Philadelphia: WB Saunders, pp. 534-37.

Piermattei D L, Flo G L and DeCamp C E. 2006. Brinker, Piermattei, and Flo's Handbook of small animal orthopedics and fracture repair. 4th ed. St. Louis, Mo.: *Saunders/Elsevier*, pp. 191-94.

Pittenger M F and Martin B J. 2004. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circulation Research* **95**(1): 9-20.

Pool A R. 2001. Cartilage in health and diseases. In: Koopman W T (Eds.) *Arthritis and Allied Conditions*. Lippincott Williams and Wilkins, Philadelphia, pp. 226-84.

Poole A. 1994. An introduction to the pathology of osteoarthritis. *Front Bioscience* **4**: 662-70.

Punwar S and Khan W S. 2001. Mesenchymal stem cells and articular cartilage repair: Clinical studies and future direction. *Open Orthopedic Journal* **5**(2), 296-301.

Rebelatto C K, Aguiar A M, Moretao M P, Senegaglia A C, Hansen P, Barchiki F, Oliveira J, Martins J, Kuligovski C, Mansur F, Christofis A, Amaral V F, Brofman P S, Goldenberg S, Nakao L S and Correa A. 2008. Dissimilar differentiation of mesenchymalstem cells from bone marrow, umbilical cord blood, and adipose tissue. *Experimental Biology and Medicine (Maywood)* **233**(7): 901-13.

Reich M C, Raabe O, Wenisch S, Bridger S P, Kramer M, Arnhold S. 2012. Isolation culture and chondrogenic differentiation of canine adipose tissue- and bone marrow- derived mesenchymal stem cells- a comparative study. *Veterinary Research Communications* **36**: 139-42.

Renberg W C. 2005. Pathophysiology and management of osteoarthritis. *Veterinary Clinic North America Small Animal Practice* **35**(5): 1073-91.

Renzi S, Lombardo T, Dotti S, Dessi S S, Blasio P and Ferrari M. 2012. Mesenchymal stromal cell cryopreservation. *Biopreservation and Biobanking* **10**(3): 276-81.

Rialland P, Bichot S, Moreau M, Guillot M, Lussier B, Gauvin D, Martel-Pelletier J, Pelletier J P and Troncy E. 2012. Clinical validity of outcome pain measures in naturally occurring canine osteoarthritis. *BMC Veterinary Research* **8**: 162.

Ringden O, Uzunel M, Rasmusson I, Remberger M, Sundberg B, Lonnies H, Marschall HU, Dlugosz A, Szakos A, Hassan Z, Omazic B, Aschan J, Barkholt L and LeBlanc K. 2006. Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* **81**(10): 1390-97.

Roemer F W, Crema M D, Trattnig S and Guermazi A. 2011 Advances in imaging of osteoarthritis and cartilage. *Radiology* 260: 332-54.

Roemer F W, Guermazi A, Felson D T, Niu J, Nevitt M C,Crema M D, Lynch J A, Lewis C E, Torner J and Zhang Y. 2011. Presence of MRI- detected joint affusion and synovitis increases the risk of cartilage loss in knee without osteoarthritis at 30 month follow up: The most study. *Annals of Rheumatics Diseases* **70**(10): 1804-09.

Runge J J, Biery D N, Lawler D F, Gregor T P, Evans R H, Kealy R D, Szabo S D and Smith G K. 2008. The effects of lifetime food restriction on the development of osteoarthritis in the canine shoulder. *Veterinary Surgery* **37**(1): 102-07.

Russell K A, Chow N H C, Dukkoff D, Gibson T W G, LaMarre J and Betts D H. 2016. Characterization and immunomodulatory effect of canine adipose tissue and bone marrow derived mesenchymal stromal cells. *PLoS ONE* **11**(12): 15-18.

Sandorson S L. 2012. The epidemic of canine obesity and its role in osteoarthritis. *Israel Journal of Veterinary Medicine* **67**(4): 15-17.

