PROLIFERATION AND CYTOTOXICITY ANALYSIS OF CANINE MESENCHYMAL STEM CELLS CULTURED ON FUNCTIONALIZED CARBON NANOTUBE

Thesis

Submitted to the
DEEMED UNIVERSITY
ICAR-Indian Veterinary Research Institute
Izatnagar - 243 122 (U.P.), India

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Physiology)

June, 2016

भाकृअनुप — भारतीय पशु.चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
इज़्ज़तनगर . २४३१२२, (उ.प्र.), भारत

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Dated: 29/06/16

Certificate

This is to certify that the research work embodied in this thesis entitled "Proliferation and cytotoxicity analysis of canine mesenchymal stem cells cultured on functionalized carbon nanotube" submitted by Dr. Madhusoodan.A.P. Roll No. M-5523, for the award of Master of Veterinary Science degree in Veterinary Physiology at ICAR - Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that Dr. Madhusoodan.A.P., Roll No. M-5523, has worked for more than 21 months in the institute and has put in more than 150 days attendance under me from the date of registration for the Master of Veterinary Science degree in this Deemed University, as required under the relevant ordinance.

(Dr. Sadhan Bag)
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Certificate

We the undersigned members of Advisory Committee of **Dr. Madhusoodan.A.P., Roll No. M-5523,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Physiology,** *agree that the thesis entitled* **"Proliferation and cytotoxicity analysis of canine mesenchymal stem cells cultured on functionalized carbon nanotube"** *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *degree of the Deemed University, ICAR - Indian Veterinary Research Institute, Izatnagar.*

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Dedicated to……

All those who stood with me in my difficult conditions…

Acknowledgement

I thought it is a right platform for giving due acknowledgement to all those people who molded my 25 years of life so memorable such that I can ruminate these events in rest of my life.

*Foremost I would like to thank my mother (**Smt.Nirmala**) and father (**Sri.Parameshwarappa**) who has been sacrificed their entire lives for providing education to me and my brother **(Deepu)**. They are true role models for me and their struggling journey is a path for me to walk rest of life.*

*My relatives especially 'Mama' (**Sri.Siddesh**) and 'Aunty' (**Smt.Suma**) showed me the value of relationship as well as their care and concern is indebtable.*

*My life changed at 6th standard as I got selected to prestigious 'Jawahar Navodaya Vidyalaya' (JNV). The main reason for this is my '**Gurus**', '**Sri. Mohankumar**' and his wife '**Smt. Kalavati**', who considered me as their own family member and helped me in several difficult situations and made me to crack the JNVST exam which changed my life and provided the right platform to aspire and achieve the goals. So, I would like to give due acknowledgement to my 'Gurus' and would like to remember their help and be faithful to them till my last breath. Whatever I achieved is because of their blessings.*

*In my 7 years of Navodaya life, I was fortunate to meet some of the inspirational teachers, motivational principals, true friends, and kind mess and hostel workers. Among the teachers, I would like to thank my Kannada teachers **Sri. Prakash.B.J.** and **Sri. Dharmanna Chitta** who inculcated me the leadership qualities, taught me to take responsibilities, importance of hardwork which helped me to handle the unimaginable situations. I would like to remember my maths teacher **Sri. B. P. N.***

Acknowledgement

***S wamy**, Science teacher **Sri.Ravi** and Biology madam **Smt. Rajani**, who helped me to gain lot of interest in Science (especially Biology) as well as in Maths. My histoty teacher, **Sri.Ishwar Singh** was true inspirational personality to me. My proud Navodaya friends **Kishor, Suresh, Nirmala, Vinutha** have taught me the value of friendship and I'm glad to remember them.*

*In my next of life which is veterinary life, I would like to thank my lecturer's namely (**Drs.**) **Kotresh.A.M., Adarsh, Sukaputra, Venkatesh** who shaped my professional life as well as motivated me to pursue higher studies by their inspirational stories and timely advice whenever the situation was demanding.*

*I would like to duly acknowledge '**Pragati Grameena Bank**', for providing me educational loan to lessen my financial burden as well as '**Sitaram Jindal Foundation**' for providing me the scholarship during my undergraduation.*

*My veterinary friends (**Drs**). **Anand, Prajwal, Ashoka, Pradeep, Girish** are worthy to remember as they helped me to achieve precious goal.*

*My seniors (**Drs**) **Prashanth, Karthik.Nagaraj** and Juniors (**Drs**) **Ramith, Karthik, Abhijeet** shaped my both academic and personal life by constantly supporting and guiding me in right pathway by understanding my feelings and emotions.*

*I would like to remember the contributions of librarian **Kamble sir** and office worker **Sri. Raghavendra** at my 5 years of undergraduation.*

*Two years of my post-graduation at IVRI is dream come true experience for me. I would like to thank **ICAR, IVRI, Director, Joint Director(s)** for providing financial support through **JRF** which reduced my financial dependence.*

*I would like to thank Head, **Dr.G.Taru Sharma** and scientists of my division **Dr. Puneet Kumar, Dr.V.P.Maurya, Dr.Mihir Sarkar, Dr.Gyanendra Singh, Dr.Vikash Chandra, Dr.V.S.Chouhan**, and **Dr. Hary Abdul Samad** for providing me the resourceful and learning environment which aided me in rising interest in the field of Veterinary Physiology and Climatology.*

*I would like to thank my advisory committee members **Dr.Mihir Sarkar**, Principal*

Scientist, P&LC division, ***Dr.S.K.Bhure****, Principal Scientist, Animal Biochemistry division,* ***Dr. V.S.Chouhan****, Scientist, P&LC division,* ***Dr.A.C.Saxena****, Veterinary Surgery division, for their kind suggestion and useful criticism to my research work.*

I would like to thank ***Dr. Ajaykumar****, Scientist, Animal Biochemistry division and* ***Dr. S.K.Ghosh****, Principal Scientist, Veterinary Parasitology division for providing facilities in their lab to carry out my research work.*

My colleagues (Drs) ***Bibhu, Tanmay, Chandu, Sourav, Nipuna and Vidyalaxmi*** *are true source of inspiration and I was mesmerized by their dedication and urge to achieve their dreams. Thank you guys for your invaluable memories.*

My seniors of the division namely (Drs) ***Mukesh, Jaya, Reshma, Sriti, Girijesh, Tejaswi, Avishek, Dileep, Irfan, Pankaj, Indu, Rajesh, Anjali, Matin, Mehtab, Smriti*** *were kind enough and were ready to help at anytime. I got to learn lot of deeds from the seniors. At the same time smiley juniors namely* ***Sheelendra, Amit, Anand, Megha, Lakshmi, Amrapali, Yawing*** *were cheerful.*

' ***Kannada Balaga****' in IVRI campus taught me the meaning of brothership. I would like to thank my seniors (Drs)* ***Anantakrishna, Badami, Sajjan, Yathish, Chethan, Harish, Priyanka, Dileep, Suman, Madhu, Chandramohan, Anjaneya, Chethan Sharma, Rakesh, Avinash, Balaraju, Manjunathachar, Santhosh Shinde, Rami Reddy, PJ, Sagar, Goutham, Sunitha*** *and Juniors (Drs)* ***Nikhil, Madhu, Sathish, Sharath, Manohar, Suraj, Vinay, Suhas, Vidyadhar, Darshan, Binduja, Manu****. I would like to mention our memorable after dinner hilarious and joyful walk with my friends and lovable juniors. I would like to thank my mess workers* ***Bharath, Chandan and Rahul*** *for serving delicious food.*

Value and beauty of friendship can't be put into lines. In this regard, my friends of Kannada Balaga namely ***Bhanu's(Chota & Bada), Chethu, Shivaraju, Saddam, Nitish, Partha, Chandan, Mohan, Sunil, Vikram, Arijith, Shruthi, Kavya, Nirupama, Prutvishri*** *were brilliant and gems of Kannada Balaga. Definitely I would miss this homely environment.*

Acknowledgement

Small things matters a lot. In this regard I would like to remember the work of ***Sri. Umashankar*** *and* ***Smt. Kalavati*** *for providing neat and clean aseptic glassware's to my research without which my research work would not have yielded this fruitful result.*

My lab seniors (Drs) ***Sanjhali and Purnima*** *were down to earth personalities from whom I learnt valuable lessons.*

I would like to remember the support of Young Scientist ***Dr. Kuldeep Kumar*** *for providing me the necessary help and advices.*

My laboratory alphabet learning was undertaken by ***Dr. Bhabesh Milli****, who was a eminent personality and soft spoken, well acquitted with scientific skills.*

My research skills and techniques are developed by the ***Dr. Kinshuk Das****, without whom my work would not have reached the target on stipulated time. I got to learn lot of qualities from him but the most impressed one is his systematic approach and sincerity to the work, which I would like to imbibe in my professional life.*

I'm blessed enough to be guided by ***Dr. Sadhan Bag****, Principal Scientist, P&C division, who was kind hearted enough to understand my situation and helped me in every aspects to make my stay at IVRI so comfortable and memorable. The working environment and facilities created by him in the laboratory was so amazing. I'm so glad to work under him. He is a true icon of scientific skills and knowledge. I'm finding shortage of words and phrases to describe him. 2 years of journey in the RP & ETT lab was amazing and marvelous.*

Still so many persons helped me in my journey directly and indirectly for whom I would like to thank at this moment.

Madhusoodan. A.P.

(Madhusoodan.A.P.)

Date: 30/06/2016

Place: IVRI, Izatnagar

Abbreviations

%	:Percentage
°C	: Degree Celsius
µg	: Microgram
µm	: Micrometre
3-D	: Three Dimensional
ALP	: Alkaline Phosphatase
ANOVA	: Analysis of Variance
BME	: Beta Mercaptoethanol
BSA	: Bovine Serum Albumin
cBM-MSCs	: Canine Bone Marrow Mesenchymal Stem Cells
CD	: Cluster of Differentiation
cDNA	: Complementary Deoxyribonucleic Acid
cm^2	: Square Centimetre
cMSCs	: Canine Mesenchymal Stem Cells
CNFs	: Carbon Nanofibers
CNTs	: Carbon Nanotubes
CO_2	: Carbon Dioxide
COOH	: Carboxyl
C_t	: Cycle Threshold
DAPI	: 4, 6-Diaminidino-2-Phenylindole
DMEM	: Dulbecco's Modified Eagle's Media
DPBS	: Dulbecco's Phosphate Buffered Saline
ECM	: Extracellular Matrix
EDTA	: Ethylene Diamine Tetraacetic Acid
ERK	: Extracellular signal-Regulated Kinases
ESCs	: Embryonic Stem Cells
FACS	: Fluorescent Assisted Cell Sorting

Abbreviations

FAK	: Focal Adhesion Kinase
FBS	: Fetal Bovine Serum
FITC	: Fluorescent Isothyocynate
h	: Hour
hBMMSCs	: Human Bone Marrow Mesenchymal Stem Cells
HLA	: Human Leucocyte Antigen
hMSCs	: Human Mesenchymal Stem Cells
hNSCs	: Human Neuronal Stem Cells
iPSCs	: Induced Pluripotent Stem Cells
m	: Metre
MAPK	: Mitogen-Activated Protein Kinases
mESCs	: Murine Embryonic Stem Cells
min	: Minutes
ml	: Millilitre
mM	: Millimole
MSCs	: Mesenchymal Stem Cells
MTT	: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MWCNTs	: Multi Walled Carbon Nanotubes
ng	: Nanogram
NGF	: Nerve Growth Factor
nmol	: Nanomole
NPCs	: Neural Progenitor Cells
O.D.	: Optical Density
OH	: Hydroxyl
PBS	: Phosphate Buffered Saline
PCR	: Polymerase Chain Reaction
PEG	: Poly Ethylene Glycol
PFA	: Paraformaldehyde
PI	: Propidium Iodide
PSCs	: Progenitor Stem Cells
RNA	: Ribonucleic Acid
ROCK	: Rho-associated Protein Kinase
rpm	: Rotation Per Minute

Abbreviations

RT	: Room Temperature
RT-PCR	: Real Time Polymerase Chain Reaction
SEM	: Scanning Electron Microscope
SWCNTs	: Single Walled Carbon Nanotubes
TEM	: Transmission Electron Microscope
TGF-ß	: Transforming Growth Factor-Beta

List of Tables

List of Figures

CONTENTS


Introduction

Introduction

Stem cells are the cells with a specific function with the ability of self-renewal, possess varied potency and differentiate into multilineages (Wei *et al*., 2013). Because of clinical applications and biological importance, stem cells become a prominent subject in modern research era (Ullah *et al*., 2015).

These cells are categorized as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells. Mesenchymal stem cells (MSCs) are adult stem cells which can be isolated from human and animal sources. MSCs are the non-haematopoietic, multipotent stem cells with the capacity to differentiate into mesodermal lineage such as osteocytes, adipocytes and chondrocytes as well ectodermal (neurocytes) and endodermal lineages (hepatocytes). MSCs express cell surface markers like cluster of differentiation (CD)29, CD44, CD73, CD90, CD105 and lack the expression of CD14, CD34, CD45 and HLA (human leucocyte antigen). MSCs for the first time were reported in the bone marrow and till now they have been isolated from various tissues, including adipose tissue, amniotic fluid, endometrium, dental tissues, umbilical cord and Wharton's jelly which harbours potential MSCs. MSCs have been cultured long-term in specific media without any severe abnormalities. Furthermore, MSCs have immunomodulatory features, secrete cytokines and immunereceptors which regulate the microenvironment in the host tissue (Ullah *et al*., 2015).In regenerative medicine and tissue engineering, MSCs are one of the best primary adult stem cell with continual proliferation and multipotent differentiation potential.

Nanoscience and nanotechnology are the study and application of extremely small particles and can be used across all the other fields of science such as chemistry, physics, materials science, engineering and medicine. The intersection of nanotechnologies with stem cell research in recent years has enthralled the scientific community to explore the new avenues like stem cell microenvironment and tissue engineering, stem cell tracking and imaging, stem cell transfection, isolation, sorting and molecular detection**.**

Introduction

A nanoscaffold is a three-dimensional structure with nanometer (10^{-9} m) scale composed of polymer fibers inherently provide a large surface area and high porosity structure with tenable chemical and physical properties that imparts unique physical and performance characteristics at the macroscopic level. The aim of designing different scaffolds is to simulate the best structural and environmental pattern for extracellular matrix. It is hypothesized that a close intimation to the natural ECM could provide scaffold with a more conductive environment to support the adhesion, migration, proliferation and differentiation of stem cells (Seyedjafari *et al.,* 2011). Recently, designing biocompatible nanomaterial based scaffolds along with the combination of MSCs is a trend in regenerative medicine and tissue engineering (Zhao *et al*., 2013; Akhavan *et al*., 2013; Ghoraishizadeh *et al*., 2014). And carbon nanotubes (CNTs) play a vital role in nanomaterial research due to their mechanical, optical, electrical and structural properties. In the field of regenerative medicine, these nanotubes are becoming increasingly attractive as they can be modified and used a scaffold material to be integrated into human bodies for promoting tissue regeneration and stem cell therapy.

One of the most effective ways to control the fate of stem cells is by changing the properties of the cell culture substrates, which can provide dynamic microenvironmental and morphological cues for stem cell proliferation and differentiation. CNT has emerged as a new potential cellular culture substrate that could alter the behavior of stem cells (Hazetline *et al*., 2013).

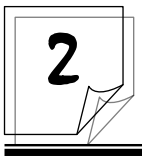
Although, nanotechnology is being used for last many years in stem cell based therapeutics in human, the application of this technology is in nascent stage in veterinary medicines and very few nanomaterials have been tested for culture and differentiation of stem cells in livestock species. As in human stem cell research, varieties of nanomaterial need to be explored for the advancement of cell based tissue regenerative in livestock. Since carbon nanotubes have been proved to be the one of the preferred nanomaterials in human stem cell based therapeutics, the potentiality of this in veterinary regenerative medicine should also be explored.

The present study is, therefore, aimed at studying the cellular behaviour, proliferation, differentiation potentiality including cytotoxicity of canine bone marrow derived mesenchymal stem cells in presence of hydroxylated carbon nanotubes (SWCNT and MWCNT) on culture substrate which will help to develop a carbon nanotube based composite scaffold for tissue regeneration in this species.

Objectives of the study:

1. To study the effect of OH-functionalized CNT on proliferation and differentiation of canine MSC.
2. To study the cytotoxicity of OH-functionalized CNT on canine MSC.


Review
of
Literature



Review of literature

2.1 Tissue engineering

Tissue engineering is defined by Williams (2009) as "the creation or formation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals".

To regenerate new tissues, the two most essential components are cells and scaffolds. The cells, which may be a part of an engineered tissue or recruited in vivo with the help of scaffolds, constitute the "prototype" of the living tissue to generate and to synthesize matrices for repopulation; the scaffolds provide an appropriate environment to facilitate intercellular contact and signaling, and consequently enable the cells to effectively accomplish their missions. In some cases, cells and scaffolds could collaborate with differentiation inducing factors, such as growth factors and chemical inducers, which function as "switches" for facilitating and committing the cells to differentiate into the respective cell lineages and attain the fully functionalized new tissue (Zhao *et al*., 2013).

2.2 Scaffolds in tissue engineering

In most native tissues, cells are contained within a tissue specific, three-dimensional (3-D) extracellular matrix (ECM), which comprises a complex network of nanoscale fibers forming highly structured local microenvironments (Gelain *et al*., 2006). Cellular communication, transport of oxygen and nutrients, removal of wastes and cellular metabolism require such an environment, where cellular orientation can be polarized and movement of contents can be directional. Hence, in tissue engineering, a 3-D framework is needed to organize the cells into a higher ordered assembly so as to achieve the desired tissue function. In addition,most of the engineered tissues/organs need support, named "scaffold", for their formation from cells. These scaffolds usually serve the purposes of assisting cell proliferation and differentiation, enabling diffusion of vital cell nutrients and expressed products, and exerting certain mechanical and biological

influences to the cells. It is generally hypothesized that a close imitation to the natural ECM could provide scaffold with a more conducive environment to support the adhesion,migration, proliferation and differentiation of stem cells (Liang *et al*., 2007; Seyedjafari *et al*., 2011).

Many physicochemical properties of ECM can exert subtle effects on the surrounding cells biological cues. For one, we should notice the nanostructure intrinsic to the natural ECM: (1) many tissues basement membranes exhibit enormous nanotopographies, which affect numerous cellular behaviors including adhesion, proliferation, migration and differentiation (Abrams *et al*., 2000); (2) ECM molecules such as collagen and hydroxyapatite crystals in bone exhibit rich nanostructures, which are hypothesized to contribute to cell matrix signaling (Bettinger *et al*., 2009). In order to better mimic the nanostructure in natural ECM, engineered nanomaterials, which are defined by the size of materials with at least one dimension less than 100 nm, have recently emerged as promising candidates in producing scaffolds that resemble the ECM and efficiently replace defective tissues (Lock and Liu, 2011).

Carbon-based nanomaterials can be used in scaffold design to recreate the specialized local microenvironment for optimal cell growth and differentiation to a specific cell type. Carbon-based nanomaterials can be beneficial tools for stem cell research involving multipotent and pluripotent stem cells, which are likely to play important roles in future regenerative medicine (Pryzhkova, 2013).

2.3 Carbon Allotropes

Carbon is an element involved in a number of natural processes on Earth. Carbon can form minerals and is an important constituent of the atmosphere. It is an indispensable component of chemical processes in living organisms and has routine applications in diverse, nonbiological areas of daily life. The numerous carbon forms (allotropes) identified to date include naturally occurring minerals (such as graphite, diamond, and coal) and fullerenes (such as buckyballs, graphene, and carbon nanotubes), which can be artificially synthesized and have more recently been found in nature (Pierson, 1993).

2.4 Carbon nanotube

CNT consists of a sheet/sheets of graphitic structure rolled into a cylinder. Due to its hexagonal structure and π electrons conjugation, CNT possesses high mechanical strength, flexibility, and electrical conductivity (Ijima *et al*., 1992; Hummer *et al*., 2001). Mainly three conventional methods have been used for synthesis of CNT such as arc discharge, laser ablation, and chemical vapor deposition methods (Zhang *et al*., 2011). CNT can be categorized into single-walled CNT (SWCNT), multi-walled CNT (MWCNT), and functionalized CNT. SWCNT is made up of a single sheet of graphene rolled up, and its ends are closed with fullerene caps. MWCNT is made up of multiple sheets of graphene cylinder. Functionalized CNT is modified CNT with specific organic groups attached on its surface. Its properties can be controlled in many ways as CNT can be easily functionalized (Fischer, 2002).

2.5 Mesenchymal stem cells

It is commonly believed that stem cell is involved in continuous maintenance and repair of most tissue types. Taken into consideration that stem cells are capable of self renewal and they share the ability to differentiate in multiple lineages. This class of cells is of paramount importance for an organism, not only during development, but also during adulthood with respect to cellular homeostasis. Stem cells for non-hematopoietic tissues, which can be derived from bone marrow, are commonly termed mesenchymal stem cell (MSC). MSCs are considered a readily accepted source of stem cells because such cells have already demonstrated efficacy in multiple types of cellular therapeutic strategies, especially in bone tissue regeneration strategies (Petite *et al*., 2000).

The multiline differentiation potential of MSC populations derived from a variety of different species has been extensively studied in vitro since their first discovery in the 1960s (Friedenstein *et al*., 1966). MSC has the capacity to differentiate into all connective tissue cell types, including bone, cartilage, tendon, muscle, marrow, fat and dermis. In vitro studies showed successfully differentiation in a variety of cell lineages, including osteoblasts, chondrocytes, adipocytees, fibroblasts, myoblasts and cardiomyocytes, hepatocytes, tenocytes, cenocytes, and even neurons (Jiang *et al*, 2002; Makino *et al*., 1999).

2.6 Mechanism of interaction between stem cells and nanomaterial scaffold

Stem cells play an important role in tissue engineering and regenerative medicine because

of their ability to self-renew and differentiate. Controlling the fate of stem cells is one of the most studied issues in tissue engineering. As a culture substrate, CNT has drawn tremendous interests in tissue engineering as it has the ability to dynamically direct stem cells lineage. For example, CNT has a high binding affinity to biological molecules such as extracellular matrix (ECM) proteins. Due to its high binding affinity to ECM proteins such as fibronectin, CNT can efficiently control cellular behavior (Namgung *et al*., 2011).

Cellular sensing of extracellular nanoscale architecture and dynamics of cell-ECM adhesions initiates downstream intracellular mechanotransductive events, resulting in a alteration cellular behaviours, including cell adhesion, morphology, proliferation, gene expression, self-renewal, and differentiation (Kingham and Oreffo, 2013).

The molecular mechanism of stem cell and its anchoring with nanoscaffold have been postulated to chiefly due to mechanotransduction, a process by which physical stimulate is converted to biochemical signalling pathway followed by cellular response. Integrins, the transmembrane receptors when bind to ECM proteins like fibronectin involved in a signal cascade that converts external mechanical signal to the cells into functional reaction by activating mainly ERK/MAPK and RhoA/ROCK signalling pathways (Biggs *et al.*, 2009). Although not properly established, Focal Adhesion Kinase (FAK) could be considered as a central regulator of this integrin mediated signalling since it facilitates cellular connection and spreading process. Activated FAK triggers the signalling of ERK/MAPK which upregulates gene transcription leading to cell cycling and replication (Chen *et al*., 2010). The signalling events of ERK/MAPK and RhoA/ ROCK pathways can induce osteogenic differentiation of MSCs (Zhao *et al*., 2013). Enhanced proliferation of neural progenitor cells (NPCs) on aligned collagen nanofibre also shows the involvement of activated endogenous MAPK (Wang *et al*., 2011).