Sasaki A, Mizuno M, Ozeki N, Katano H, Otabe K And Tsujil K.2018. Canine mesenchymal stem cells for synovium have a higher chondrogenic potential that those from infrapatellar fat pad, adipose tissue and bone marrow. *PLoS ONE*, p. 13.

Schachner E R and Lopez M J. 2015. Diagnosis, prevention and management of canine hip dysplacea: A review. *Veterinary Medicine* **6**: 181-92.

Schlueter A E and Orth M W . 2004. Equine osteoarthritis: a brief review of the disease and its causes. *The International Journal of Animal Exercise Physiology Biomechanics and Nutrition* **1**(4): 22-31.

Shahid M, Manchi G, Slunsky P,Naseer O, Fatima A,Leo B, Raila J. 2017. A systemic review of existing serological possibilities to diagnose canine osteoarthritis with a particular focus on extracellular matrix proteoglycans and protein. *Polish Journal of Veterinary Science* **20**(1): 189-201.

Shivakumar S B, Bharti D, Jang S, Hwang S, Park J,Shin J, Byun J, Park B and Rho G. 2015. Cryopreservation of human wharton's jelly-derived mesenchymal stemcells following controlled rate freezing protocol using different cryoprotectants: A comparative study. *International Journal of Stem Cells* **8**(2): 155-69.

Skovrlj B, Qureshi S and Kern S. 2015. Mesenchymal stem cells for intervertebral disc repair and regeneration. *Seminars in Spine Surgery* **27**(2): 76-81.

Smith G K, Erin E R, Powers M Y, Lawler D F, Biery D N, Shofer F S, Mckelvie P J and Kealy R D. 2006. Lifelong diet restriction and radiographic evidence of osteoarthritis of the hip joint in dogs. *Journal of the American Veterinary Medical Association* **229**(5): 690-93.

Smith G K, Mayhew P D and Kapatkin A S, McKelvie P J, Shofer F S and Gregor T P 2001. Evaluation of risk factors for degenerative joint disease associated with canine hip dysplasia in German Shepherd dogs, Golden Retrievers, Labrador Retrievers, and Rottweilers. *Journal of American Veterinary Medicine Association* **219**(12): 1719-24.

Smith R K. 2008. Mesenchymal stem cell therapy for equine tendinopathy. *Disability and Rehabilitation.* **30**(20-22): 1752-8.

Stashak T S. 2006. Wound infection: Contributing factors and selected techniques for prevention. *AAEP Proceedings* **52**: 270-80.

Swagerty D L. 2001. Radiographic assessment of osteoarthritis. *American Family Physician* **64**(2): 279-87.

Takahashi M, Naito K, Abe M, Sawada T, Nagano A. 1998. Relationship between radiographic grading of osteoarthritis and the biochemical markers for arthritis in knee Osteoarthritis. *Arthritis Research Therapy* **6**(3): 208-12.

Tang Y, Li Y, Chen H, Wu Q, Yin G and Zhou D. 2006. Growth and osteogenesis characteristics of cultured canine mesenchymal stem cells under osteogenic induction. *Sheng Wu Yi Xue Gong Cheng Xue Za Zhi* **23**(1): 142-46.

Teshima R, Matsumoto H, Michishita M, Matsuoka A, Shiba M, Agashima T and Koyama H. 2017. Allogenic adipose tissue-derived mesenchymal stem cells ameliorate acute hepatic injury in dogs. *International* **2017**: 3892514.

Tharasanit T, Phutikanit N, Wangdee C, Soontornvipart K, Tantrajak S, Kaewamatawong T Suwimonteerabutr J, Supaphol P, Techakumphu M. 2011. Differentiation potential of canine bone marrow mesenchymal stem cells. *The Thai Veterinary Medicine* **41**(1): 79-86.

Toma C, Pittenger M F, Cahill K S, Byrne B J and Kessler P D. 2002. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105** (1): 93-98.

Udehiya R K, Amarpal., Aithal H P, Kinjavdekar P, Pawde A M, Singh R and Sharma G T. 2013. Comparison of autogenic and allogenic bone marrow derived mesenchymal stem cells for repair of segmental bone defects in rabbit. *Research in Veterinary Science* **94**: 743-52.