2.7 Cellular behaviour and proliferation on carbon nanotube based scaffold

One of the most effective ways to control the fate of stem cells is by altering the cell culture substrate property, which can provide dynamic microenvironment and morphological cues for stem cell proliferation and differentiation. As the mechano-transduction plays a key role in stem cell proliferation and differentiation it can be reasoned that different physiochemical properties of nanomaterial scaffolds may influence the fate of it. Surface chemistry, roughness, stiffness, size and alignment of

nanomaterials have profound effect on stem cell behaviour, proliferation and differentiation (Zhao *et al*., 2013). Recently, the interactions between CNTs and various types of stem cells have begun to be studied. CNT has emerged as a new potential cellular culture substrate that could alter the behaviour of stem cells (Hazeltine *et al*., 2013).

Adhesion and growth experiments on pluripotent P19 mouse embryonic stem cell demonstrate that the cells adhered more strongly to MWCNTs than to uncoated glass surface but the rate of cell proliferation was slow. The cells appear to become somewhat more flattened after four days, and many of the cells exhibit more irregular outlines, reflecting the presence of filopodial cell extensions (Holy *et al*., 2011).

Cell proliferations on carbon nanotube scaffolds were investigated (Kitahara *et al.,* 2010) for the embryonic stem cells (ES cells) of mouse and the bone marrow-derived mesenchymal stem cells (MSC) of rat. Although ES cells were well attached and proliferated on gelatin-coated plastic dishes, they did not attach on the CNT-coated scaffold, and formed the spheroids growing in the culture medium. For MSC, they attached on the CNT-coated scaffold and grew to form mineralized particles as on the plastic dishes of control. The growth rate of the MSC was lower than control but showed the higher expression of alkaline phosphatase activity per cell in 2 weeks and degree of mineralization per cell for 3 and 4 weeks.

Tay *et al*., (2010) compared the human mesenchymal stem cell proliferation, behaviour on a SWCNT film resulting in larger cell area, significant cell spreading and focal adhesion distribution on the film resulting in larger cell area and have higher occurrence of filopodia (microspikes) at the cell boundaries as compared to cover slip. These cytoskeleton arrangements suggest that nano roughness alone is sufficient to modulate cellular behaviour although the proliferation rate was found to be slow.

Namgung *et al*., (2011b) reported that human mesenchymal stem cells (hMSCs) grown on CNT networks could recognize the arrangement of individual CNTs in the CNT networks, which allowed controlling the growth direction and differentiation of the hMSCs. Furthermore, hMSCs on aligned CNT networks exhibited enhanced proliferation and osteogenic differentiation compared to those on randomly oriented CNT networks.

2.8 Differentiation of MSCs co-culture on carbon nanotube based scaffold

2.8.1 Osteogenic differentiation

Having a density similar to graphite and much lower than that of other metallic bone scaffold materials, CNTs have been viewed as excellent stimulant scaffolds for

growth and proliferation of osteoblasts, the bone forming cells (Zanello *et al.,* 2006). Among the many application studies of CNTs in the culture system (Aoki *et al.,* 2005; 2007), a remarkable observation is that a cell line Saos-2, which is rather differentiated osteoblastic cells, strongly attached and extremely extended upon CNTs as a culture substrate (Aoki *et al*., 2006).

Nayak *et al*., 2010 investigated the effects of a thin film of pegylated multiwalled carbon nanotubes spray dried onto preheated cover slips in terms of their ability to influence human mesenchymal stem cells' proliferation, morphology, and final differentiation into osteoblasts. Interestingly, cell differentiation occurred even in the absence of additional biochemical inducing agents, as evidenced by multiple independent criteria at the transcriptional, protein expression, and functional levels. Kitahara *et al*., 2010 reported a less attachment of mouse ES cells on CNT-coated scaffold whereas rat MSC attached well and grew to form mineralized particles. Although the growth rate of MSC was lower than control but showed a higher expression of alkaline phosphatase (ALP) activity per cell in 2 weeks.

Osteogenic differentiation potential of rat MSCs were studied by Baktur *et al*., 2013 on different varieties of MWCNT-collagen scaffolds, differentiation thereon is investigated by monitoring the transcription factor RunX2 (RunX), transforming growth factor β (TGF-β), alkaline phosphatase, osteocalcin, and mineralized nodules of extracellular matrix (ECM). The MWCNT-collagen scaffolds induced significant increases in ALP activity and ECM mineralization due to the increased stiffness and strength of the scaffold by entrapping MWCNTs.

When human preosteoblasts and murine embryonic stem cells were cultured on coverslips sprayed with COOH–SWCNTs, mineralization of the ECM was noticed along with increased osteogenic differentiation noticed by amplified expression of classical bone marker genes and an increase in the secretion of osteocalcin. This result of Durán *et al*., 2014 predicated that COOH–SWCNT scaffold might further promote osteogenic differentiation in vivo.

In contrary COOH-functionalized SWCNT or MWCNT have been reported to cause a dose-dependent decrease in mesenchymal stem cells viability, proliferation and differentiation toward the osteogenic or adipogenic lineage (Mooney *et al*., 2008; Liu *et al*., 2010) although carboxylated MWCNT were able to inhibit apoptosis and to promote myogenic differentiation from myoblast cells (Zhang *et al.,* 2012).

2.8.2 Chondrogenic differentiation

The cell type which is highly responsible for cartilage regeneration is chondrocytes. Mesenchymal stem cells are increasingly being considered as a promising alternative to differentiated chondrocytes for use in cell-based cartilage repair strategies (Chen *et al*., 2006). Mixture of carbon nanotubes into the sheets of polycarbonate urethane and an FDA-approved polymer, which has a rough surface and also readily conducts electricity allowed to grow the chondrocytes more densely on the roughened surface compared to a smooth polycarbonate surface (Humphrius, 2008). The effect of SWCNT surface functionalization (-COOH or -PEG) on chondrocyte viability and biochemical matrix deposition was examined by Chahine *et al*., 2014. Outcome included cell viability, histological and SEM evaluation, gene expression quantification including ECM markers and surface adhesion protein. Findings indicated that chondrocytes tolerated functionalized CNT well with minimum toxicity. Mooney *et al*., (2008) studied the differentiation potential of hMSC on COOH-SWCNT and OH-MWCNT suspended media. In comparison to the adipogenesis and osteogenesis assay, there was a slight decrease in chondrogenesis.

2.8.3 Neuronal Differentiation

Nanotopographical materials have been widely demonstrated for promoting neural lineage commitments of PSCs. In a study by Xie *et al.*, (2009) uniaxially aligned nanofiber substrates were used to induce mouse ESC differentiation into different neural lineages, such as neurons, oligodendrocytes, and astrocytes.

Mattson *et al*., (2000) provided the first evidence that MWCNTs can be used to support neuronal cell attachment and growth. CNT have a much higher electron mobility potential (Durkop *et al*., 2004), and therefore theoretically much higher conductivities (Zhou *et al*., 2000). In addition, CNT have chemically inert surfaces that can be specifically modified to promote desired cell responses and nanoscale roughness and porosity created by CNT materials offer a unique neuronal interface that can be beneficial for recording fine neural signals in dynamic systems (Johansson *et al*., 2006).

The passive conductance of CNT makes them attractive as substrate coatings for neural culture; however, the effects of conductivity on neural cell growth are not fully understood (Voge and Stegemann, 2011). The addition of the nanotubes conferred conductivity to the nanofibers and promoted mESC neural differentiation as evidenced by an increased mature neuronal markers expression (Kabiri *et al.*, 2012).

Malarkey *et al* (2009) compared cell culture substrates coated with PEI to surfaces coated with hydrophilic SWNT-polyethylene glycol (PEG) films. It was shown that thinner, less conductive SWNT films resulted in longer neurite processes, while thicker, more conductive films produced larger cell bodies, compared to controls.

Furthermore, studies have shown that CNTs chemically functionalized with various bioactive molecules can improve neural regeneration activity including neurite branching, outgrowth and attachment of growth cones (Hu *et al*., 2005). In addition, the high electrical conductivity of CNTs can enhance neuronal circuit network activities. Lovat *et al*., (2005) reported that the electrical signal transfer on neuronal networks can potentially be improved by using purified MWCNTs. In addition, neuron cell viability, adhesion and differentiation on a freestanding layer-by-layer assembled SWCNT/polymer thin-film membrane were tested (Gheith *et al*., 2005). Results revealed that SWCNT/ polymer films favorably induced NG108- 15 cell attachment and differentiation guided neurite outgrowth and directed more elaborate branches than on control culture dishes.

Park *et al*., (2011) demonstrated a structural-polarization-controlled neuronal differentiation of hNSCs using the patterns of CNT monolayer coating. The result of the cell viability assay (>97%) suggested a good biocompatibility of CNT patterns for hNSC growth and differentiation. CNTs could induce the adhesion and growth of hNSCs even better than conventional cell-culture substrates such as bare glass. Importantly, the structural-polarization-controlled neuronal differentiation was demonstrated at the level of an individual axon or neurite.

Sridharan *et al.,* (2009) studied the ability of a combination collagen/single-walled carbon nanotube (SWCNT) substrate to direct the differentiation of human embryonic stem cells. Their analysis indicated that the collagen/CNT formed rigid fibril bundles, which polarized the growth and differentiation of hESCs, resulting in more than 90% of the cells to the ectodermal lineage in Day 3 in the media commonly used for spontaneous differentiation. Holy *et al*., 2011 studied the neuronal differentiation of Pluripotent P19 mouse embryonic stem cell on thin film of MWCNT. The authors found that the expression patterns of some neuronal differentiation markers were increased in cells grown on MWCNTs.

The nanostructure of the LBL-assembled SWNT-polyelectrolyte composite, which gives rise to the composite's structural flexibility, chemical stability, and physical

properties, showed no adverse affect on the differentiation of NSCs. NSCs behaved similarly to those cultured on the standard and widely used PLO substratum in terms of cell viability, the development of neural processes, and the appearance and progression of neural markers (Jan and Kotov, 2007).

The use of carbon nanotubes (CNTs) to promote neural differentiation is well known but interestingly carboxylated MWCNT substrates may provide a method of post- transplantational spontaneous neural differentiation. Chen and Hsiue, (2013) demonstrated that carboxylated multiwalled carbon nanotubes (MWCNTs) can induce and maintain neural differentiation of human bone marrow mesenchymal stem cells (hBMMSCs) without any exogenous differentiating factors, as evidenced by the protein expression. The low cytotoxicity of carboxylated MWCNTs was also shown by a proliferation assay. Collagen hydrogel provides excellent 3-D conditions for rat MSC growth, and a small incorporation of CNTs within the hydrogel significantly stimulates MSC expression of neural markers GAP43 and βIII tubulin and secretion of neurotrophic factors like NGF and brain derived neurotrophic factor (Lee *et al.,* 2014).

Table 1: Different CNT based scaffolds used in stem cell research/therapy

Components	Cell type	Applications	References
SWCNTs	hMSCs	Neural tissue engineering	Tay *et al*., 2010
Laminin/SWNTs	hNSCs	Neural tissue engineering	Kam *et al*., 2009
Poly(methacrylic acid) grafted CNTs	hESCs	Neural tissue engineering	Chao *et al.,* 2010
Poly(acrylic acid) grafted CNTs	hESCs	Neural tissue engineering	Chao *et al*., 2009
PLGA/c-MWCNT	Rat MSCs	bone tissue engineering	Bao *et al*., 2011
Collagen-MWCNT	Rat primary os-teoblasts (ROBs)	bone tissue engineering	Hirata *et al*., 2010
Surface coated CNT	h-chondrocytes	Cartilage tissue engineering	Humphrius, 2008
PLA nanofibres modified with CNT and gelatin (GEL)	h-chondrocytes	Cartilage tissue engineering	Markowski *et al.,* 2015
Highly purified multiwall carbon nanotube	Myoblastic mouse cells (C2C12)	Adipose-like lineage	Tsukahara and Haniu, 2011

2.9 Cytotoxicity of Carbon Nanotubes on cells

Since CNTs and CNFs (carbon nanofibers) are being extensively explored for various applications, becoming commercially available and being mass produced, assessing health risks of those nanomaterials is of extreme importance. One of the greatest concerns about toxicity effects of CNTs/CNFs is their risk in the workplace and the environment (Tran *et al*., 2009).

Pristine, as-produced CNTs tend to agglomerate and making it difficult to use them in biological systems and have cytotoxic effect to some extent as reported by Tian *et al*., 2006 working on human fibroblast cell. The cytotoxicity of pristine CNTs is due to the residual metal catalysts and also the insolubility of pristine CNTs (Bianco *et al*., 2005).

Therefore, to integrate CNTs into biological systems, CNTs need to be functionalized. Functionalization can make CNTs soluble and improve their biocompatibility properties and some researchers claim that functionalised CNTs are less toxic and only slightly affect cells viability (Andersen *et al*., 2012; Ursini *et al*., 2012). The PLA-CNT nanofibres degraded in a biological environment with the time, and it might be expected to expose the surface of carbon nanotubes. This biological test on human osteoblast-like MG 63 cells indicated that the samples containing carbon nanotubes were not toxic (Wieche´c *et al*., 2012). Homogeneous layer of functionalized nanotubes did not show any cytotoxicity and accelerated hMSC cell differentiation to a higher extent than uncoated cover slips, by creating a more viable microenvironment for stem cells (Nayak *et al*., 2010).

To assess the influence of the nanofibrous scaffolds upon human chondrocytes, tests for cytotoxicity and genotoxicity were performed (Markowski *et al*., 2015). The work revealed that the nanofibrous structures studied were neither genotoxic nor cytotoxic, and their microstructure, physical and mechanical properties create promising scaffolds for potential use in cartilage repairing**.**

Functionalized CNTs have been shown in many studies to be able to cross cell membranes (Pantarotto *et al*., 2004; Shi Kam *et al*., 2004) which allowed them to become of particular high interest for drug delivery and gene delivery strategies (Tran *et al*., 2009).

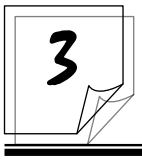
Tian et al., (2006) tested the cytotoxicity of 5 forms of carbon on human fibroblast cells, there was decreased cell survival and up regulation of apoptotic genes

(p16,bax,p53,hrk). They concluded that the material with lowest surface area have highest toxicity.

Cytotoxicity of MWCNT's with neuroblastoma cells assessed by incubating cells with 3 different purity MWCNT's. Results indicated cell viability is not affected and apoptosis and ROS are not induced. With prolonged incubation, cell viability was minimal for pure MWCNT's (99%). (Vittorio,O., *et al*., 2009).

Cytotoxicity of pristine,-OH & -COOH functionalised MWCNT's on respiratory alveolar epithelial cells analyzed. Results indicated that P-MWCNT's and COOH-MWCNT's induce significant cytotoxicity but OH-MWCNT do not induce a significant increase in cytotoxicity. (Ursini *et al.,* 2016).


Materials and
Methods



Materials and Methods

3.1 Chemicals and Media

All media and chemicals of M/S Sigma chemicals Co. St. Louis, MO, USA were used unless otherwise mentioned. 0.22µm, 0.45µm filters from Millipore corporation, Bed ford, MA, USA and disposable Petri dish, tissue culture flask (T-25, T-75, 6well, 12well) and 15 ml graduated tube of 'Trueline' brand product were used. All the procedures were carried out in highly sterile condition under laminar air flow cabinet to avoid any bacterial or fungal contamination. Water used for media preparation was from reverse osmosis milli Q water system. All the prepared media excluding DPBS were kept at least 3-4 hours in CO_2 incubator at 37^0C and 5% CO_2, 95% relative humidity for quenching before use.

3.2 Collection of canine bone marrow

Bone marrow content was collected from domestic dog (*Canis lupus familiaris*) at the Referral Veterinary Polyclinic, IVRI, Izatnagar campus. Briefly, the bone marrow was aspirated from the lateral aspect (either side) of superior iliac crest area of anesthetized dogs with the help of an 18 gauge bone marrow biopsy needle in an aseptic manner. For the collection of bone marrow (BM), the needle was inserted through the skin and the muscle with little force. Once the needle made contact with the bone, it was advanced by rotating it slowly until the bone cortex was penetrated. The stylet of the needle removed and 4-5 ml bone marrow was drawn/ aspirated into a hypodermic needle containing heparin. The needle was then removed and the same procedure followed in the contra- lateral bone to collect another bone marrow aspirate in the same syringe. Thus a total quantity of 8-10 ml of bone aspirate was collected from each animal.

3.3 Isolation of mesenchymal stem cells from bone marrow

The bone marrow sample was mixed with an equal amount of Dulbecco's phosphate buffered saline (DPBS) and layered onto 4 ml of Ficol- Histopaque and made

the total volume upto 9-10ml. The mixture is then subjected to centrifugation at 3000 rpm for 30 minutes and the nucleated cells were collected from the interface. The cells were mixed with DPBS followed by centrifugation (1500 rpm for 5 minutes each) and the cell pellet was re-suspended with a small volume of DPBS. The cells were counted and plated at the rate of $2x10^5$ cells/cm^2 in tissue culture flasks. The cells were maintained in DMEM (low glucose) culture medium supplemented with 15% fetal bovine serum with antibiotics (100 units/ml of penicillin and 100 µg/ml of streptomycin) in an atmosphere of 5 % CO_2 with 95% humidity at 37° C for 48 hours. After 48 hours of primary culture, all the non adherent cells were removed by changing the medium. Thereafter, the media was supplemented every third day.

3.4 Propagation and characterization of canine mesenchymal stem cells

Once the cells attained 80-90% confluency, they were propagated several times to increase the population. After aspirating the culture media, cell layer was washed twice with DPBS. Later, trypsin /EDTA (0.25%) was added into the flask and incubated at 37° C for 5 minutes or until the cells were separated from the bottom of the flask. Double the volume of fresh culture medium was added to the flask and mixed gently with strokes, transferred into a tube and centrifuged at 1000 rpm for 5 min at room temperature. The cell pellet was re-suspended in 1ml DMEM culture media, counted and seeded in an optimum density (usually 1;3 ratio) to a new flask containing fresh media. Cells were observed under inverted phase contrast microscope at a regular interval for their viability, morphological features and confluence.

Characterization of mesenchymal stem cell (MSC) was done by:

3.4.1 Immunocytochemistry: Endoglin (CD 105),Thy 1(CD90), CD73, CD45, Oct4, Nanog.

3.4.2 FACS: surface markers: CD45, CD73, CD 90, CD105

3.4.1 Immunocytochemistry of mesenchymal stem cells

The immunocytochemistry of MSCs was done for the MSC markers, endoglin (CD 105),Thy 1(CD90) CD73, CD45, Oct4, Nanog as per the methods followed by the National Institutes of Health (NIH), 2009 resources for stem cell research, using fluorescent conjugated antibody.

Immunostaining Protocol

1. MSCs monolayer was washed once briefly with 1x PBS and fixed for 20 min. with 4% paraformaldehyade (PFA) in 1x PBS at 4°C.
2. MSCs monolayer were washed 3 times with 1x PBS at room temperature for 10 min. each.
3. For intracellular epitopes, cells were incubated for 15 min in 0.25% Triton X-100.
4. MSCs monolayer were washed 3 times with 1x PBS at room temperature for 10 min. each.
5. Nonspecific binding sites blocked with 2% BSA for 40 min.
6. MSCs monolayer were again washed 3 times with 1x PBS at room temperature for 10 min. each.
7. Primary antibody were diluted in a primary antibody diluting solution and incubated for overnight at 4°C.
8. MSCs monolayer were washed 3 times with 1x PBS at room temperature for 10 min. each.
9. The appropriate secondary antibodies were diluted in a secondary antibody diluting solution and incubated for 2 hrs at RT in dark room.
10. MSCs monolayer were washed 3 times with 1x PBS at room temperature for 10 min. each.
11. MSCs monolayer was incubated with combined DAPI with antifading agent for 10 min in dark room.
12. The stained cells were observed under fluorescent Zeiss microscope immediately or later.

Control: An another MSC monolayer was run side by side as control where primary antibody was not added.

3.4.2 FACS Protocol

1. Canine MSCs were detached from culture flask with trypsin-EDTA.
2. The isolated cells pellet was prepared in DMEM with 10% FBS.
3. To the pellet, 4% Paraformaldehyde added and contents were gently mixed and kept for 30 min.
4. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
5. Cells were resuspended in 1 ml PBS.
6. Cells were pelleted by centrifugation for 5 min at 1000 rpm.

1. To the pellet, 0.25% Triton-X added and contents were gently mixed and kept for 15 min.
2. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
3. Cells were resuspended in 1 ml PBS.
4. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
5. To the pellet, 2% BSA in PBS added and contents were gently mixed and kept for 40 min.
6. The cells were stained/labelled with primary antibodies against CD-90(Thy-1), CD-105(Endoglin) , CD-73, CD- 45 and kept for overnight incubation at 4˚C .
7. Next day cells were resuspended in 1 ml PBS.
8. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
9. To the pellet, respective secondary antibodies added and covered with aluminium foil to avoid light exposure and kept for 4 hours incubation at room temperature.
10. The cell were pelleted and washed with 1 ml PBS.
11. Supernatant gently removed leaving 200-300µl of PBS at the bottom and contents were gently mixed and transferred to FACS tubes.
12. Blank MSCs were used as negative control and for calibration of the FACS machine.
13. The cells were examined in FACS Calibur (B D- Becton Dickinson,USA) and the data were analyzed using WinMDI cell Quest software (BD Biosciences, USA).

3.5 Preparation and characterization of scaffolds of functionalized carbon nanotubes

5 mg of each -OH SWCNT and MWCNT were separately dispersed in 13ml of absolute ethanol. They were probe sonicated for 30 minutes at 3 intervals of 10 minute each. The dispersed nanotubes were sprayed onto 14mm preheated coverslip with a spray gun. Coated coverslips were allowed to dry in air and were used for cell culture after sterilization with UV irradiation for 30 minutes. Characterization of scaffold was done by scanning electron microscopy(SEM) at IIT Kanpur.

3.6 Effect of functionalized OH-Carbon nanotube on cellular behaviour

The freshly cultured MSCs at 3-4 passages were utilised for the study. The MSCs were cultured in normal tissue culture dish as well as in OH-functionalised CNT scaffolds. The culture of cells was observed as follows:

3.6.1 Cellular Morphology

1. The canine MSCs ($1x10^3$) were cultured on standard tissue culture plate (control) as well as on OH functionalised SWCNT (T1) and MWCNT (T2) scaffolds (treatments) under standard cell culture conditions (37° C, 5% CO_2) in 12 well tissue culture plate.
2. Cell morphology was observed by phase contrast microscopy at 2, 4, 6 days of culture.
3. Cell area was measured using 'Image J' software at 2, 4, 6 days of culture.
4. About 200 numbers of cells were evaluated for each group having at least three replicates.

3.6.2 Colony forming assay

1. Very low density canines MSCs were cultured on standard tissue culture plate (control) as well as on CNT scaffolds (treatments) under standard cell culture conditions (37° C, 5% CO2).
2. The media was changed on every 48 h interval.
3. After 12-14 days of incubation, the cell colonies were stained with 0.04% crystal violet stain.
4. A colony having more than >20-30 numbers of cells were counted for each group using microscope.

3.6.3 The cell proliferation study-MTT assay

1. For cell proliferation study, MTT assay was done as per the manufacturer's protocol.
2. The canine MSCs were cultured on standard tissue culture plate (control) as well as on CNT scaffolds (T_1 and T_2) under standard cell culture conditions (37° C, 5% CO2) using 12 well tissue culture plates.
3. In each well about 5x 10^4 number of cells were cultured.
4. The MTT assay was done on day 2, 4 & 6 of culture.

3.7 Osteogeneic Differentiation:

The canine MSCs were cultured on standard tissue culture plate (control) and OH-functionalised SWCNT and MWCNT scaffolds (T_1 and T_2) under differentiation conditions as per the protocol of Filioli Uranio *et al* (2011).

1. The canine MSC at P3/P4 cells were seeded over control and scaffold using 12 well tissue culture dish.
2. The cells were cultured with MSC culture media till attainment of 60-70% confluency.

3. Then the MSC culture media was replaced with osteogenic differentiation media which consist of DMEM medium containing 10% FBS and antibiotics and 100 nmol –Dexamethasone, 10mmol - β-glycerophosphate, 0.3 mmol L- ascorbic acid.
4. The cells were maintained in this media upto 14 and 21 days with adding fresh differentiation media at every 48-72 h interval.

3.7.1 To evaluate the occurrence of osteogenic differentiation

1. At the end of differentiation period, the induced cells were fixed with 4% paraformaldehyde or methanol for 20 minutes.
2. The fixed cells were stained with **alizarin red** for 30 minutes at room temperature.
3. After 30 minutes of incubation, the plates were washed four times with distilled water and inspected macro- and microscopically in order to examine the mineralized matrix.
4. For further confirmation of differentiation, RNA extraction was done from differentiated cells and RT-PCR analysis of osteogenic gene expression viz. Osteocalcin/Osteopontin/COL1A1 was done.

3.7.2 Flowcytometry

1. Briefly, differentiated cells were detached from control as well as scaffold with trypsin-EDTA after 21 days of culture.
2. The isolated cells pellet was prepared in DMEM with 10% FBS.
3. To the pellet, 4% Paraformaldehyde added and contents were gently mixed and kept for 30 min.
4. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
5. Cells were resuspended in 1 ml PBS.
6. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
7. To the pellet, 0.25% Triton-X added and contents were gently mixed and kept for 15 min.
8. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
9. Cells were resuspended in 1 ml PBS.
10. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
11. To the pellet, 2% BSA in PBS added and contents were gently mixed and kept for 40 min.
12. The cells were stained/labelled with antibodies against Osteocalcin (contains mixture of both primary and secondary antibodies) and kept for overnight incubation at 4˚C .
13. Next day cells were resuspended in 1 ml PBS.

14. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
15. Supernatant gently removed leaving 200-300µl of PBS at the bottom and contents were gently mixed and transferred to FACS tubes.
16. Blank MSCs were used as negative control and for calibration of the FACS machine.
17. The cells were examined in FACS Calibur (B D- Becton Dickinson,USA) and the data were analyzed using WinMDI cell Quest software (BD Biosciences, USA).

3.8 Chondrogenic Differentiation

The canine MSCs were cultured on standard tissue culture plate (control) and OH functionalised SWCNT and MWCNT scaffolds (T_1 and T_2) under differentiation conditions as per the protocol of Filioli Uranio *et al* (2011).

1. The canine MSC at P3/P4 cells were seeded over control and scaffold using 12 well tissue culture dish.
2. The cells were cultured with MSC culture media till attainment of 60-70% confluency.
3. Then the MSC culture media was replaced with chondrogenic differentiation media which consist of DMEM medium containing 10% FBS and antibiotics and 100 nmol –Dexamethasone, 1mmol sodium pyruvate, L- ascorbic acid(50µg/ml), TGF-ß1 (10ng/ml) and ITS(1%).
4. The cells were maintained in this media upto 14 and 21 days with adding fresh differentiation media at every 48-72 h interval.

3.8.1 To evaluate the occurrence of chondrogenic differentiation

At the end of culture period, the induced cells were stained with alcian blue stain. For further confirmation of differentiation, RNA extraction was done from differentiated cells and RT-PCR analysis of chondrogenic gene expression viz. Aggrecan/COL2A/ Sox-9 was done.

3.8.2 Flowcytometry

1. Briefly, differentiated cells were detached from control as well as scaffold with trypsin-EDTA after 21 days of culture.
2. The isolated cells pellet was prepared in DMEM with 10% FBS.
3. To the pellet, 4% Paraformaldehyde added and contents were gently mixed and kept for 30 min.
4. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
5. Cells were resuspended in 1 ml PBS.

6. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
7. To the pellet, 0.25% Triton-X added and contents were gently mixed and kept for 15 min.
8. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
9. Cells were resuspended in 1 ml PBS.
10. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
11. To the pellet, 2% BSA in PBS added and contents were gently mixed and kept for 40 min.
12. The cells were stained/labeled with primary antibody against Aggrecan and kept for overnight incubation at 4˚C .
13. Next day cells were resuspended in 1 ml PBS.
14. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
15. To the pellet, secondary antibody added and covered with aluminium foil to avoid light exposure and kept for 4 hours incubation at room temperature.
16. The cells were pelleted and washed with 1 ml PBS.
17. Supernatant gently removed leaving 200-300µl of PBS at the bottom and contents were gently mixed and transferred to FACS tubes.
18. Blank MSCs were used as negative control and for calibration of the FACS machine.
19. The cells were examined in FACS Calibur (B D- Becton Dickinson,USA) and the data were analyzed using WinMDI cell Quest software (BD Biosciences, USA).

3.9 Neuronal differentiation

The canine MSCs were cultured on standard tissue culture plate (control) and CNT scaffolds (T_1 and T_2) under *ex vivo* differentiation conditions as per the protocol of Woodbury *et al*.,(2000).

1. The canine MSC at P3/P4 cell were seeded over control and scaffold using 12 well tissue culture dish.
2. The cells were cultured with MSC culture media till attainment of 60-70% confluency.
3. When cells attain 70% confluency, 24 hours prior to neuronal induction, media was replaced with preinduction media consisting of DMEM/20% FBS/1 mM ß-mercaptoethanol (BME).
4. After 24 hours of preinduction, cells were transferred to serum free medium containing 1-4mM BME for 7 days.

5. After 7 days of incubation, the differentiated cells were characterized for generation of neuronal cells.

3.9.1 To evaluate the occurrence of neurogenic differentiation

For confirmation of differentiation, RNA extraction was done from differentiated cells and RT-PCR analysis of neurogenic gene expression viz. Nestin/ß-Tubulin/MAP-2 was done.

3.9.2 Flowcytometry

1. Briefly, differentiated cells were detached from control as well as scaffold with trypsin-EDTA after 7 days of induction culture.
2. The isolated cells pellet was prepared in DMEM with 10% FBS.
3. To the pellet, 4% Paraformaldehyde added and contents were gently mixed and kept for 30 min.
4. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
5. Cells were resuspended in 1 ml PBS.
6. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
7. To the pellet, 0.25% Triton-X added and contents were gently mixed and kept for 15 min.
8. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
9. Cells were resuspended in 1 ml PBS.
10. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
11. To the pellet, 2% BSA in PBS added and contents were gently mixed and kept for 40 min.
12. The cells were stained/labeled with primary antibody against ß-Tubulin and kept for overnight incubation at 4˚C .
13. Next day cells were resuspended in 1 ml PBS.
14. Cells were pelleted by centrifugation for 5 min at 1000 rpm.
15. To the pellet, secondary antibody added and covered with aluminium foil to avoid light exposure and kept for 4 hours incubation at room temperature.
16. The cells were pelleted and washed with 1 ml PBS.
17. Supernatant gently removed leaving 200-300µl of PBS at the bottom and contents were gently mixed and transferred to FACS tubes.
18. Blank MSCs were used as negative control and for calibration of the FACS machine.
19. The cells were examined in FACS Calibur (B D- Becton Dickinson,USA) and the data were analyzed using WinMDI cell Quest software (BD Biosciences, USA).

3.10 Cytotoxicity study of OH-functionalized CNT on canine MSCs

For analysis of toxicity of OH functionalized SWCNT and MWCNT, canine MSC were cultured in normal tissue culture dish as well as on scaffold. About $1.5x10^4$ number of cells were cultured in control and scaffold (T_1 and T_2) in standard culture condition using MSC medium upto a period of 6 days. The toxicity of OH functionalized CNT, was evaluated on day 4 and day 6 of culture using flow cytometry as well as molecular method. The toxicity analysis was based on apoptotic cells as well as expression of apoptotic related gene expression.

3.10.1 Flowcytometry

1. Flowcytometric analysis of apoptosis cells was done by Annexin V FITC/Propidium Iodide staining as per manufacturers' protocol.
2. Briefly, canine MSCs were detached from control as well as scaffold with trypsin-EDTA after 4 and 6 days of culture.
3. Cell pellet made using DMEM with 10% FBS.
4. The cells were counted by neubauer chamber.
5. The isolated cells were prepared at a concentration of $1\text{-}5\times10^5$/ml in DMEM with 10% FBS by centrifugation for 5 min at 1000 rpm.
6. Resuspended the cells in 300 µl of 1x binding buffer.
7. Cells will be pelleted by centrifugation for 5 min at 1500 rpm.
8. The cells were stained with Annexin-V FITC/PI stain.
9. The cells were incubated at room temperature for 5 min. in the dark.
10. The cells were examined in FACS Calibur (B D- Becton Dickinson,USA) and the data obtained was analyzed using WinMDI cell Quest software (BD Biosciences, USA).

3.10.2 Relative expression of apoptotic genes

1. Canine mesenchymal cells were harvested from control and scaffold on day 4 & 6 of culture.
2. Cellular RNA content was isolated by using Trizol method and cDNA synthesised as per manufacturer's protocol.
3. Relative expression of apoptotic genes were analysed by using gene specific primers.
4. The transcript level of all genes were quantified using the relative quantification method based on comparative threshold cycles values (Ct). The abundance of gene was determined relative to the abundance of the housekeeping gene.

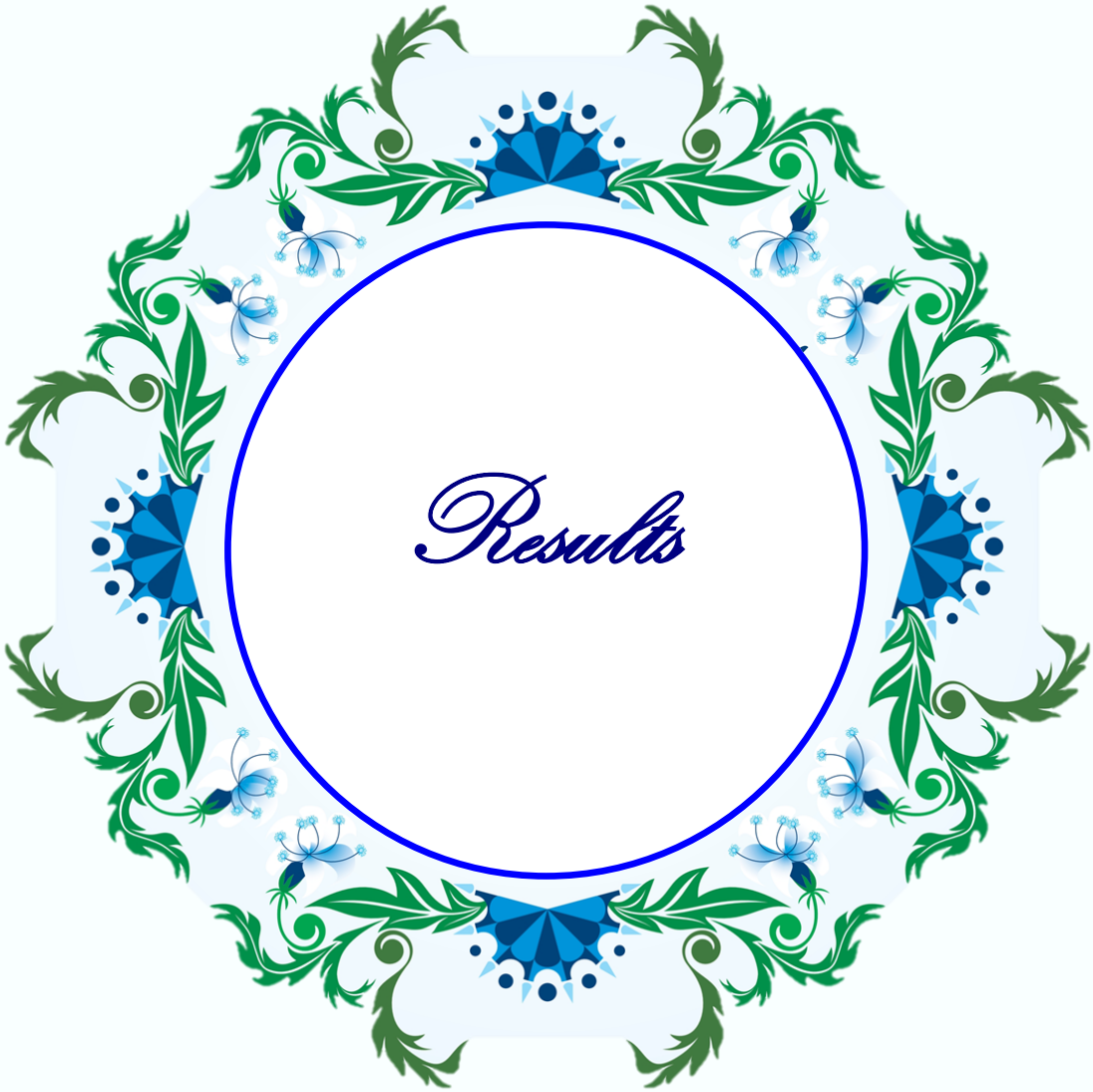
5. The relative expression of genes was analysed by Paffal method.

Statistical analysis

Software basis statistical analysis by One way ANOVA was done to evaluate the data.

Table 2: List of Primers and conditions used for real time PCR analysis.

S.No	Target Gene	Primer sequence 5'-3'	An-nealing Temp (^{o}C)	Prod-uct (bp)	References
1.	COL1A1	F: TAGACACCACCCTCAAGAGC R:CCAGTCGGAGTGGCACAT	60	118	Vieira *et al.,* 2010
2.	Osteopontin	F:CATTGATGGCCGAGGTGATAG R:AAGTGATGTGAAGTCCTCCTC	60	114	Vieira *et al.,* 2010
3.	Osteocalcin	F: GAGGGCAGCGAGGTGGTGAG R: TCAGCCAGCTCGTCACAGTTGG	60	134	Umehara *et al*., 2012
3.	Caspase 3	F: TCATTATTCAGGCCTGCCGAGG R:TTCTGACAGGCCATGTCATCCTCA	60	86	Del Puerto *et al.*, 2010
4.	Caspase 8	F: GATGCAGATGCGTTGAGT R:ACTGTGGTCCATGCTTTG	57	120	XM 005640429.1
5.	Caspase 9	F:ACGAGACTCACACCAGAGG R:TCGTCCAGAACCATTGTC	57	150	NM 001039 98.1
6.	MAP-2	F:GAAGTTCAGGCCCACTCTCC R:CCTGTTGCTGTGGTTTTCCG	58	107	XM 005640598.1
7.	Tubulinβ III	F:AGCCAAGTTCTGGGAAGTCA R:CCCACTCTGACCAAAGATGAA	57	238	Wilcox *et al.,* 2011
8.	GAPDH	F:CCATCTTCCAGGAGCGAGAT R:TTCTCCATGGTGGTGAAGAC	60	97	Vieira *et al.,* 2010
9.	Nestin	F: GAGAACCAGGAGCAAGTGAA R: TTTCCAGAGGCTTCAGTGTC	57	328	Valenzuela *et al*.,2008
10.	SOX9	F: GCTCGCAGTACGACTACACTGAC R: GTTCATGTAGGTGAAGGTGGAG	60	101	Vieira *et al.,* 2010
11.	COL2A	F: GAAACTCTGCCACCCTGAAT R: GCTCCACCAGTTCTTCTTGG	62	156	Vieira *et al.,* 2010
12.	Aggrecan	F: ATCAACAGTGCTTACCAAGACA R: ATAACCTCACAGCGATAGATCC	62	122	Vieira *et al.,* 2010


Results

Results

4.1 Culture and characterization of cBM-MSCs

Immediately after collection of bone marrow, buffy coat was separated using Ficol-Hypaque density gradient method. Nucleated cells were washed twice and then seeded in T25 culture flask in DMEM –Low glucose supplemented with 15% FBS **(Fig.1)**. After 48h, the non-adherent cells were carefully removed and fresh culture media was supplemented to continue the cell attachment and proliferation. The process was repeated on every 48h for up to 96h to get rid of maximum number of other cell types. After 96h of culture, the cells of primary culture (P_0) were washed gently with phosphate-buffer saline(PBS) and fresh culture media was added every 3rd day. Adherent cells exhibits fusiform or spindle shape with 40-50% confluent on day six **(Fig.1)** and attend characteristics individual fibroblastic colonies like morphology with homogenous population by day 10-12 with maximum confluence **(Fig.1).** The cBM-MSCs were maintained their characteristic spindle shape in subsequent passages **(Fig.1)** and confluent cells were propagated easily by trypsinization method.

4.2 Characterization of cBM-MSCs

4.2.1 Immunophenotyping of cBM-MSCs

The immunocytochemistry was conducted to localize the MSCs specific markers on the cBM-MSCs. The MSCs at 3rd passage were cultured on cover slip and allowed to attain monolayer of cells with 60-70% confluence. The monolayer of MSCs was immunostained using MSC positive markers namely, CD105, CD90 and CD73 and negative marker CD45 with pluripotent markers Nanog and Oct4. **Fig.2** showed the immune positive markers CD105, CD90, CD73 and negative for CD45; a classical hematopoietic stem cells marker. All the cells were counter stained with DAPI (blue) for identification of their nuclei.

The cBM-MSCs were quantified by flowcytometric analysis against the MSCs specific markers. It was observed that among the cultured MSCs, 92.18% were positive for CD73,

97.7% for CD90, 96.5% for CD105 and 0.58% were positive for negative surface marker, CD45, indicating that the cell population were of predominantly the mesenchymal stem cells **(Fig.3)**, **(Table.3)** .

The differentiation potential of cBM-MSCs into adipocytes was investigated and it was observed that after co-culture with specific induction media for 21 days, there were small lipid droplets like appeared within the cytoplasm. The lipid droplets took reddish color in Oil Red O staining **(Fig.4)**, which indicated the accumulation of lipid in differentiated cells while control cells were found to be negative.

For osteogenic differentiation potential of cBM-MSCs, the cells were cultured with osteogenic induction media. After 7-8 days of co-induction, cellular aggregates were observed which gradually increased with time. These aggregates were observed to look brownish red coloration with Alizarin Red staining and control cells were negative for staining indicating the absence of calcium aggregates **(Fig.4)**.

4.3 Characterization of scaffold

Scanning Electron Microcopy images of the scaffold reveals that, both -OH functionalized SWCNT and MWCNT formed the mesh like scaffold where individual cylinder like nanotubes could be distinguished **(Fig.5)**.

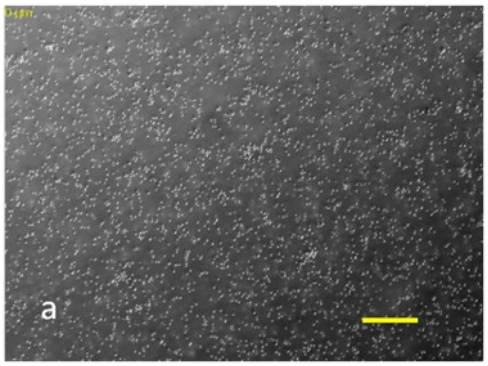
4.4 Effect of functionalized OH-Carbon nanotube on cellular behavior

4.4.1 Cellular morphology

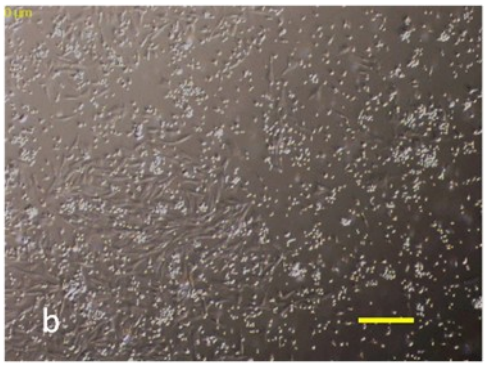
On assessment of cellular morphology, as such there was no difference in appearance of actin fibres by Phalloidin staining **(Fig.7)** . But while assessing the cellular surface area it was found that cells over the scaffolds were possessing filopodia or microspikes **(Fig.8)** which aids in increase in cell surface area. It is evident by measurement of cell surface area on day 2 of culture indicating that the cell surface area was significantly ($P<0.05$) higher among the cMSCs cultured over SWCNT scaffold compared to control and MWCNT scaffold. On day 4 and 6 days of culture, compared to control, among both scaffolds, cell surface area was significantly ($P<0.05$) higher **(Table.4)**.

4.4.2 Colony forming assay

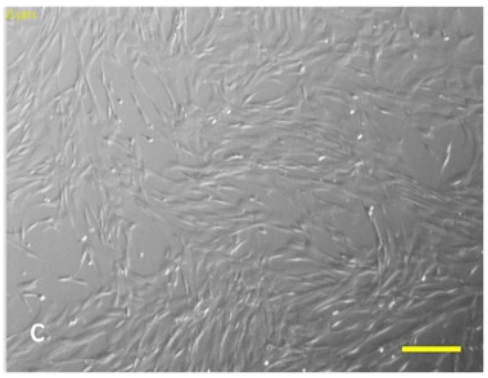
Colony forming assay demonstrates cultures containing subpopulation of cells capable of generating new colonies from single cell. Colony forming ability was significantly ($P<0.05$) lower among both SWCNT and MWCNT scaffold compared to control **(Fig.9)**.



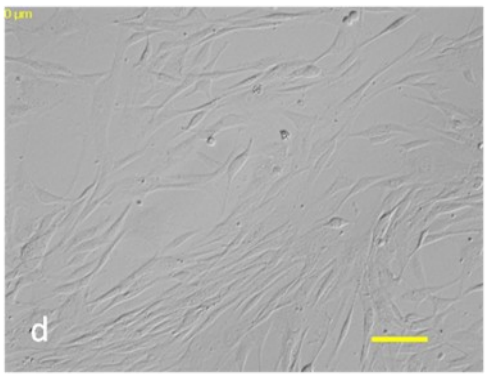
a. Primary culture on day 0.
(Scale bar-100µm)



b. Primary culture on day 6.
(Scale bar-100µm)



c. The cells of 3rd passage showing typical spindle shaped morphology (4x) (Scale bar-100µm).



d. The cells of 3rd passage (10x) (Scale bar-50µm).