Vieira N M, Brandalise V, Zucconi E, Secco M, Strauss B E, and Zatz M. 2010. Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. *Cell Transplantation* **19** (3): 279-89.

Vilar J M, Morales M, Santana A, Spinella G, Rubio M, Cuervo B, Cugat R, Carrillo J M. 2013. Controlled, blinded force platform analysis of the effect of intra articular injection of autologus adipose derived mesenchymal stem cells associated to PRGF-endoret in osteoarthritic dogs. *BMC Veterinary Research* **9**: 131.

Wahl J M, Herbst S M, Clark L A and Murphy K E. 2008. A review of hereditary diseases of the German shepherd dog. *Journal of Veterinary Behavior* **3**(6): 255-65.

Wakitani S, Goto T, Pineda S J,Young R G,Mansour J M, Caplan A I and Goldberg V M. 1994. Mesenchymal cell based repair of large, full-thickness defects of articular cartilage. *Journal of Bone Joint Surgery American* **76**(4): 579-92.

Wang J S, Shum-Tim D, Galipeau J, Chedrawy E, Eliopoulos N, Chiu R C. 2000. Marrow stromal cells for cellular cardiomyoplasty: feasibility and potential

clinical advantages. *Journal of Thoracic Cardiovascular Surgeries* **120** (5): 999-1005.

Whitehair J G, Vasseur P B and Willits N H. 1993. Epidemiology of cranial cruciate ligament rupture in dogs. *Journal of the American Veterinary Medical Association* **203**: 1016-19.

Worth A J, Bridges J P, Cave N J and Jones G. 2012. Seasonal variation in the hip score of dogs as assessed by the New Zealand veterinary association hip dysplasia scheme. *New Zealand Veterinary Journal* **60**(2): 110-14.

Yaneselli K M, Kuhl C P, Terraciano P B, de Oliveira F S, Pizzato S B, Pazza K, Magrisso A B, Torman V and Rial A. 2018. Comparison of the characterstics of canine adipose derived mesenchymal stem cell extracted from different sites and different passage numbers. *Journal of Veterinary Science* **19**(1): 13-20.

Yanez R, Lamana M L, Garcia-Castro J, Colmenero I, Ramirez M and Bueren J A. 2006. Adipose tissue-derived mesenchymal stem cells have *in vivo* immunosuppressive properties applicable for the control of the graft-versus-host disease. *Stem Cells* **24**(11): 2582-91.

Yingling G L and Nobert K M. 2008. Regulatory consideration related to stem cell treatment in horses. *Journal of the American Veterinary Medical Association* **232**(11): 1657-61.

Yip H K, Lee M S, Sun C K, Chen K H, Chai H T, Sung P H, Lin K C, Ko S F, Yuen C M, Liu CF, Shao P L and Lee F Y. 2017. Therapeutic effects of adipose-derived mesenchymal stem cells against brain death-induced remote organ damage and post-heart transplant acute rejection. *Oncotarget* **8**(65): 108692-711.

Yusaf E. 2016. Pharmacologic and non-pharmacologic treatment of osteoarthritis. *Current Treatment Options in Rheumatology* **2**(2): 111-25.

Zhan X S, El-Ashram S, Luo D Z, Luo H N, Wang B Y, Chen S F, Bai Y S, Chen Z S, Liu CY and Ji H Q. 2019. A comparative study of biological characteristics and transcriptome profiles of mesenchymal stem cells from different canine tissues. *International Journal of Molecular Science* **20**(6): E1485.

Zuk P A, Zhu M, Mizuno H, Huang L, Futrell L W, Katz A L, Benbaim P, Lorenz H P and Hedrick M H. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Engineering* **7**(2): 211-28.

Zuk P A, Zhu M, Ashjian P, De Ugarte D A, Huang J I, Mizuno H, . Alfonso Z C, Fraser J K, Benhaim P, and Hedrick M H. 2002. Human adipose tissue is a source of multipotent stem cells. *Molecular biology of the cells* **13**(12): 4279-95.

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