Fig.1: Primary and subsequent culture of canine bone marrow derived mesenchymal stem cells (cBM-MSCs)

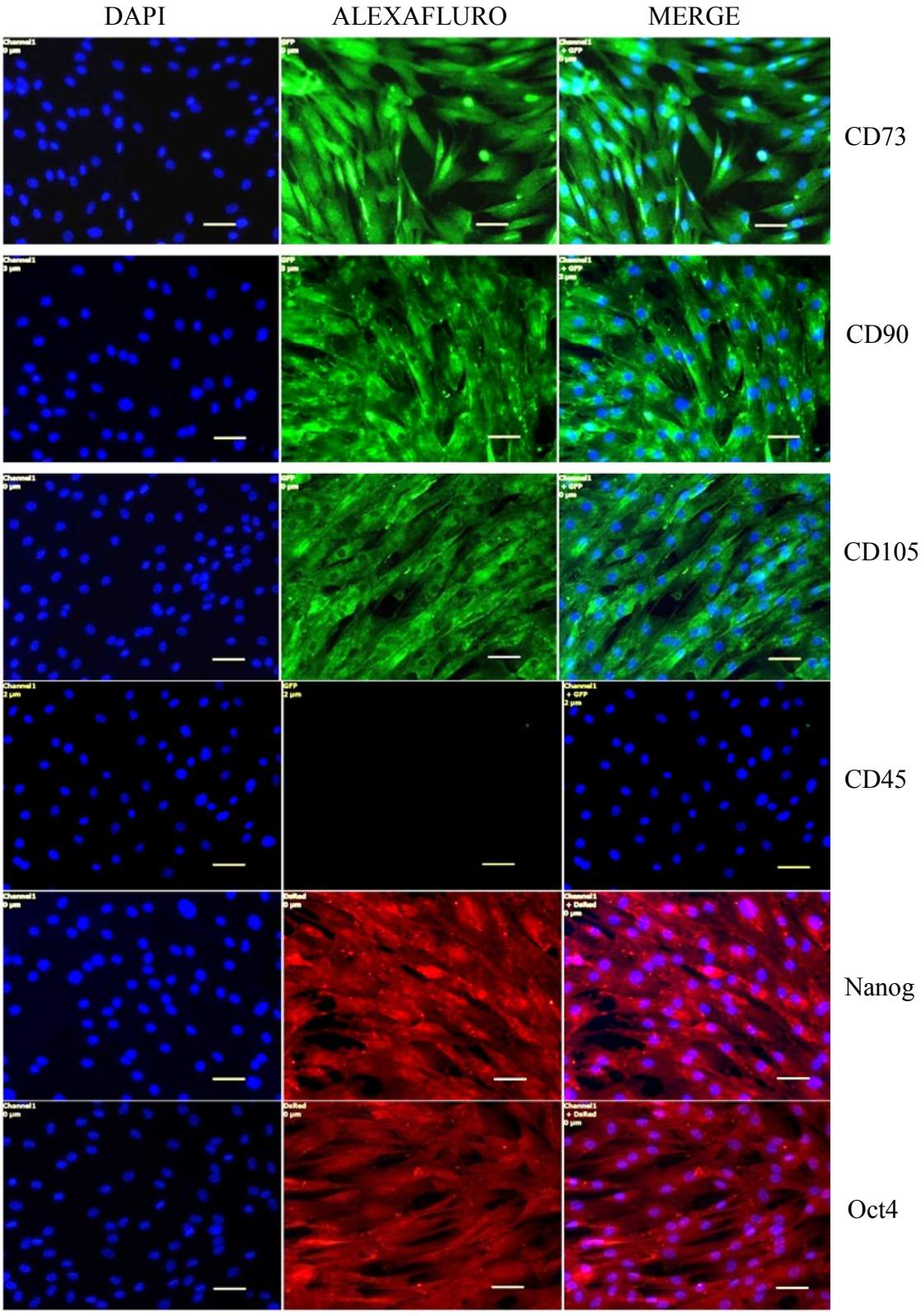


Fig. 2: Immunophenotyping of surface markers in bone marrow derived cell monolayer. The results indicated that cells were positive for CD73, CD90, CD105 while negative for CD45. The cells also express pluripotent markers Nanog and Oct4. (Scale bar-50µm).

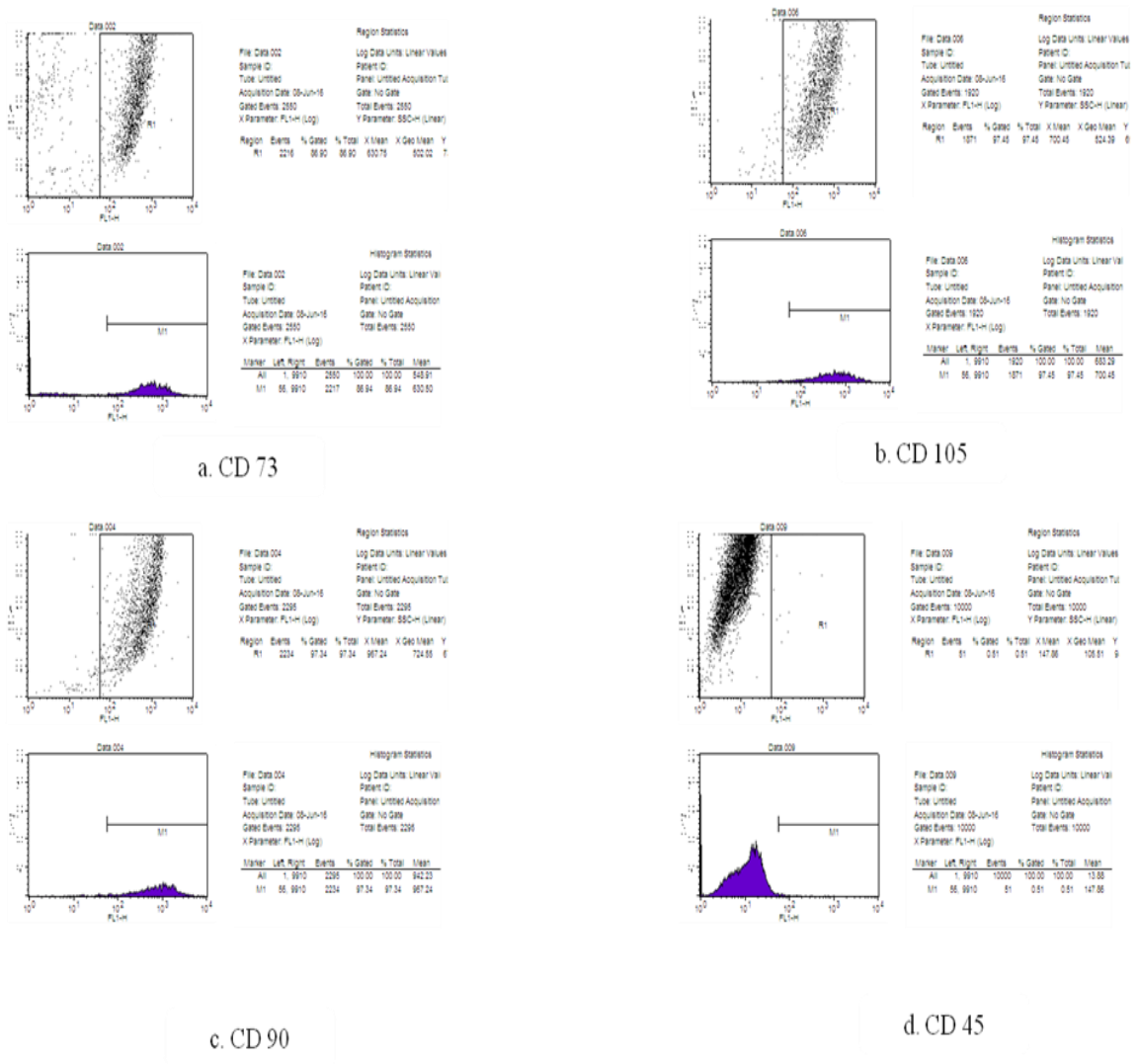
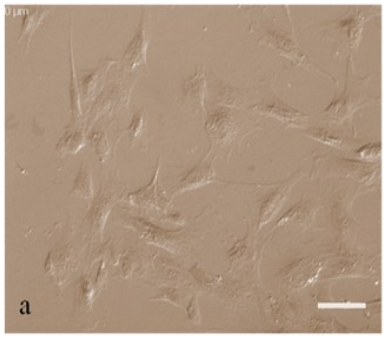


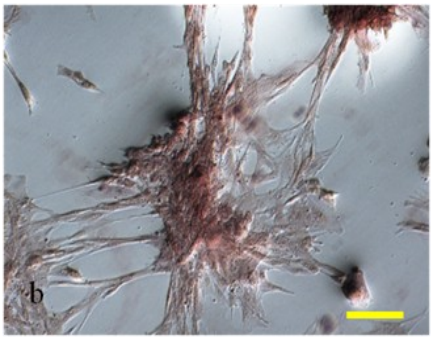
Fig. 3: Flowcytometry analysis of canine bone marrow derived cells using MSC surface markers. a. CD73. b. CD105. c. CD90. d. CD45.

Table 3 : Quantification of surface markers of canine MSCs

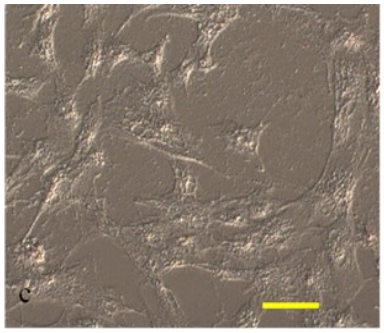
S.No	MSC Surface Markers	%
1	CD 73	92.18±1.86
2	CD 90	97.70± 2.12
3	CD 105	96.51± 1.34
4	CD 45	0.58 ±0.14



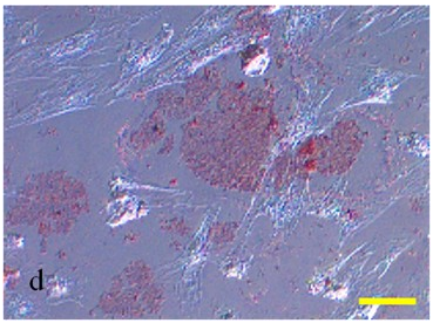
a. Control
(Lack of mineral deposits)



b. Osteocyte specific reddish brown mineral deposition in Alizarin red staining.



c. Control
(Lack of oil droplets)



d. Adipocyte specific reddish colored oil droplets in Oil Red O staining.

Fig. 4: Differentiation of canine bone marrow derived MSCs into osteocytes and adipocytes.

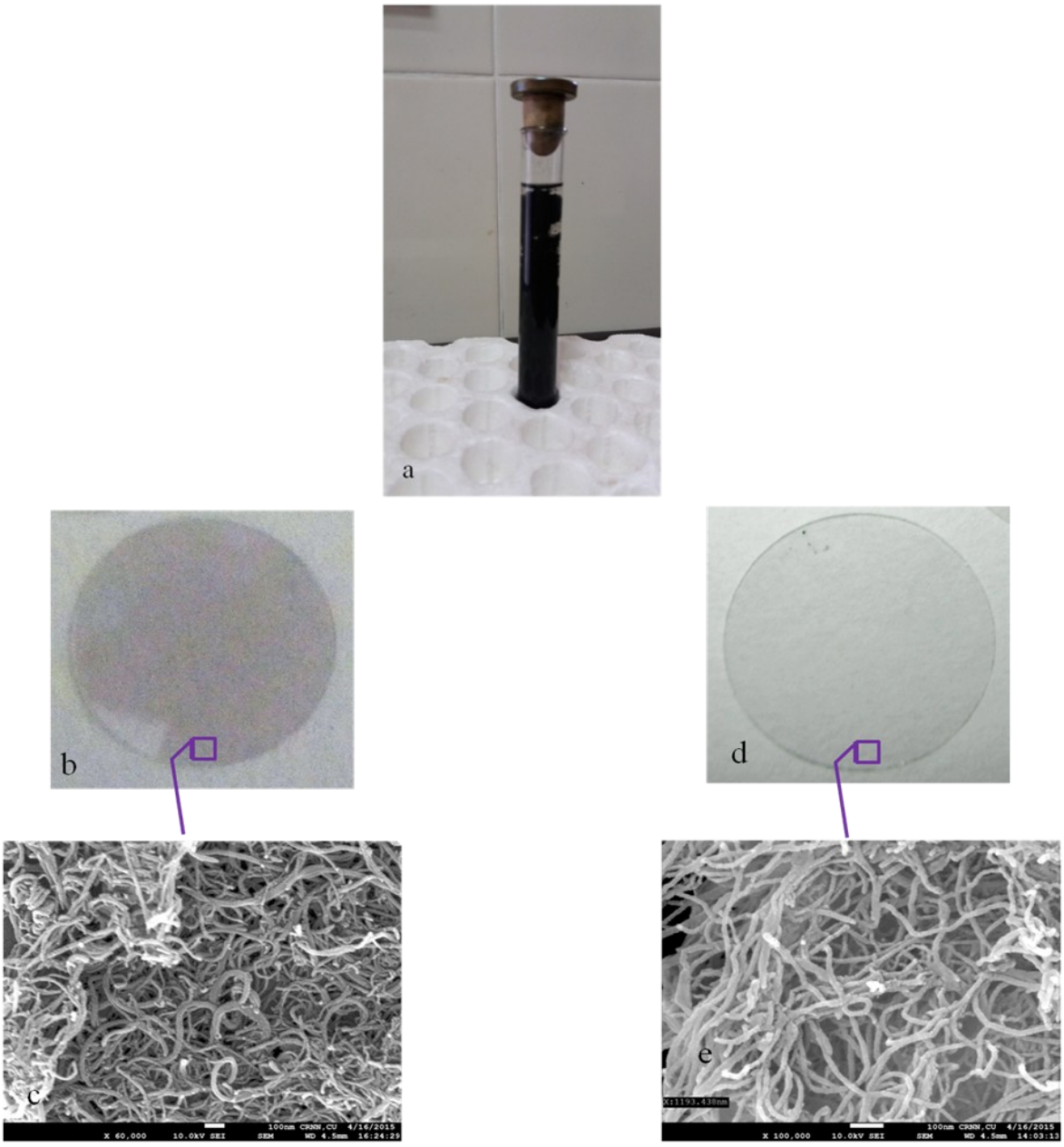


Fig. 5: Hydroxylated (-OH) Carbon nanotube (CNT) dispersion and scaffold.

a. CNT dispersion after Sonication.

b. SWCNT scaffold over 14mm coverslip.

c. FESEM images of SWCNT scaffold.

d. MWCNT scaffold over 14mm coverslip.

e. FESEM images of MWCNT scaffold.

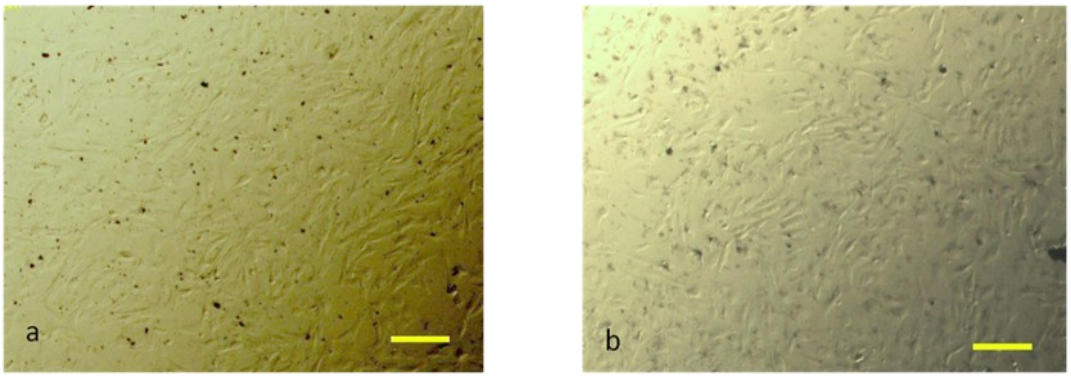


Fig. 6: Cellular morphology of MSCs over scaffold, phase contrast microscopic image a. SWCNT b. MWCNT (Scale bar-100µm).

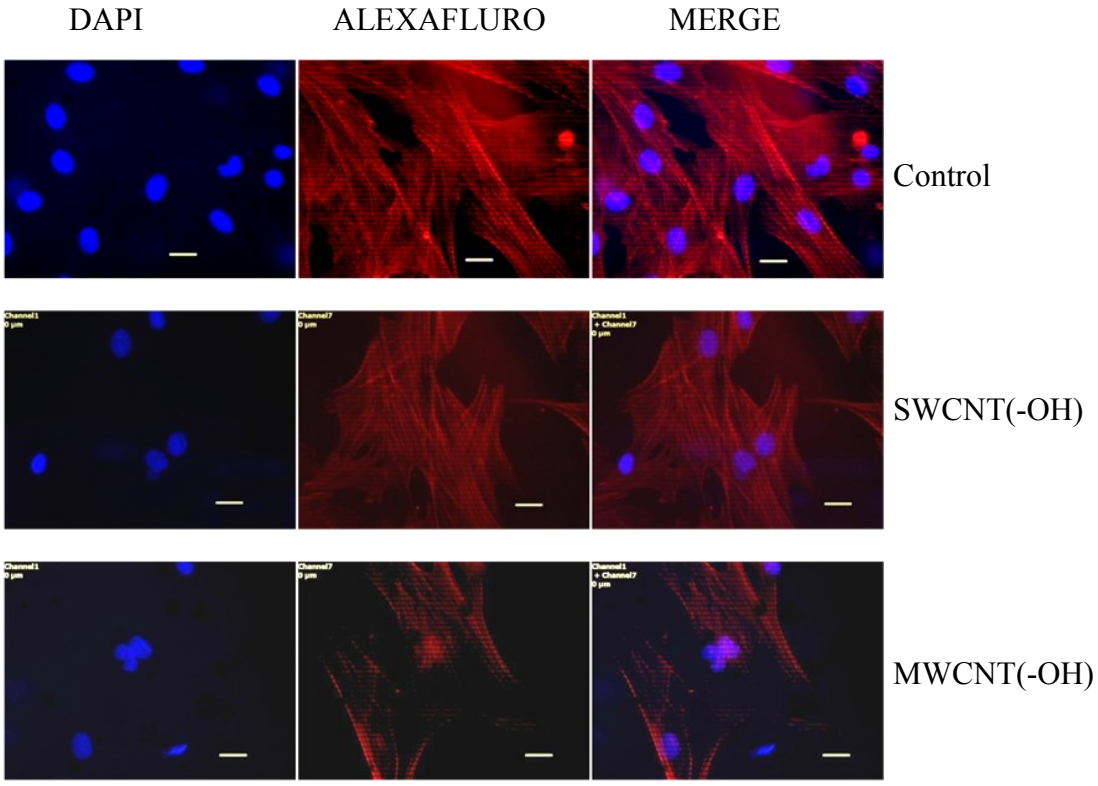


Fig. 7: Phalloidin staining of actin fibres to assess morphology of MSCs cultured over control and scaffolds. (Scale bar-20µm).

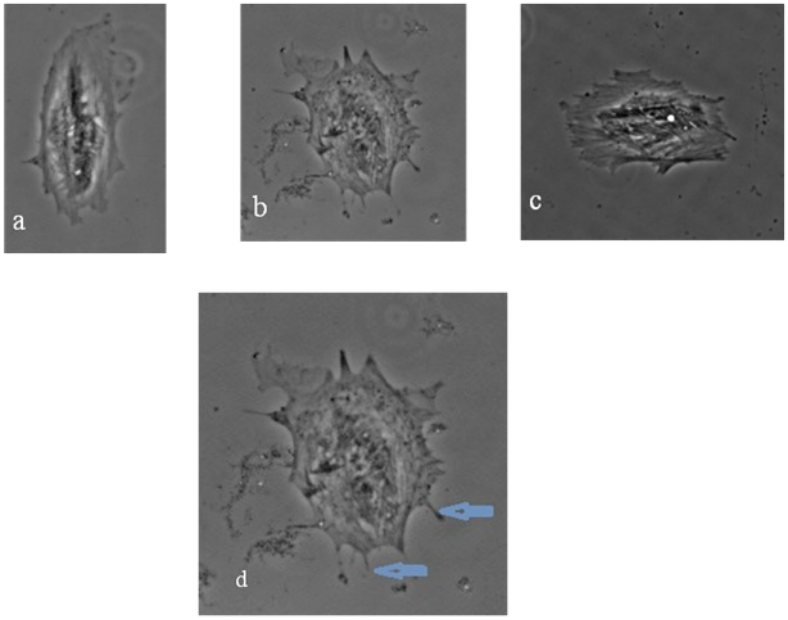


Fig. 8: Phase contrast microscopic images of MSCs cultured over a. Control. b. SWCNT scaffold. c. MWCNT scaffold to measure cell surface area. d. Enlarged image of MSC over SWCNT scaffold in which arrows indicating microspikes. (Magnification-10x)

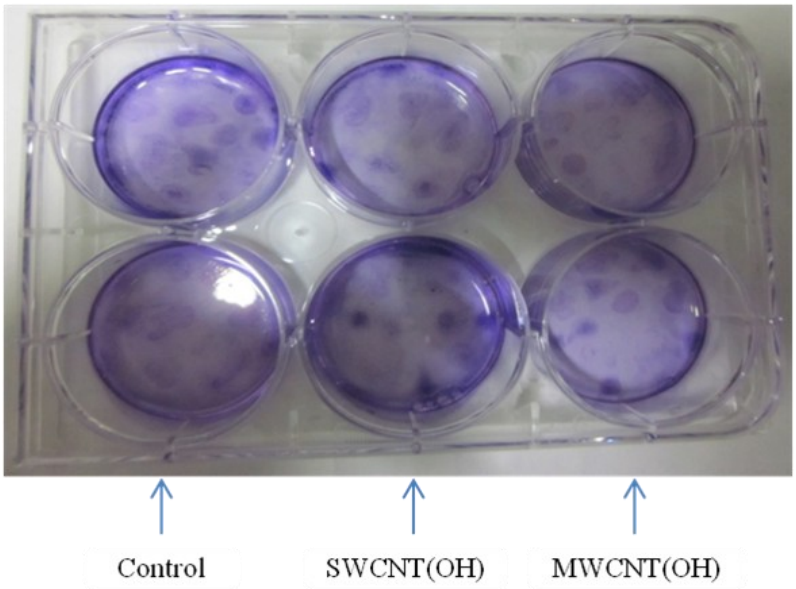


Fig. 9: Colony forming assay. 6-Well plate demonstrating colonies formed among respective columns of control, SWCNT and MWCNT scaffold. Stain used is 0.04% crystal violet.

Table 4: Surface area measurement on different days of culture over control and scaffolds.

Cell Surface Area(μm^2)			
	Day 2	**Day 4**	**Day 6**
Control	1559.87±94.34[a]	1929.49±50.50[a]	2728.57±108.48[a]
SWCNT(OH)	1850.06±87.48[b]	2508.06±82.82[b]	3458.33±92.06[b]
MWCNT(OH)	1730.34±96.14[ab]	2337.65±61.44[b]	3196.46±117.34[b]

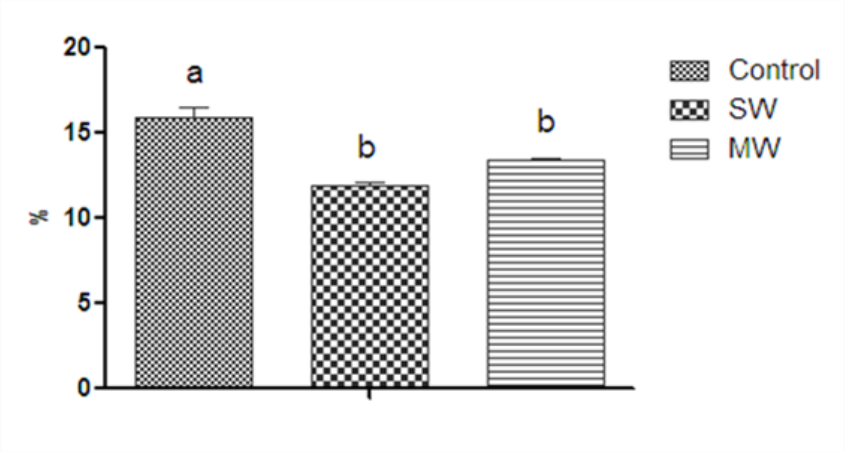


Fig. 10: Graph showing the percentage of colonies formed over control and scaffold. Each data represents a mean± standard error. The superscript a, b indicates significant difference ($P<0.05$)

Table 5: Colony forming assay over control and scaffolds.

Colony forming assay(%)	
Control	15.9±0.57[a]
SWCNT(OH)	11.91±0.24[b]
MWCNT(OH)	13.45±0.11[b]

On comparison between SWCNT and MWCNT scaffold, it was slightly higher in MWCNT **(Fig. 10), (Table.5)**.

4.4.3 The cell proliferation study-MTT assay

With respect to cell proliferation, on day 2, 4 and 6 of culture, compared to control, there was significant ($P<0.05$) decrease in the cell proliferation among the treatments **(Fig.6)**. Among the –OH functionalized CNTs, cell proliferation was better in MWCNT compared to SWCNT on day 2, 4 and 6 days of culture, which indicated cell proliferation of canine MSCs was more in MWCNT **(Fig.11)**, **(Table.6)**.

4.5 Osteogeneic differentiation

Alizarin Red staining of 21 days osteogenic culture of cBM-MSCs over the control and scaffolds indicated the brownish red colored calcium aggregates in both control and scaffolds. It indicated that osteogenic differentiation occurs in control as well as in SWCNT and MWCNT scaffold. It was observed that large calcium aggregates were found in osteogenic culture of cMSCs over MWCNT scaffold **(Fig.12)**.

Immunocytochemistry of osteogenic culture of cBM-MSCs over the control and scaffolds against Osteocalcin (OCN) indicated that in both control and scaffolds, cells were positive for OCN indicating that osteogenic differentiation occurred in control as well as in scaffolds **(Fig.13)**.

After the osteogenic differentiation of cBM-MSCs over control and scaffolds for 14 and 21 days. qPCR expression of osteocyte specific genes was as follows. Expression of Osteopontin (OPN) was significantly ($P<0.05$) higher in –OH MWCNT scaffold compared to control and -OH SWCNT scaffold on both 14 and 21 days of culture On 14^{th} day of culture, expression of OPN was much higher than 21^{st} day of culture **(Fig.14,15,16), (Table.7)**.

Expression profile of Osteocalcin (OCN) was also significantly ($P<0.05$) higher in –OH MWCNT scaffold compared to control and –OH SWCNT scaffold on both 14 and 21 days of culture. But the expression profile of OCN on 14^{th} and 21^{st} days of culture was almost same on both control and treatments **(Fig.14,15,16), (Table.7)**.

Expression profile of Col.1A1 was having contrast nature in which expression was significantly ($P<0.05$) higher in –OH SWCNT scaffold compared to control and –OH MWCNT scaffold on both 14 and 21 days of culture. Also compared to 14^{th} day of culture

expression of Col.1A1 was slightly higher in 21st day of culture **(Fig.14,15,16), (Table.7)**.

Flowcytometry analysis of OCN positive cells after 21 days of osteogenic culture was also on par with real-time expression in which cBM-MSCs cultured over –OH MWCNT scaffold were possessing significantly ($P<0.05$) more OCN compared to canine MSCs cultured over control and –OH SWCNT scaffold **(Fig.17), (Table.8)**.

4.6 Chondrogenic differentiation

Alcian Blue staining of 21 days chondrogenic culture of cBM-MSCs over the control and scaffolds indicates the bluish colored proteoglycan aggregates in both control and scaffolds. It indicated that chondrogenic differentiation occurs in control as well as in SWCNT and MWCNT scaffold. It was observed that large proteoglycan aggregates were found in chondrogenic culture of cMSCs over MWCNT scaffold **(Fig.18)**.

Immunocytochemistry of chondrogenic culture of cBM-MSCs over the control and scaffolds against Aggrecan indicates that in both control and scaffolds, cells were positive for Aggrecan indicating that chondrogenic differentiation occurred over control as well as over scaffolds **(Fig.19)**.

After the chondrogenic differentiation of cBM-MSCs over control and scaffolds for 14 and 21 days. qPCR expression of chondrocyte specific genes was as follows. Expression of COL.2A was significantly ($P<0.05$) higher in –OH MWCNT scaffold compared to control and -OH SWCNT scaffold on both 14 and 21 days of culture. On MWCNT scaffold, expression was higher on 21st day of culture compared to 14th day of culture **(Fig.20,21,22), (Table.9)**.

Expression of Aggrecan was also significantly ($P<0.05$) higher in –OH MWCNT scaffold compared to control and -OH SWCNT scaffold on both 14 and 21 days of culture. On MWCNT scaffold, expression was higher on 21st day of culture compared to 14th day of culture **(Fig.20,21,22), (Table.9)**.

On 14th day of culture, expression of SOX-9 among control and both scaffolds, there was no significant ($P<0.05$) difference but on 21st day of culture, there was significant ($P<0.05$) higher expression of SOX-9 among –OH SWCNT scaffold and –OH MWCNT compared to control **(Fig.20,21,22), (Table.9)**.

Flowcytometry analysis of Aggrecan positive cells after 21 days of chondrogenic culture was also on par with real-time expression in which canine MSCs cultured over

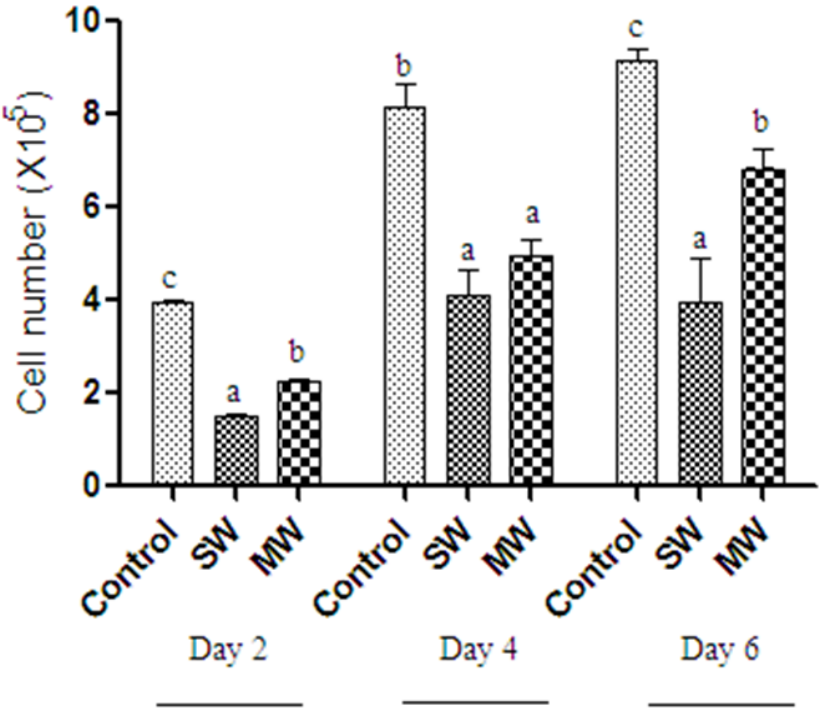


Fig. 11: Cell proliferation rate using MTT assay. Graph showing the cell proliferation rate among control and scaffold. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 6: Cell proliferation study: MTT assay.

	Cell Number($\times 10^5$)		
	Day 2	**Day 4**	**Day 6**
Control	3.94 ± 0.08^{c}	8.13 ± 0.54^{b}	9.14 ± 0.27^{c}
SWCNT(OH)	1.50 ± 0.06^{a}	4.10 ± 0.57^{a}	3.94 ± 0.97^{a}
MWCNT(OH)	2.23 ± 0.06^{b}	4.94 ± 0.38^{a}	6.83 ± 0.42^{b}

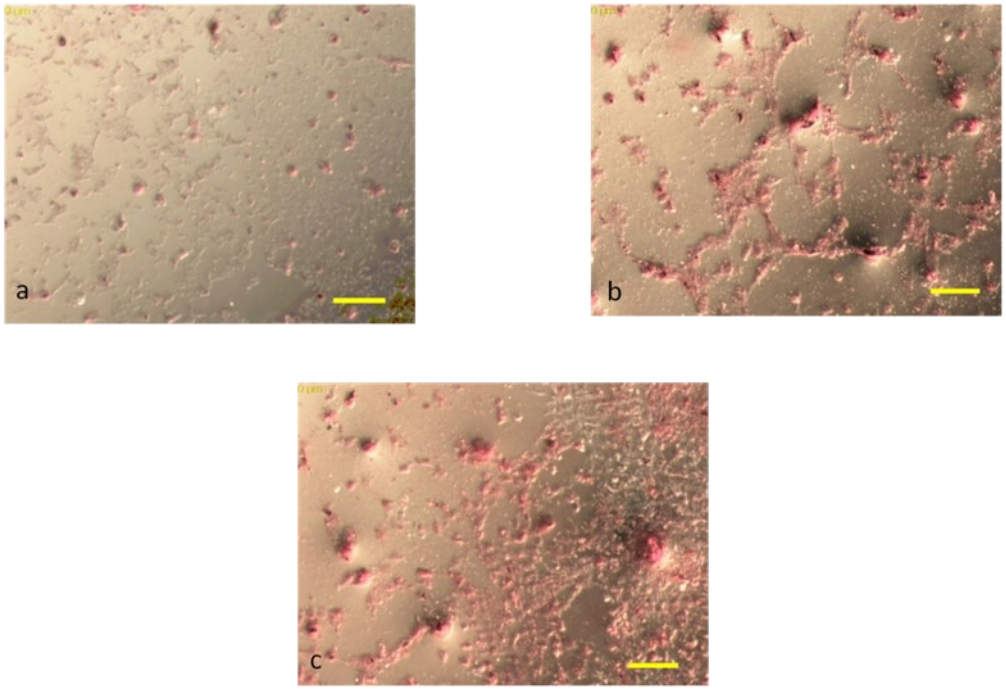


Fig. 12: Alizarin Red staining of osteogenic differentiation culture of MSCs over a. Control. b. SWCNT scaffold and c. MWCNT scaffold. (Scale bar-100µm).

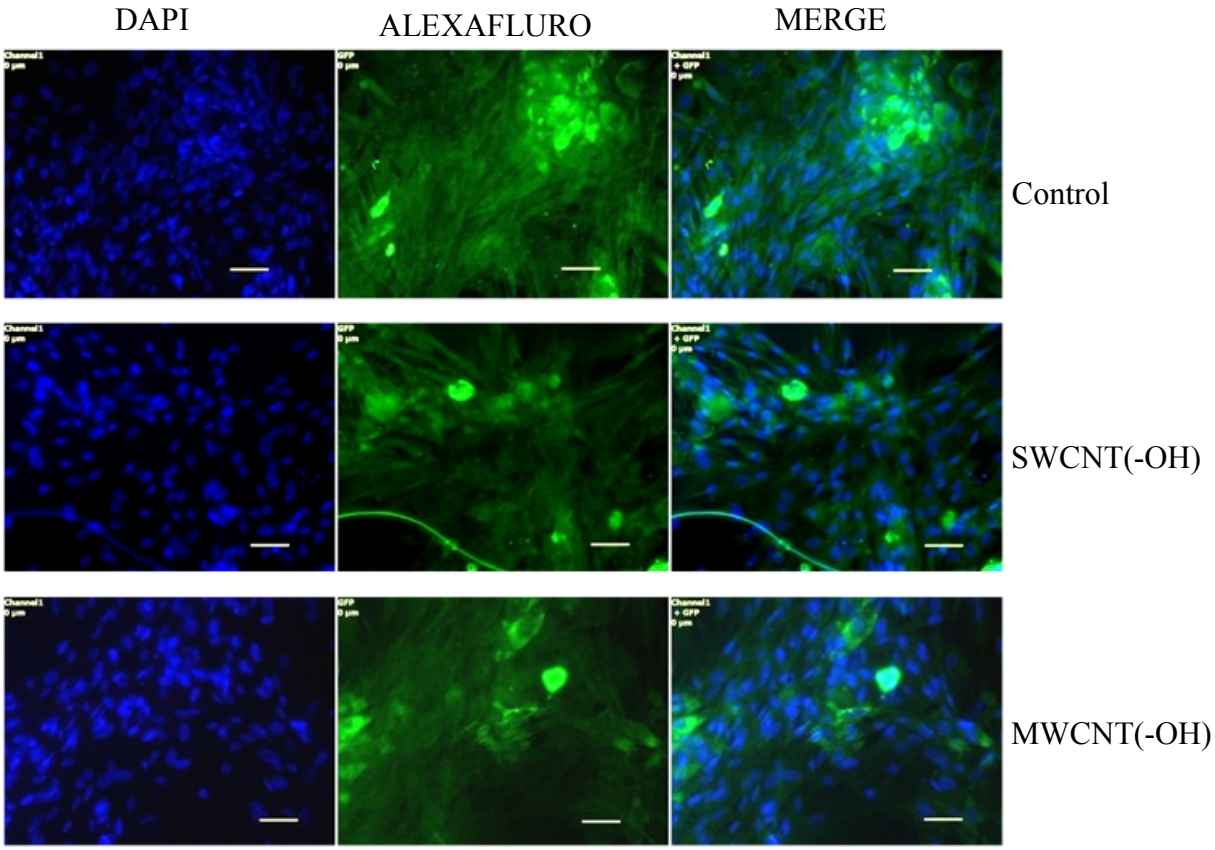


Fig. 13: Immunophenotyping of Osteocalcin (OCN) in osteogenic differentiation culture of MSCs over Control, SWCNT scaffold and MWCNT scaffold.(Scale bar-50µm).

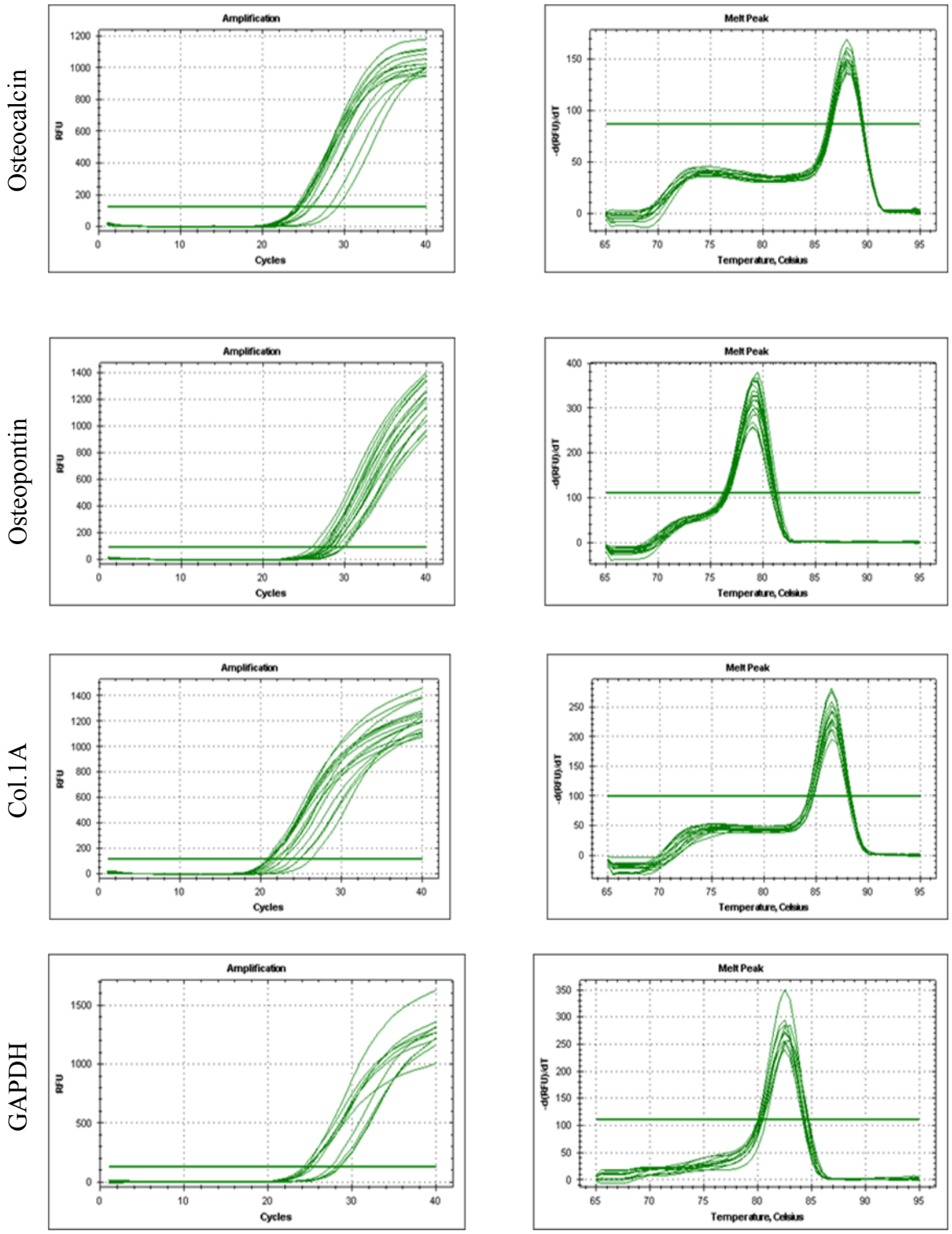


Fig. 14: Amplification curves and melting peaks of osteocyte specific genes in differentated cells: Osteocalcin, Osteopontin, Col.1A and housekeeping gene GAPDH.

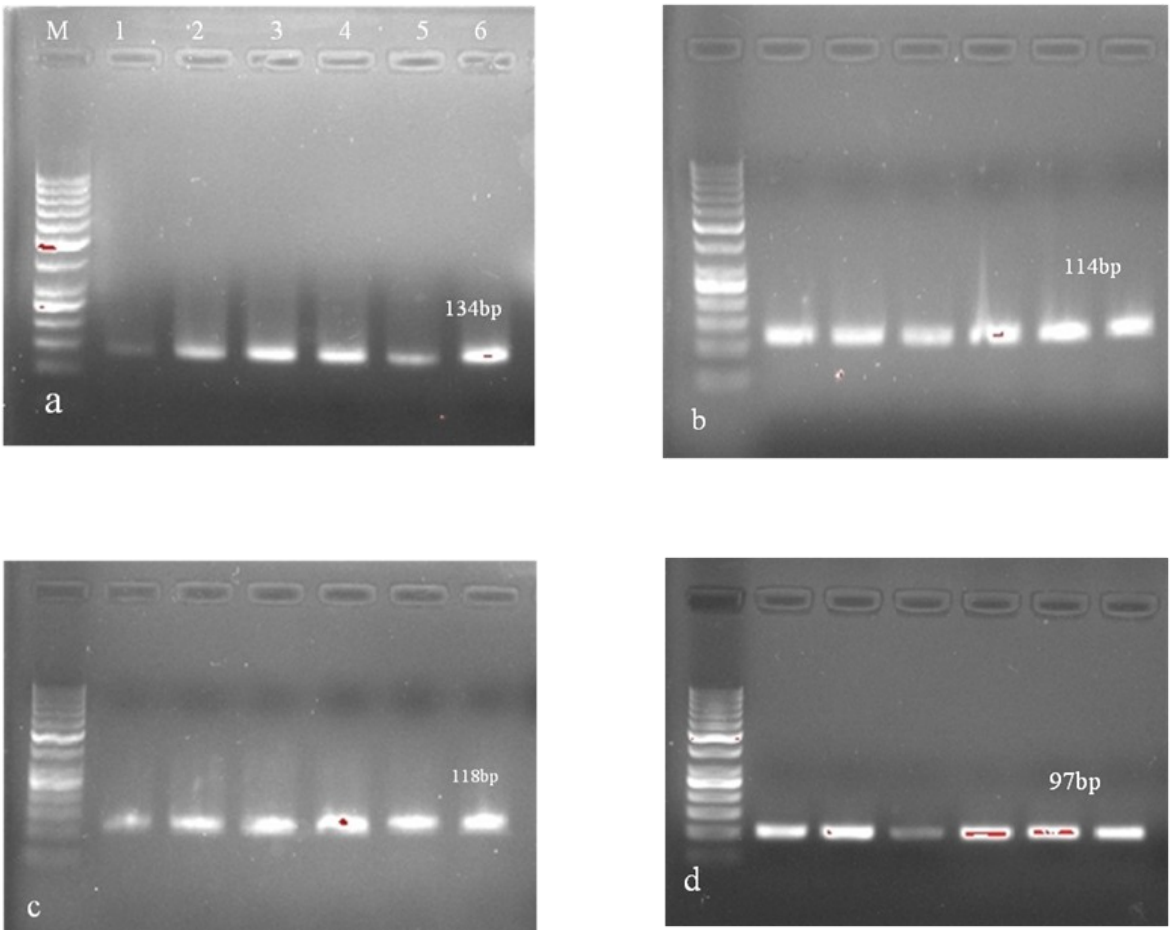


Fig. 15: Molecular characterization of differentiated cells. Gel electrophoresis of PCR amplified products of osteocyte specific genes in 2% agarose gel. a. Osteocalcin b. Osteopontin c. Col.1A d. GAPDH. Lane M: 50bp ladder, Lane 1 & 2: Control, Lane 3 & 4: SWCNT, Lane 5 & 6: MWCNT.

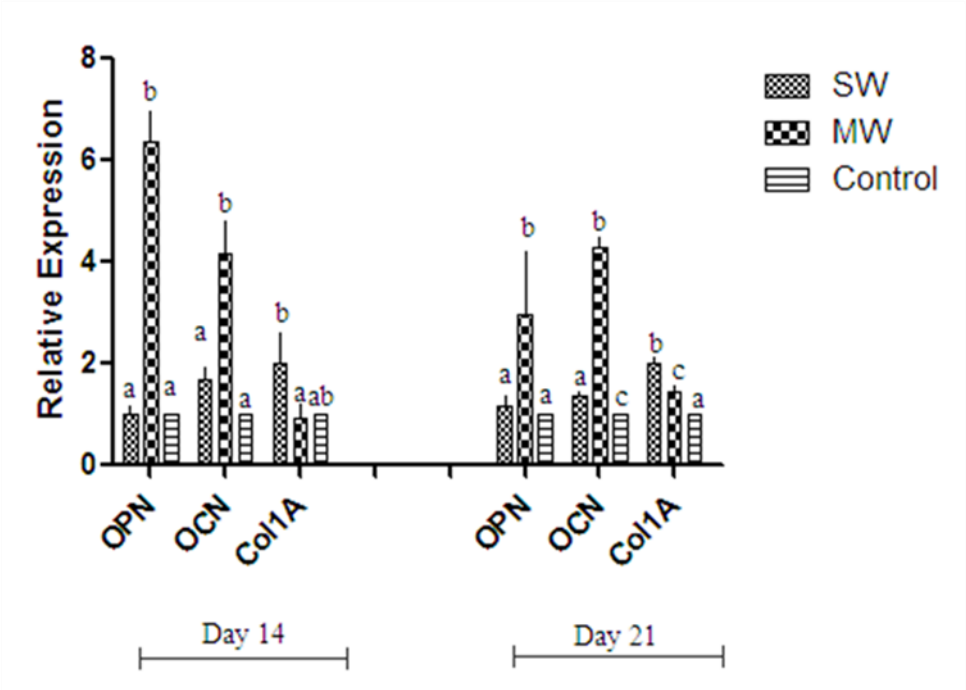


Fig. 16: Relative mRNA expression profiles of osteocyte specific genes. The graph shows the relative expression of osteocyte specific genes namely Osteopontin (OPN), Osteocalcin (OCN) and Collagen1A(Col.1A) on day 14 and 21 days of culture. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 7: Relative mRNA expression profiles of osteocyte specific genes over control and scaffolds.

	Relative expression					
	Day 14			**Day 21**		
	OPN	OCN	COL.1A	OPN	OCN	COL.1A
Control	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{ab}	1 ± 0^{a}	1 ± 0^{c}	1 ± 0^{a}
SWCNT(OH)	0.99 ± 0.15^{a}	1.66 ± 0.26^{a}	2.01 ± 0.58^{b}	1.16 ± 0.19^{a}	1.36 ± 0.07^{a}	2.00 ± 0.11^{b}
MWCNT(OH)	6.36 ± 0.62^{b}	4.15 ± 0.63^{b}	0.93 ± 0.26^{a}	2.95 ± 1.25^{b}	4.28 ± 0.18^{b}	1.44 ± 0.12^{c}

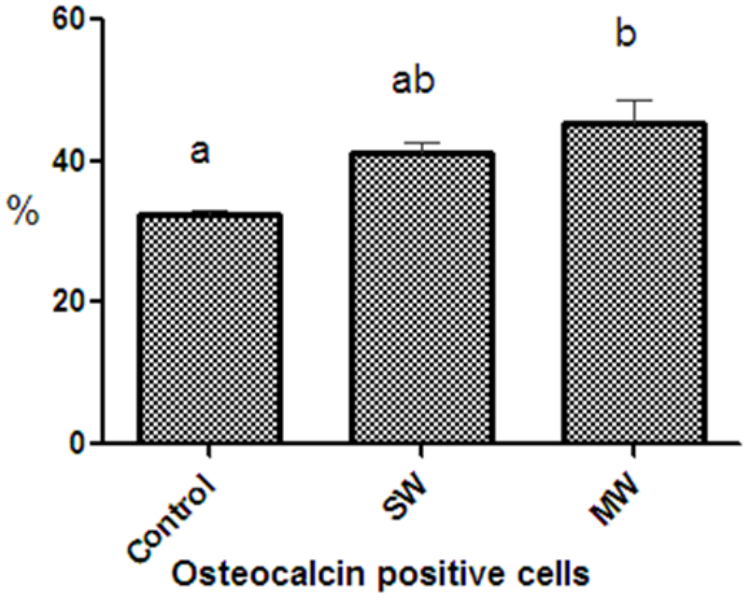


Fig. 17: Flowcytometric analysis of Osteocalcin positive cells over control and scaffolds. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 8: Flowcytometric analysis of Osteocalcin positive cells over control and scaffolds.

Osteocalcin positive cells (%)	
Control	32.45 ± 0.70^{a}
SWCNT(OH)	41.04 ± 1.66^{ab}
MWCNT(OH)	45.04 ± 3.12^{b}

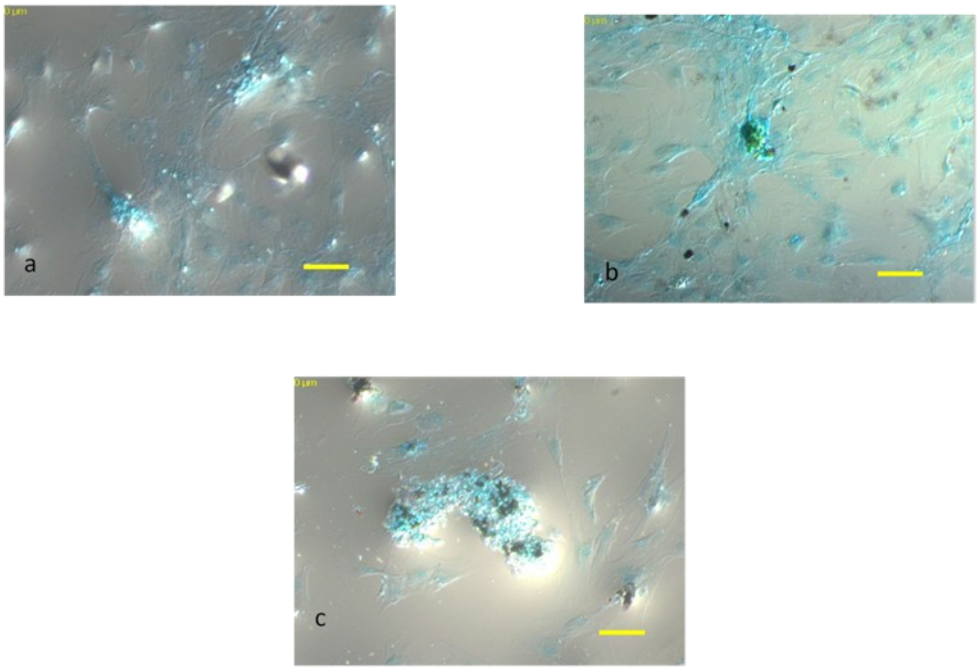


Fig. 18: Alcian Blue staining of chondrogenic differentiation culture of MSCs over a. Control. b. SWCNT scaffold and c. MWCNT scaffold. (Scale bar-50µm).

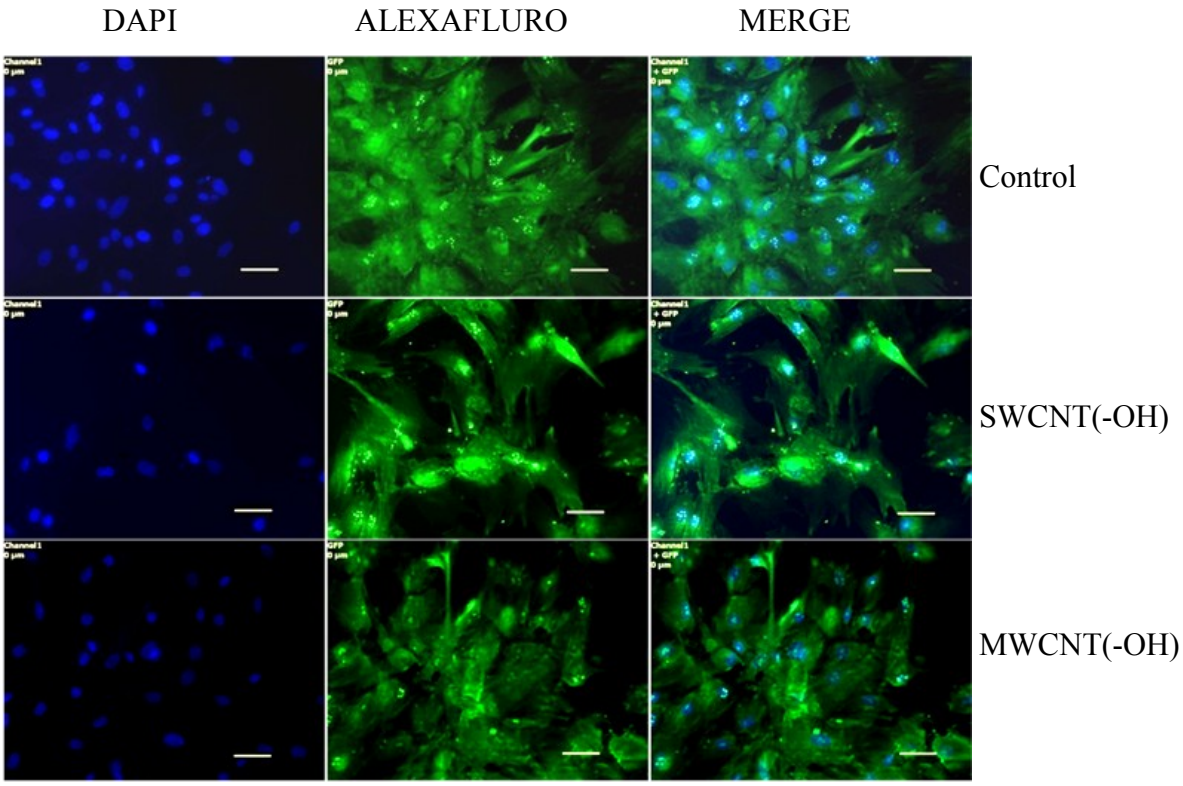


Fig. 19: Immunophenotyping of Aggrecan in chondrogenic differentiation culture of MSCs over Control, SWCNT scaffold and MWCNT scaffold. (Scale bar-50µm).

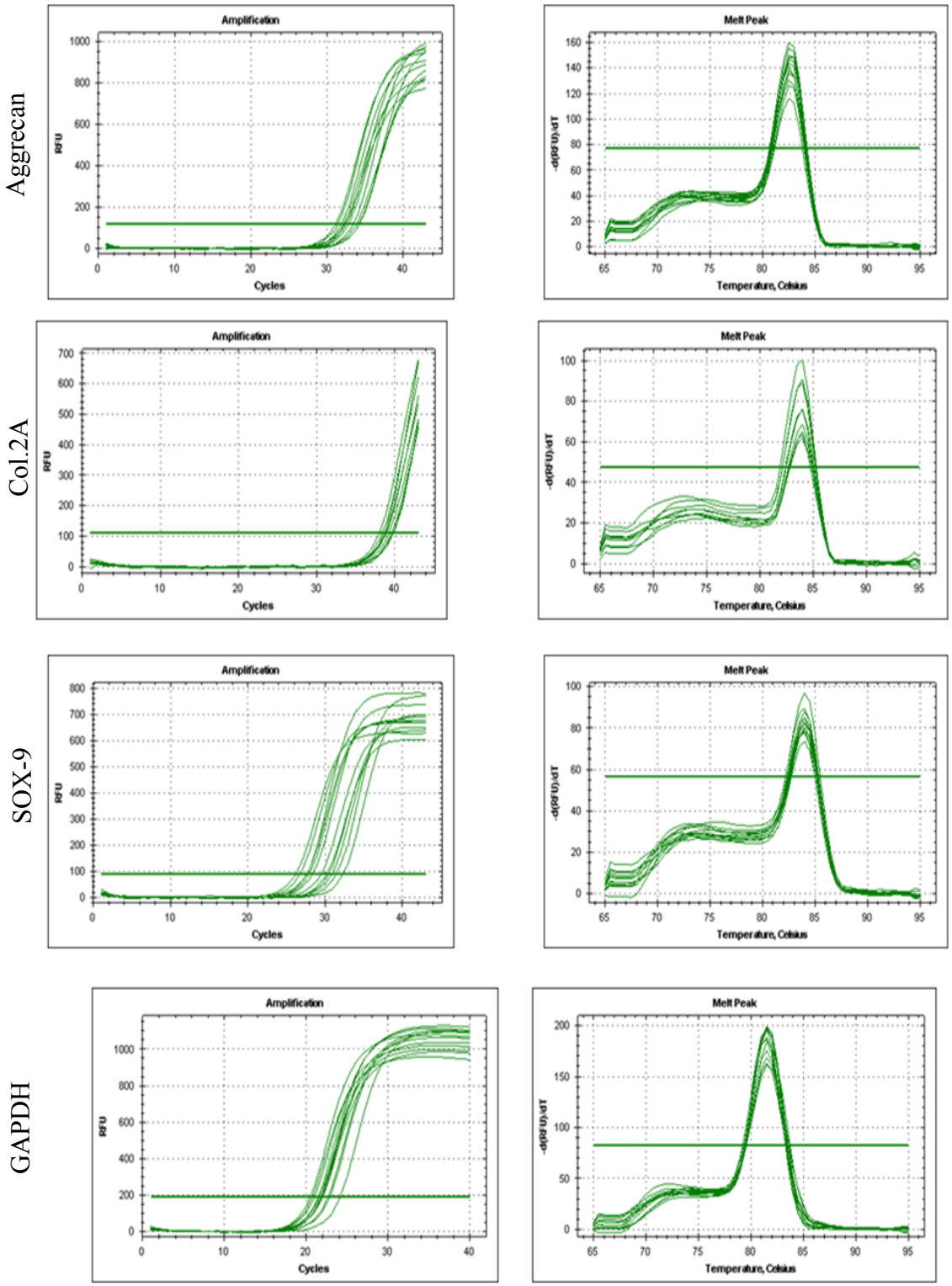


Fig. 20: Amplification curves and melting peaks of chondrocyte specific genes in differentiated cells: Aggrecan, Col.2A , SOX-9 and housekeeping gene GAPDH.

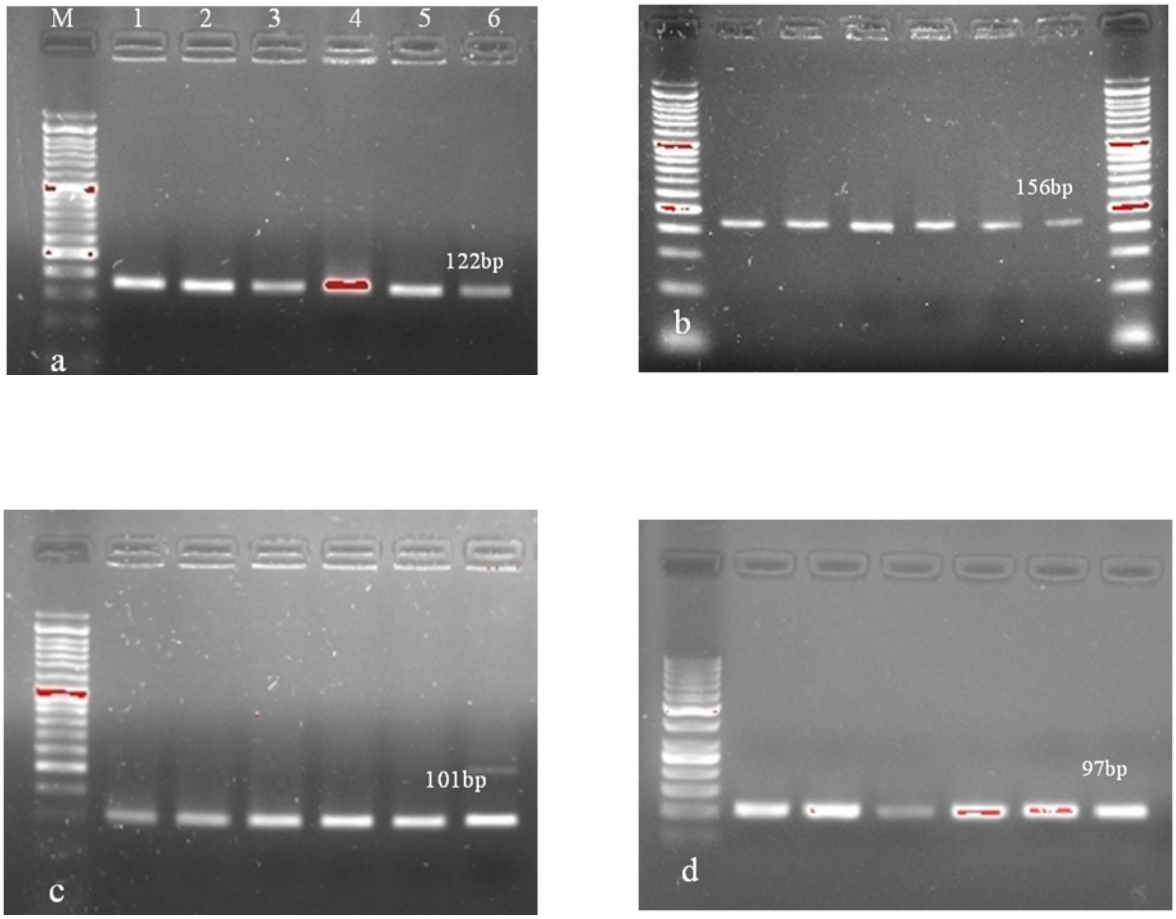


Fig. 21: Molecular characterization of differentiated cells. Gel electrophoresis of PCR amplified products of chondrocyte specific genes in 2% agarose gel. a. Aggrecan b. Col.2A c. SOX-9 d. GAPDH. Lane M: 50bp ladder, Lane 1 & 2: Control, Lane 3 & 4: SWCNT, Lane 5 & 6: MWCNT.

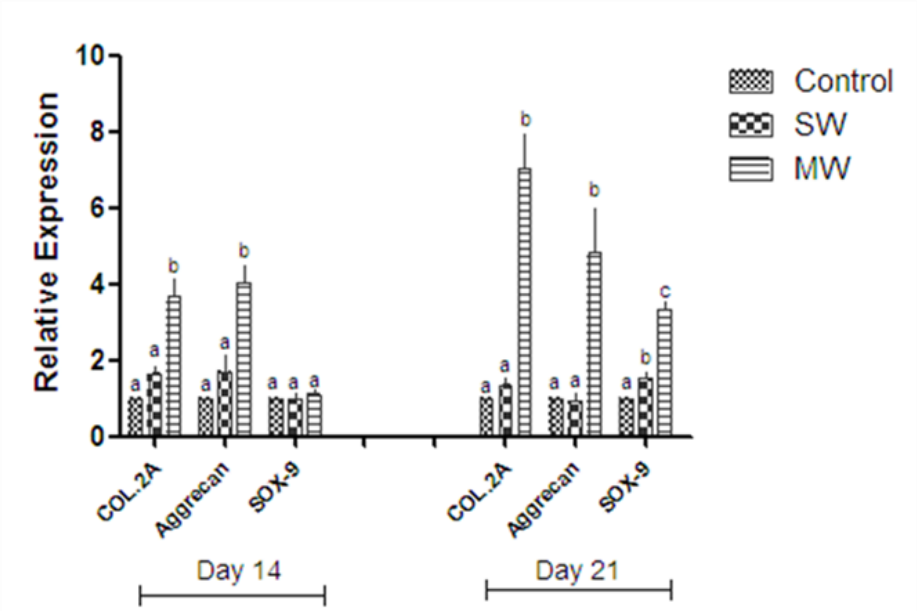


Fig. 22: Relative mRNA expression profiles of chondrocyte specific genes. The graph shows the relative expression of chondrocyte specific genes namely Collagen2A (Col.2A), Aggrecan and SOX-9 on day 14 and 21 days of culture. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 9: Relative mRNA expression profiles of chondrocyte specific genes over control and scaffolds.

Relative expression						
	Day 14			**Day 21**		
	COL.2A	**Aggrecan**	**SOX-9**	**COL.2A**	**Aggrecan**	**SOX-9**
Control	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}
SWCNT(OH)	1.65 ± 0.18^{a}	1.70 ± 0.43^{a}	0.99 ± 0.11^{a}	1.35 ± 0.16^{a}	0.94 ± 0.19^{a}	1.54 ± 0.14^{b}
MWCNT(OH)	3.67 ± 0.47^{b}	4.02 ± 0.44^{b}	1.10 ± 0.13^{a}	7.02 ± 0.91^{b}	4.84 ± 1.15^{b}	3.32 ± 0.19^{c}

–OH MWCNT scaffold were possessing significantly (P<0.05) more Aggrecan compared to canine MSCs cultured over control and –OH SWCNT scaffold **(Fig.23), (Table.10)**.

4.7 Neurogenic differentiation

Immunocytochemistry of neurogenic culture of cMSCs over the control and scaffolds against B-Tubulin III and MAP-2 indicated that in both control and scaffolds, cells were positive for B-Tubulin III and MAP-2 indicating that neurogenic differentiation had occurred in control as well as in scaffolds **(Fig.26),(Fig.27).**

After 7th day of neuronal induction of canine MSCs over control and scaffolds, qPCR expression of neuron specific genes were as follows. Expression of Nestin, MAP-2 and B-Tubulin III was significantly (P<0.05) higher in -OH SWCNT scaffold compared to control and –OH MWCNT scaffold **(Fig.28,29,30)**,**(Table.11)**.

Flowcytometry analysis of B-Tubulin III positive cells after 7 days of neurogenic culture shows that canine MSCs cultured over –OH SWCNT scaffold possessed more B-Tubulin III positive cells compared to control and –OH MWCNT scaffold **(Fig.31), (Table.12).**

4.8 Cytotoxicity study of OH-functionalized CNT on canine MSCs

Annexin-PI method of evaluating the early apoptotic, late apoptotic and necrotic cells during 4th and 6th day of culture indicated that, on day 4, percentage of early apoptotic cells were significantly (P<0.05) higher in SWCNT and MWCNT scaffold compared to control. But late apoptotic cells were significantly (P<0.05) lower among scaffold compared to control. Necrotic cells were significantly (P<0.05) higher among SWCNT and MWCNT scaffold compared to control **(Fig.32)**, (**Table.13).**

On day 6, percentage of early apoptotic cells were significantly (P<0.05) higher in SWCNT and MWCNT scaffold compared to control. But late apoptotic cells were significantly (P<0.05) lower among scaffold compared to control. Necrotic cells were significantly (P<0.05) higher among SWCNT and MWCNT scaffold compared to control **(Fig.32)**, (**Table.13).**

4.8.1 Relative expression of apoptotic genes

Relative expression of apoptotic genes on day 4 indicated, expression of Bax was significantly (P<0.05) higher among cMSCs cultured over SWCNT scaffold compared to control and MWCNT scaffold. Expression of caspase 3 was also significantly (P<0.05) higher among cMSCs cultured over SWCNT scaffold compared to control and MWCNT

scaffold. There was no significant ($P<0.05$) difference with regards to Caspase 8 expression among cMSCs cultured over control and scaffold. Expression of Caspase 9 was significantly ($P<0.05$) higher among cMSCs cultured over MWCNT scaffold compared to control and SWCNT scaffold **(Fig.33,34,35), (Table.14)**.

On day 6, expression of Bax was significantly ($P<0.05$) higher among cMSCs cultured over SWCNT scaffold compared to control and MWCNT scaffold. Expression of Caspase 3 was significantly ($P<0.05$) higher among cMSCs cultured over SWCNT scaffold compared to control and MWCNT scaffold. Expression of Caspase 8 was significantly ($P<0.05$) higher among cMSCs cultured over SWCNT scaffold compared to control and MWCNT scaffold. Expression of Caspase 9 was also significantly ($P<0.05$) higher among cMSCs cultured over SWCNT scaffold compared to control and MWCNT scaffold **(Fig.33,34,35), (Table.14)**.

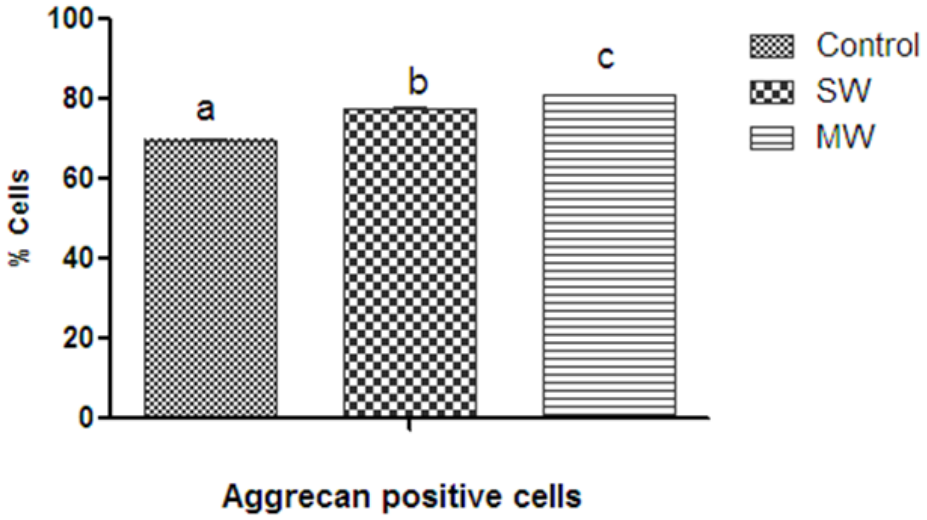
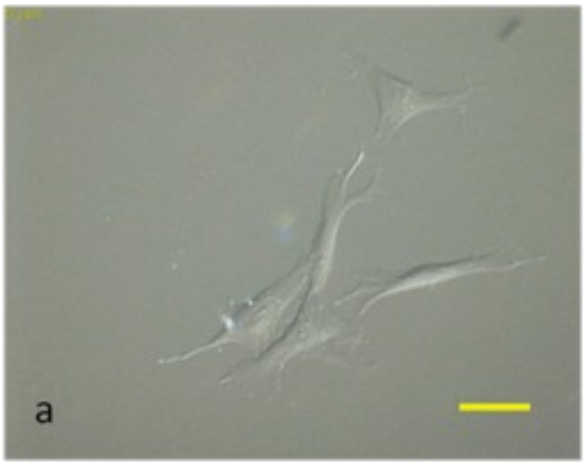
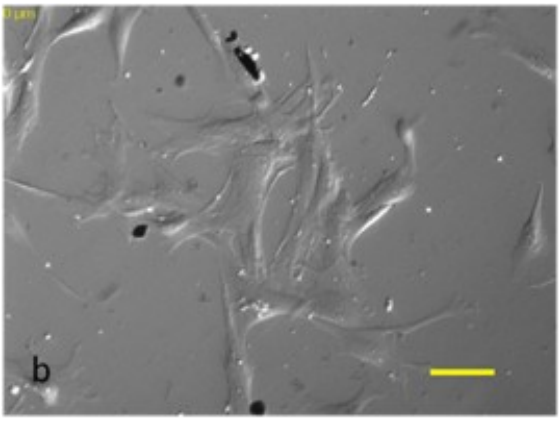


Fig. 23: Flowcytometric analysis of Aggrecan positive cells over control and scaffolds. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 10: Flowcytometric analysis of Aggrecan positive cells over control and scaffolds.

	Aggrecan positive cells (%)
Control	69.76 ± 0.32^{a}
SWCNT(OH)	77.50 ± 0.49^{b}
MWCNT(OH)	80.85 ± 0.06^{c}





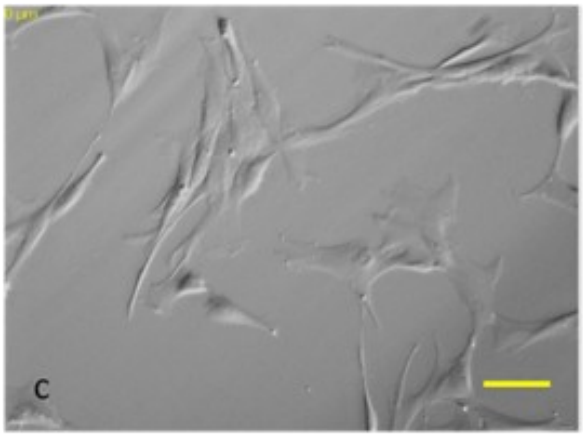
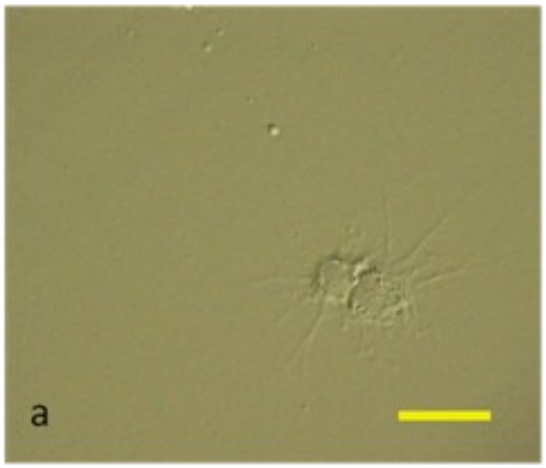
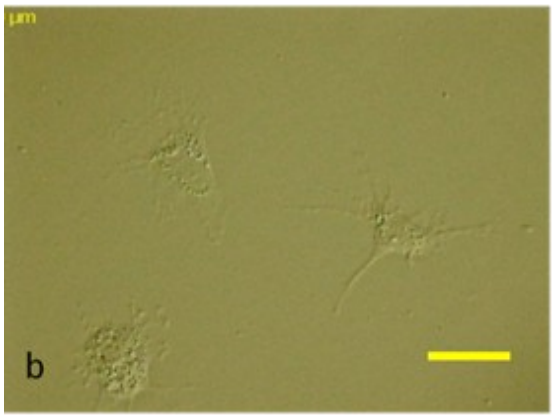


Fig. 24: Pre-induction culture of MSCs for neuron differentiation over a. Control. b. SWCNT scaffold and c. MWCNT scaffold. (Scale bar-50µm).





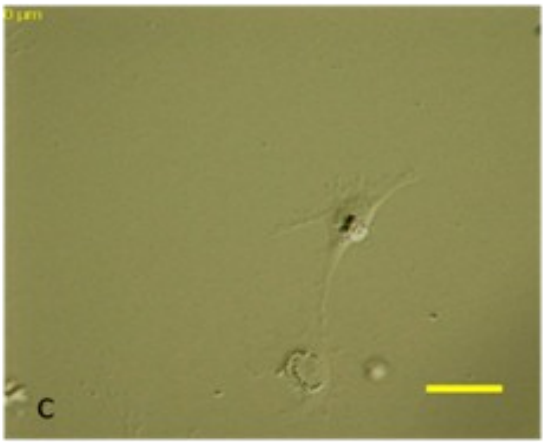


Fig. 25: 7-days induction culture of MSCs for neuron differentiation over a. Control. b. SWCNT scaffold and c. MWCNT scaffold. (Scale bar-50µm).

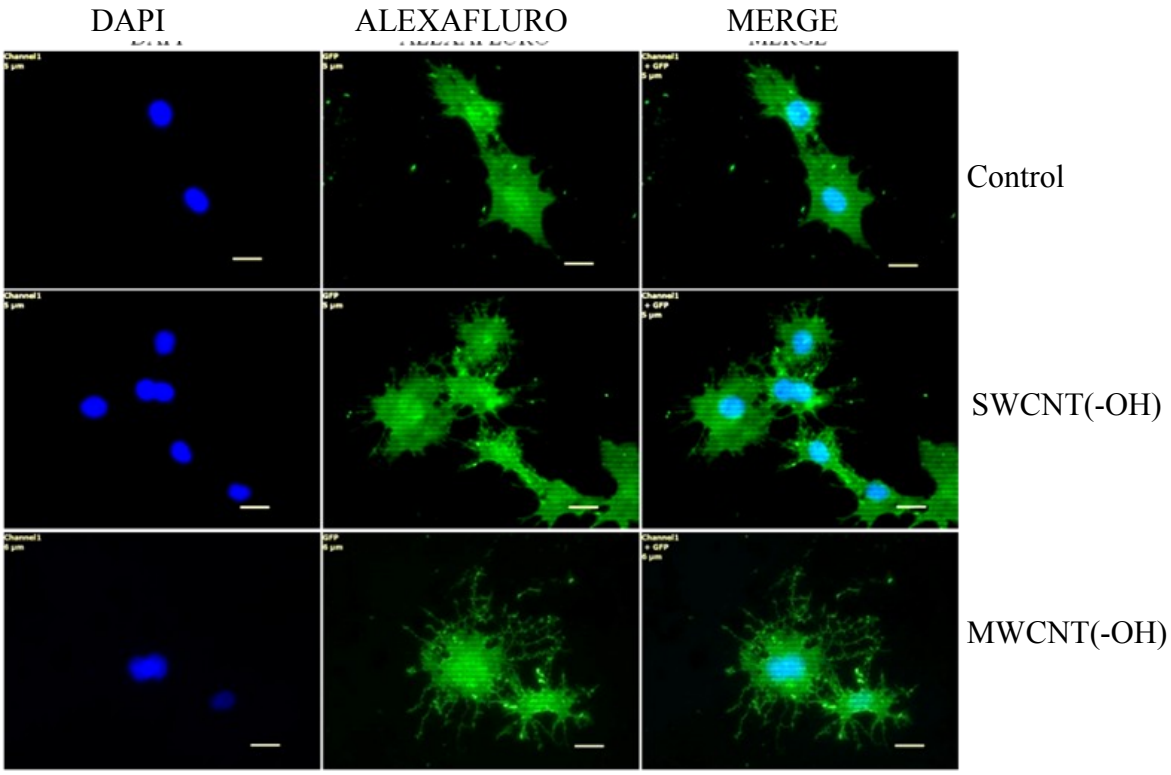


Fig. 26: Immunophenotyping of B-Tubulin III in neuron differentiation culture of MSCs over Control, SWCNT scaffold and MWCNT scaffold. (Scale bar-20µm).

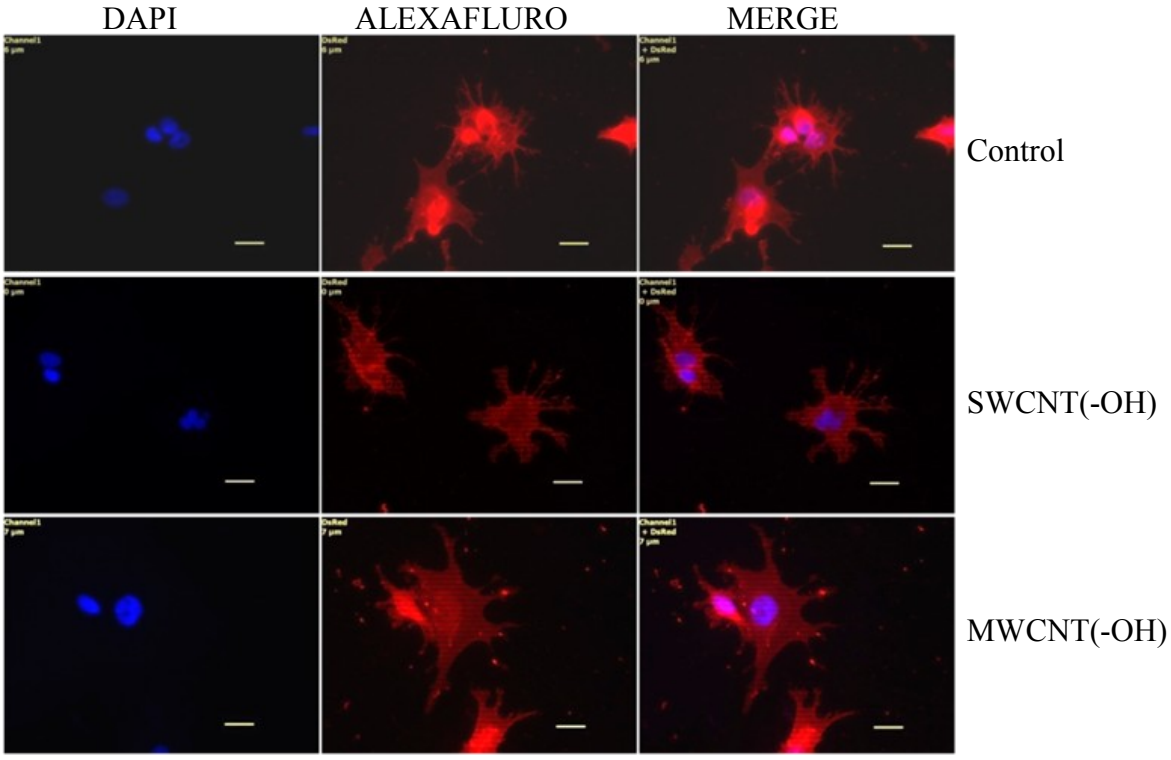


Fig. 27: Immunophenotyping of MAP-2 in neuron differentiation culture of MSCs over Control, SWCNT scaffold and MWCNT scaffold. (Scale bar-20µm).

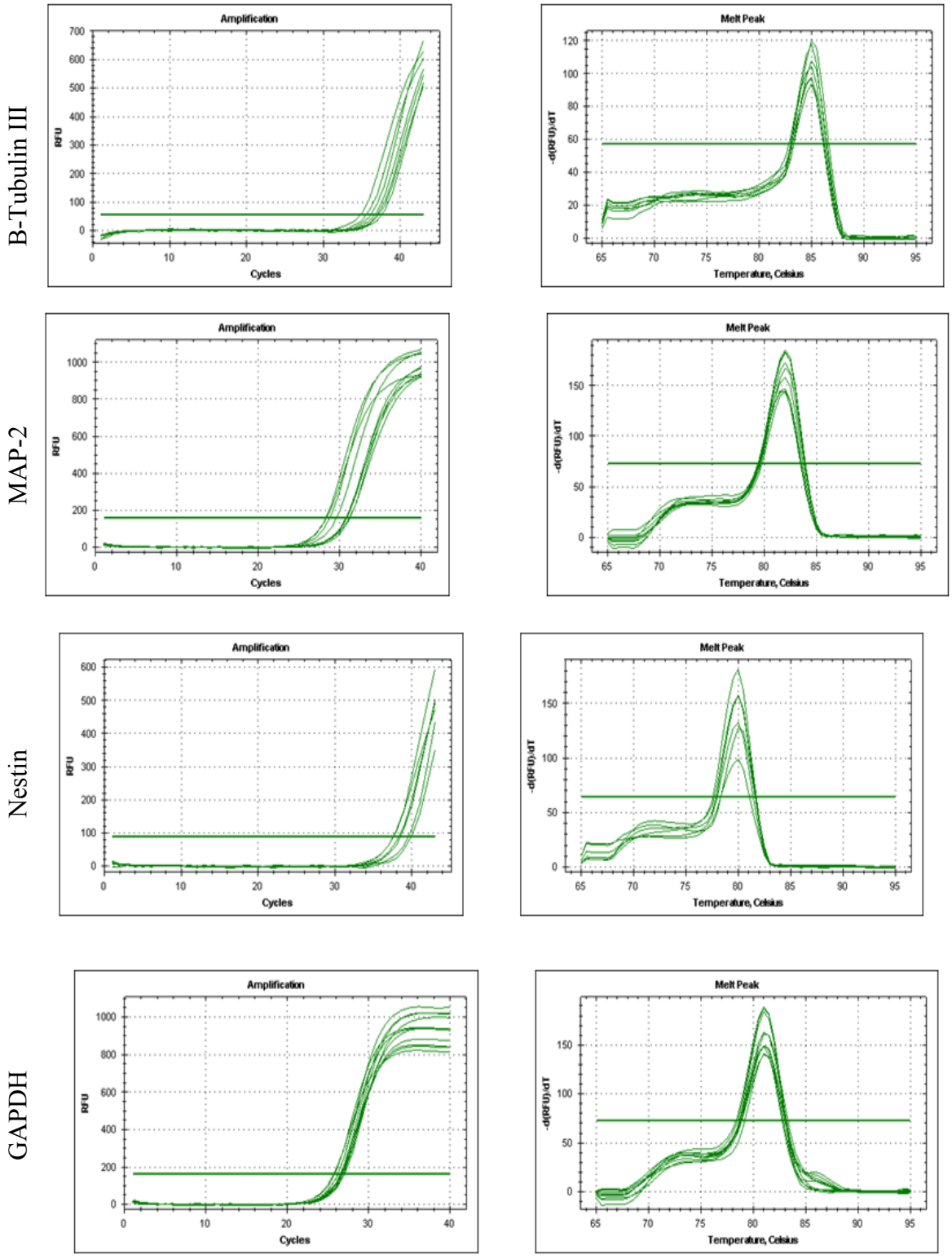


Fig. 28: Amplification curves and melting peaks of neuron specific genes in differentiated cells: B-Tubulin III, MAP-2, Nestin and housekeeping gene GAPDH.

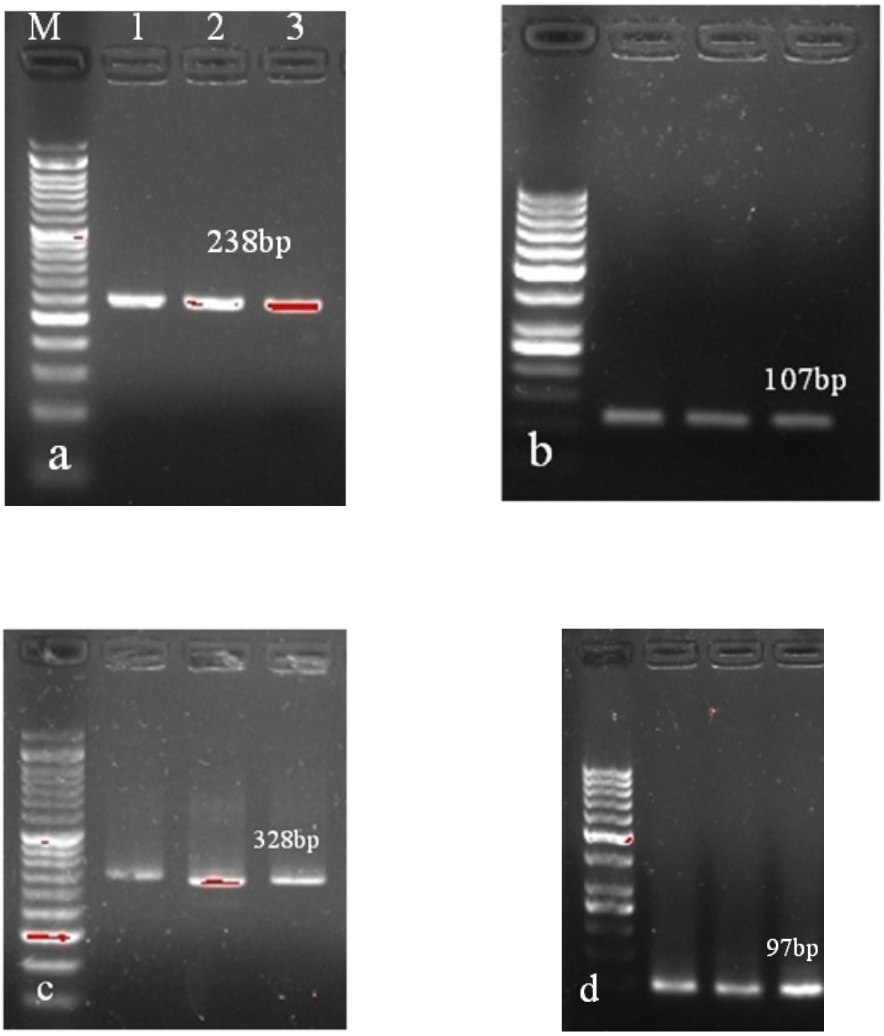


Fig. 29: Molecular characterization of differentiated cells. Gel electrophoresis of PCR amplified products of neuron specific genes in 2% agarose gel. a. B-Tubulin III b. MAP-2 c. Nestin d. GAPDH. Lane M: 50bp ladder, Lane 1: Control, Lane 2: SWCNT, Lane 3: MWCNT.

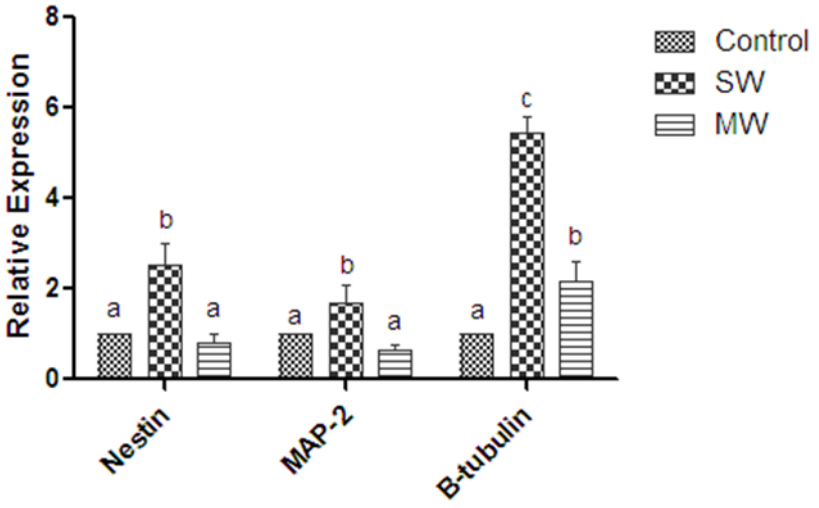


Fig. 30: Relative mRNA expression profiles of neuron specific genes. The graph shows the relative expression of neuron specific genes namely Nestin, MAP-2 and B-Tubulin III on day 7 of culture. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 11: Relative mRNA expression profiles of neuron specific genes over control and scaffolds.

Relative expression			
		Day 7	
	Nestin	**MAP-2**	**B-Tubulin III**
Control	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}
SWCNT(OH)	2.52 ± 0.49^{b}	1.69 ± 0.39^{b}	5.42 ± 0.37^{c}
MWCNT(OH)	0.82 ± 0.16^{a}	0.66 ± 0.09^{a}	2.17 ± 0.45^{b}

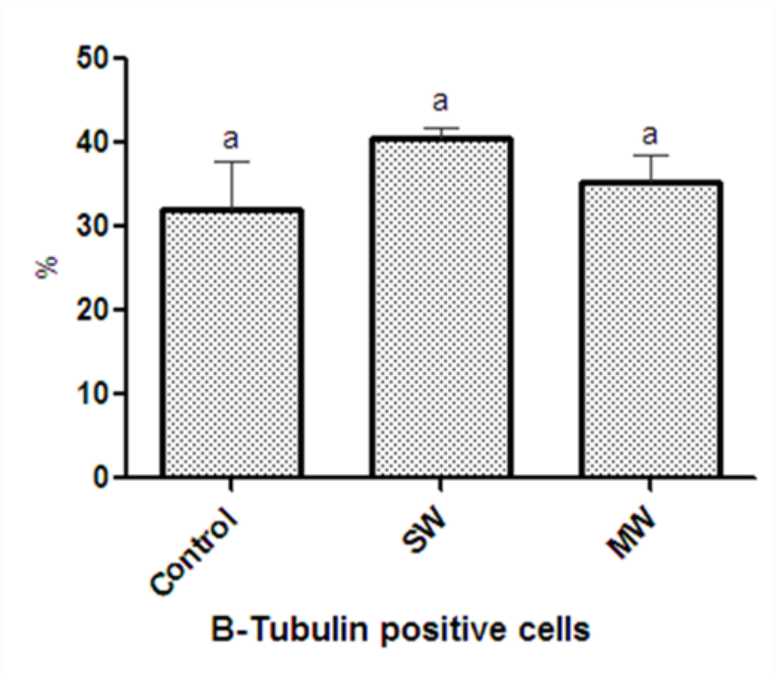


Fig. 31: Flowcytometric analysis of B-Tubulin III positive cells over control and scaffolds. Each data represents a mean± standard error. The superscript a indicates significant difference (P<0.05)

Table 12: Flowcytometric analysis of B-Tubulin III positive cells over control and scaffolds.

B-Tubulin III positive cells (%)	
Control	32.05±5.7[a]
SWCNT(OH)	40.43±1.35[a]
MWCNT(OH)	35.41±3.19[a]

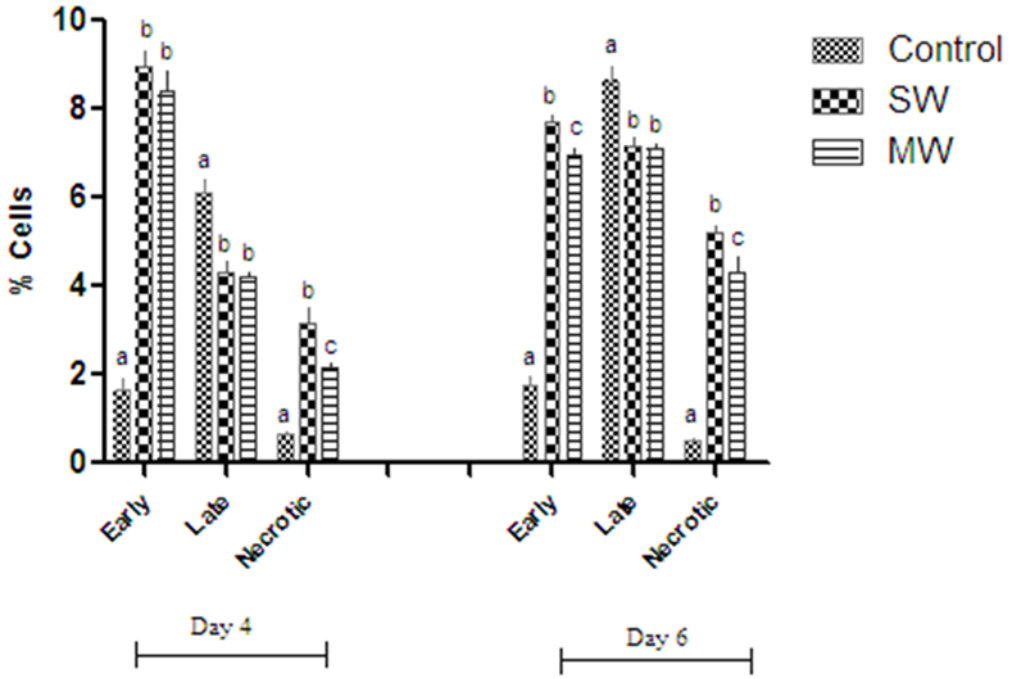


Fig. 32: Annexin-PI method of flowcytometric cytotoxic analysis of early apoptotic, late apoptotic and necrotic cells over control and scaffolds. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 13: Annexin-PI method of flowcytometric cytotoxic analysis of early apoptotic, late apoptotic and necrotic cells over control and scaffolds.

Flowcytometry cytotoxic analysis						
	Day 4			**Day 6**		
	Early	**Late**	**Necrotic**	**Early**	**Late**	**Necrotic**
Control	1.66±0.24[a]	6.09±0.28[a]	0.65±0.06[a]	1.74±0.21[a]	8.62±0.33[a]	0.51±0.05[a]
SWCNT(OH)	8.96±0.36[b]	4.30±0.24[b]	3.15±0.32[b]	7.69±0.18[b]	7.14±0.23[b]	5.25±0.16[b]
MWCNT(OH)	8.40±0.47[b]	4.20±0.10[b]	2.17±0.09[c]	6.98±0.14[c]	7.11±0.10[b]	4.29±0.38[c]

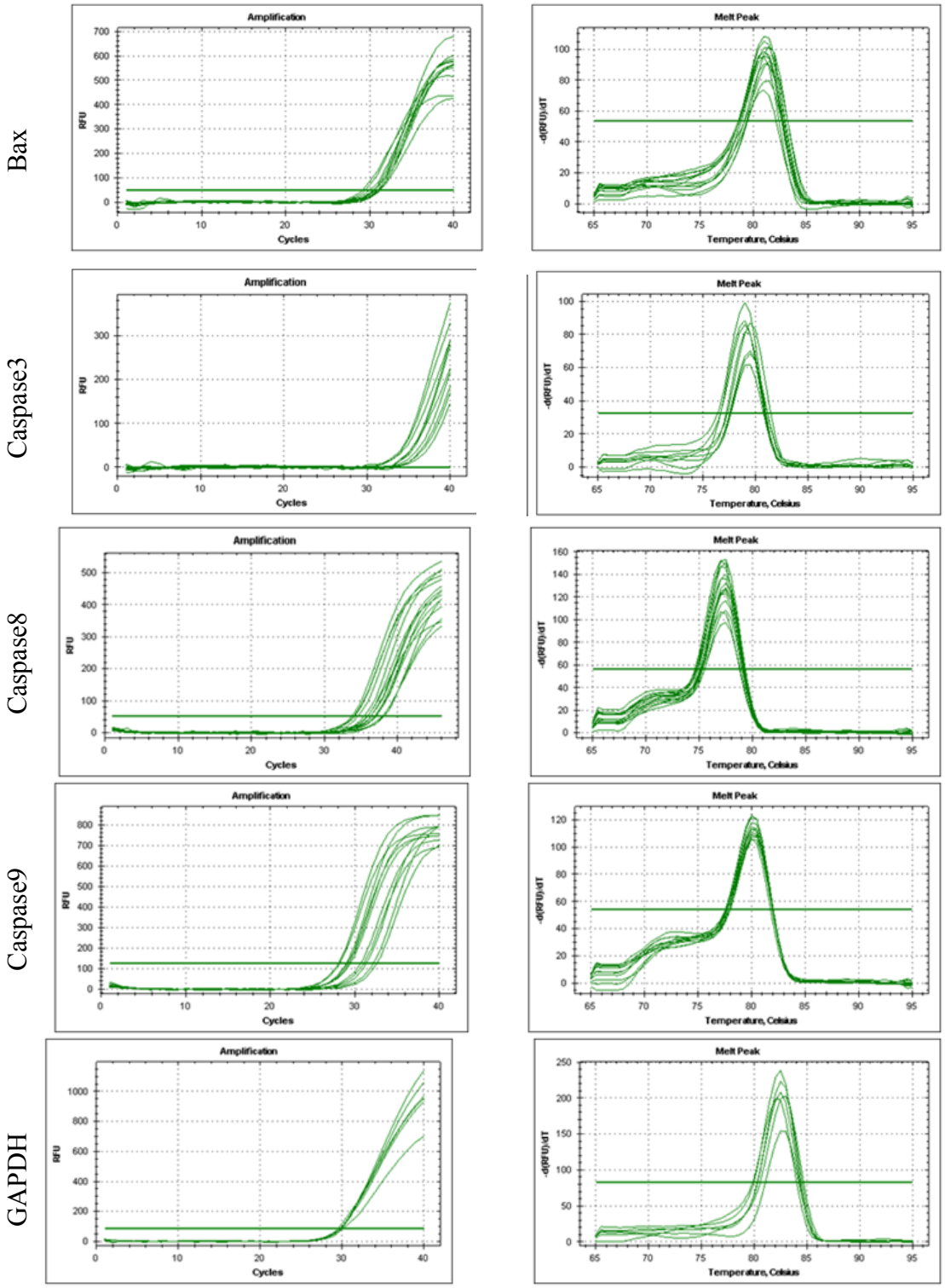


Fig. 33: Amplification curves and melting peaks of apoptotic related genes: Bax, Caspase3, Caspase8, Caspase9 and housekeeping gene GAPDH.

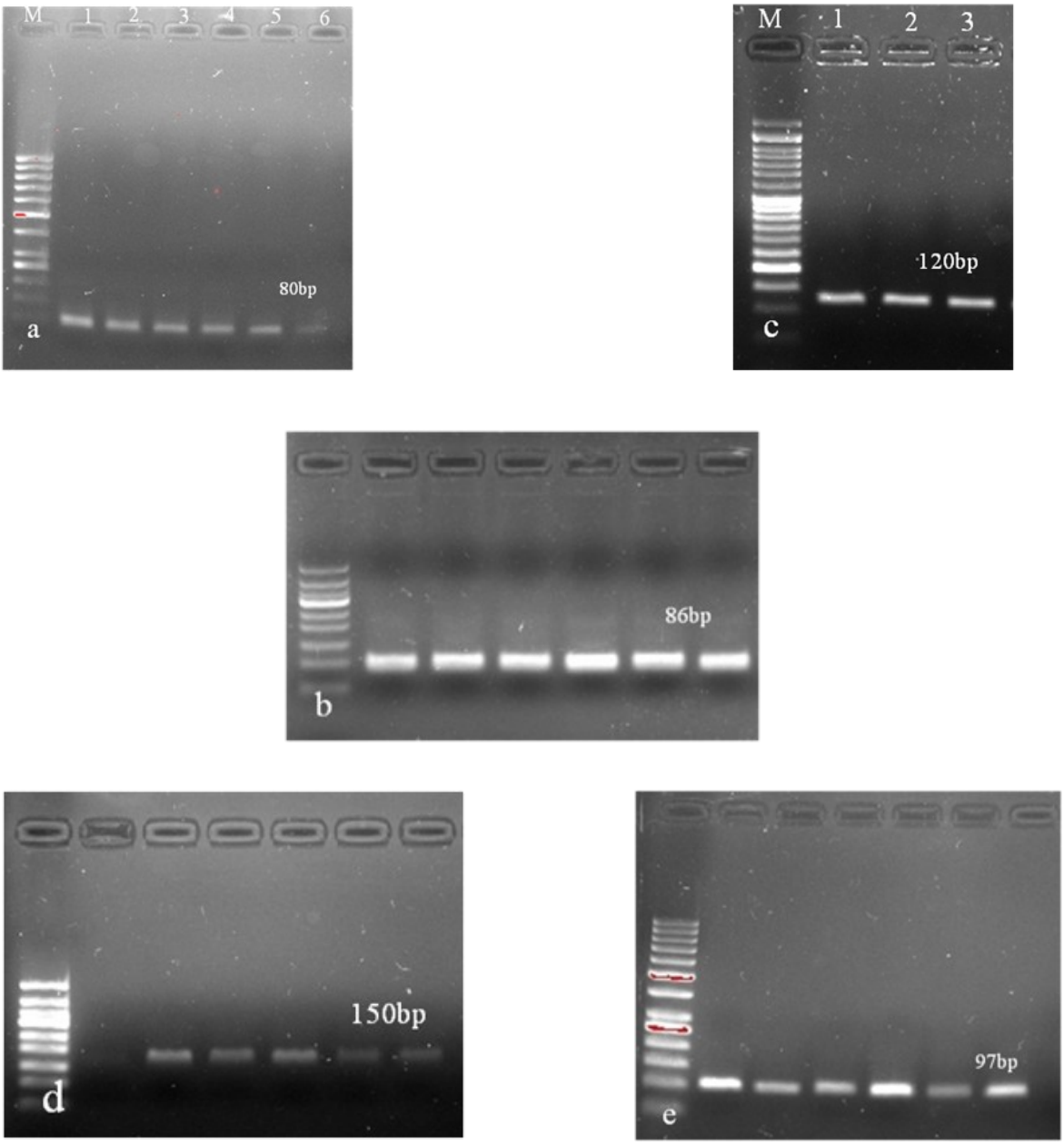


Fig. 34: Molecular characterization of apoptotic and necrotic cells. Gel electrophoresis of PCR amplified products of apoptotic related genes in 2% agarose gel. a. Bax b. Caspase3 c. Caspase8 d.Caspase9 and e. GAPDH. Lane M: 50bp ladder, Lane 1 & 2: Control, Lane 3 & 4: SWCNT, Lane 5 & 6: MWCNT.

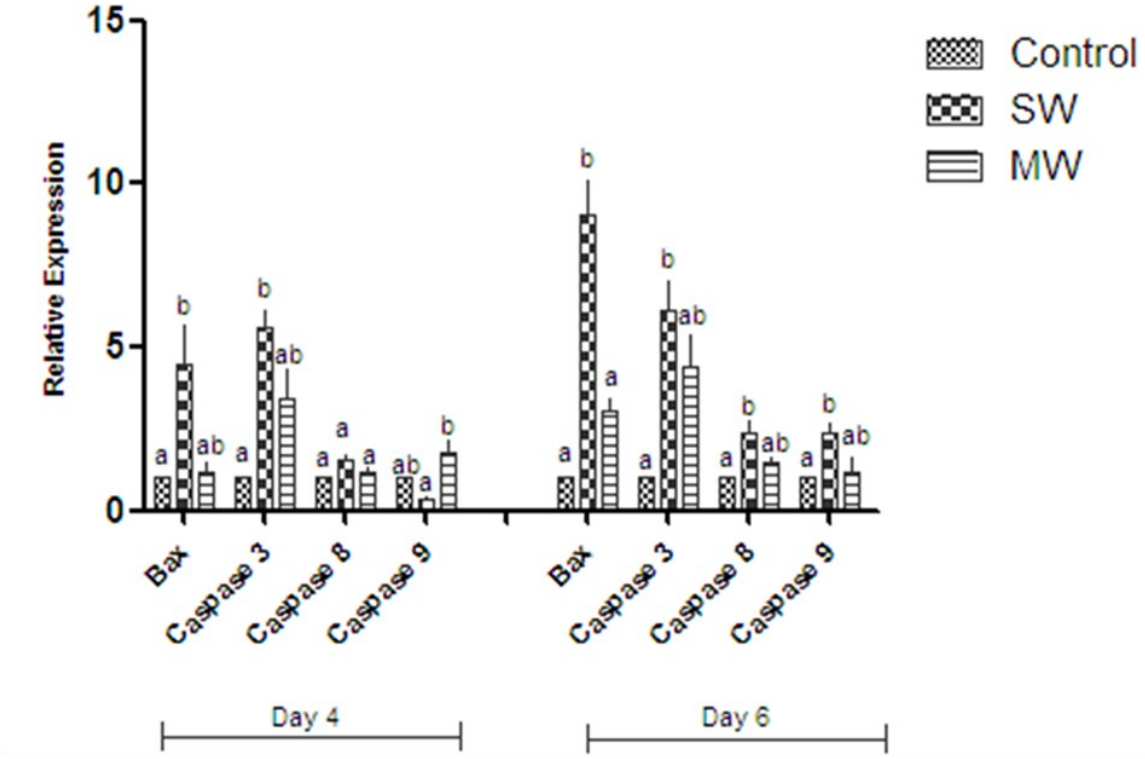
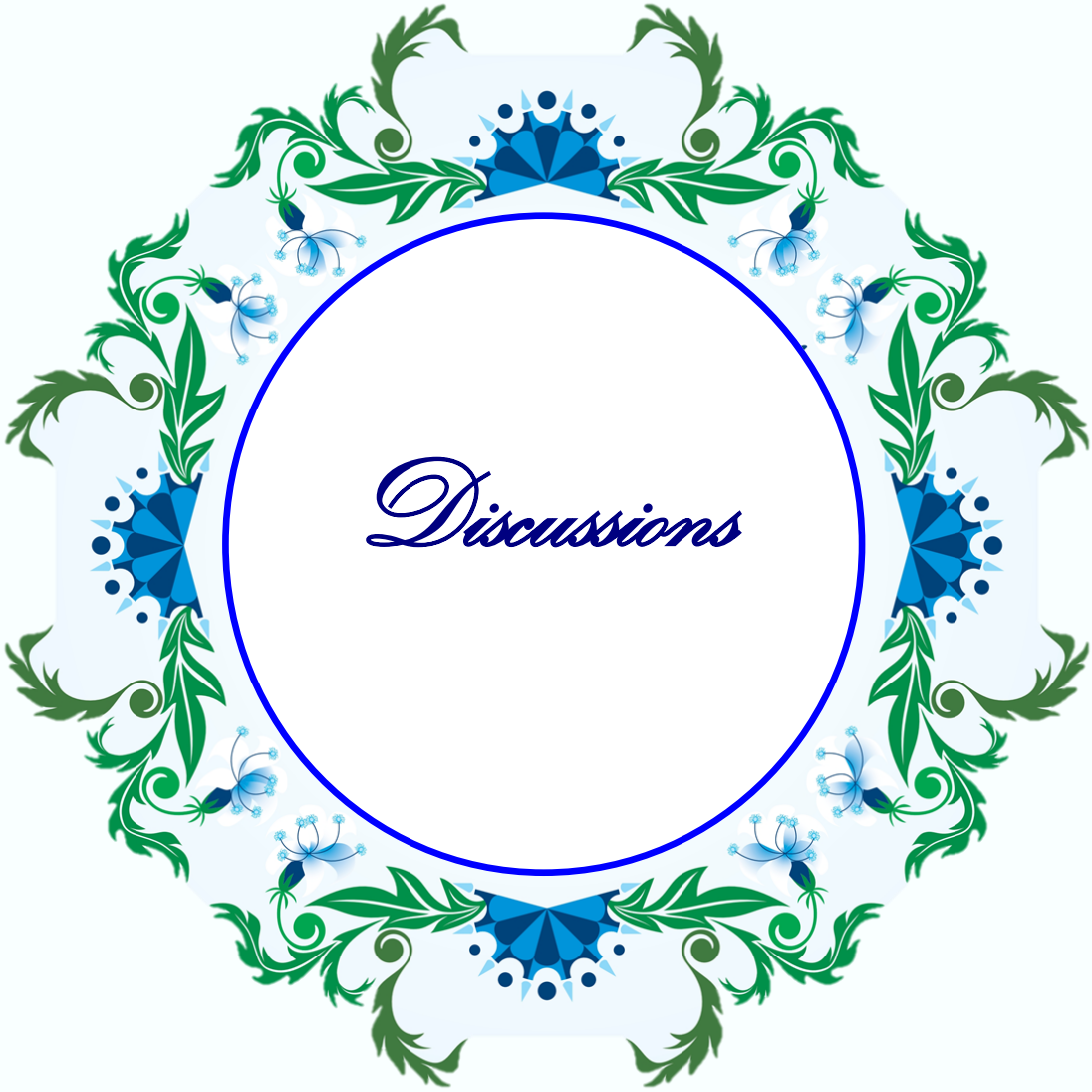


Fig. 35: Relative mRNA expression profiles of apoptosis associated genes namely; Bax, Caspase3, Caspase8, Caspase9 over control and scaffolds. Each data represents a mean± standard error. The superscript a, b, c indicates significant difference ($P<0.05$)

Table 14: Relative mRNA expression profiles of apoptosis associated genes over control and scaffolds.

	Relative expression							
	Day 4				Day 6			
	Bax	**Caspase3**	**Caspase8**	**Caspase9**	**Bax**	**Caspase3**	**Caspase8**	**Caspase9**
Control	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{ab}	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}	1 ± 0^{a}
SWCNT(OH)	4.44 ± 1.23^{b}	5.58 ± 0.56^{b}	1.53 ± 0.18^{a}	0.33 ± 0.06^{a}	9.05 ± 1.06^{b}	6.09 ± 0.94^{b}	2.39 ± 0.35^{b}	2.38 ± 0.30^{b}
MWCNT(OH)	1.19 ± 0.29^{ab}	3.41 ± 0.87^{ab}	1.20 ± 0.13^{a}	1.76 ± 0.38^{b}	3.07 ± 0.32^{a}	4.38 ± 0.98^{ab}	1.48 ± 0.10^{ab}	1.20 ± 0.39^{ab}


Discussions

Discussions

5.1 Isolation, culture and characterization of cBM-MSCs

The bone marrow derived MSC is easily accessible and possess the multi lineages differentiation potentials, represent the interesting candidates for gene therapy, tissue engineering and wide ranges of regenerative disorders (Keating, 2006) because of their multipotency, immunomodulatory properties, migratory behavior, plastic adherence properties and multi differentiation potential into chondrocytes, adipocytes and osteocytes (Fredenstein, *et al*., 1966), ectoderm origin like neurons (Woodbury *et al*.,2000)

In the present investigation, it was observed that bone marrow derived canine MSCs (cBM-MSCs) proliferated well in DMEM with low glucose media. Initially, the isolated cBM-MSCs were of non-fibroblastic morphology and later attained the typical mesenchymal phenotypes. These observations were in accordance with that of previous report by Donald *et al*, (2001), who demonstrated that fibroblast like cell morphology appear after several days of primary culture. During passaging of MSCs, the other cell populations rapidly disappeared from culture and could no longer be found from second passage onwards. Most of the cells exhibited typical MSCs characteristic, which showed spindle shape or triangular appearance. In the later passages, the MSCs population maintained their characteristic uniform spindle shape morphology.After third passage, other cell population disappeared and taken typical characteristic as demonstrated in MSCs derived from caprine bone marrow derived MSCs (Kumar *et al*., 2013).

Every cells posses certain specific unique marker, likewise the bone marrow derived MSCs of any species express surface markers that distinguished themselves from other types of stem cells. It is widely accepted that bone marrow derived MSCs express SH2 (CD 105), SH3/SH4 (CD 73), Integrin ß1 (CD 29), CD44, Thy-1 (CD 90), CD 71, vascular cell adhesion molecule-1 (CD 106), activated leukocyte cell adhesion molecule (CD 166), STRO-1, GD2, melanoma cell adhesion molecule (CD 146) etc. (Conget *et al*.,1999; Gronthos *et al*.,2003; Sordi *et al*.,2005). In consistent with general MSCs

profiles, present study demonstrated that canine bone marrow derived MSCs were positive for CD 73, CD 90 and CD 105 whereas negative for CD 45. These fulfilled the minimum criteria required for MSCs defined by International Society for Cytotherapy (ISCT). ISCT define the minimal criteria for human MSCs that includes the positive markers of CD 29, CD 44, CD 73, CD 90, CD 105, CD 166, STRO-1 and MHC class I but negative for hematopoietic markers like CD 34, CD 45 and MHC class II (Dominici *et al*.,2006).

The cBM-MSCs expressed the embryonic stem cell markers Oct 4 and Nanog and therefore probably MSCs share the differentiation capabilities of embryonic stem cells. This result was in accordance of previously reported literature on canine MSCs (Kisiel *et al*., 2012). It is well established that adult stem cells including MSCs expressed the embryonic stem cell or pluripotent markers; SSEA4, Oct 4, Nanog and Sox2. However little is known about their functional role in adult stem cells.

The cBM-MSCs were checked for in vitro differentiation potentially into mesodermal cell lineage adipocytes and osteocytes. The cBM-MSCs were successfully differentiated into osteocytes and adipocytes and differentiation were confirmed by Oil O Red staining for adipogenic cells and Alizarin Red staining for osteogenic cells. It is well documented that the MSCs posses classical multi-lineage mesodermal differentiation capacity into adipogenic, osteogenic and chondrogenic lineages (Fredenstein, *et al*., 1966).

5.2 Influence of CNT on cell surface area

Oh *et al*, (2008) reported that nanotubes elicited a dramatic stem cell elongation, which induced cytoskeleton stress and promotes differentiation. In another literature it reported that cell spread on MWCNTs toward a polygonal shape with many thin filopodia to attach to the surfaces (Deliganni *et al*., 2014). Our results are correlated in which measurement of cell surface area on day 2 culture indicated, cell surface area was significantly higher on SWCNT scaffold compared to control and MWCNT scaffold. On day 4 and 6 days of culture compared to control, among both scaffolds, cell surface area was significantly higher. Tay *et al*, (2010) noted that hMSCs cultured on untreated coverslip adopted a less well spread area morphology as compared to cells grown on SWCNTs coverslip. Cells cultured on SWCNTs coated coverslip had higher occurrence of positive subcellular features such as filopodias (microspikes).

5.3 Influence of CNT on cell proliferation

Liu *et al*., 2010 found that, -COOH SWCNT and MWCNT exhibited concentration dependent reduction in cell proliferation and viability of mice MSC. Tay *et al*, (2010) noted that, hMSCs proliferated at a slower rate on SWCNT film as compared to control. The number of hMSCs was significantly lower on SWCNT film from day 7 onwards. In our experiment, with respect to cell proliferation we have carried out colony forming assay and MTT assay, in which both tests indicated cBM-MSCs proliferation was less among scaffolds compared to control. So our results are on par with available literature regarding cell proliferation.

5.4 Influence of CNT on osteogenic differentiation of cBM-MSCs

Baktur *et al*., (2010) found that, there was a significant increase in Alkaline Phosphatase activity and ECM mineralization in osteogenic differentiation of rat MSC on MWCNT-collagen scaffolds. Duran *et al*., (2014) worked on osteogenic differentiation of murine embryonic stem cells on –COOH SWCNT and reported that there was increased osteogenic differentiation and increased secretion of osteocalcin. In the present study, the expression of Osteopontin(OPN) and Osteocalcin(OCN) was significantly higher in –OH MWCNT scaffold compared to control and -OH SWCNT scaffold on both 14 and 21 days of culture. Flowcytometry analysis of OCN positive cells after 21 days of osteogenic culture was also on par with real-time expression in which cBM- MSCs cultured over –OH MWCNT scaffold were possessing more OCN positive cells compared to cBM-MSCs cultured over control and –OH SWCNT scaffold. But, Mooney *et al*., (2008) reported a contradictory finding in which there was no significant effect on osteogenesis in hMSC cultured over –COOH and –OH CNT. Deligianni.D.D., (2014) reported that MWCNTs delay the proliferation of hBMSCs but increase their differentiation. They showed that MWCNTs substrates enhanced expression of OCN and OPN levels.

5.5 Influence of CNT on chondrogenic differentiation of cMSCs

Humphurus *et al*., (2008) noted that, there was a dense growth of human chondrocytes over the composite scaffold of CNT and polycarbonate urethane. Chahine *et al*., (2014) reported that, functionalized carbon nanotubes provide an improved molecular sized substrate for stimulation of chondrocyte growth. Chondrocytic differentiation of cBM-MSCs over –OH SWCNT and MWCNT scaffold indicated that the expression of

COL.2A was significantly higher in -OH MWCNT scaffold compared to control and –OH SWCNT scaffold on both 14 and 21 days of culture. Expression of aggrecan was also significantly higher in –OH MWCNT scaffold compared to control and SWCNT scaffold on both 14 and 21 days of culture. Flowcytometry analysis of aggrecan positive cells after 21 days of chondrogenic differentiation over control and scaffold indicated that, aggrecan positive cells were significantly higher among MWCNT scaffold compared to control and SWCNT scaffold. But, Mooney *et al*., (2008) noted the, decrease in chondrogenesis of hMSC cultured over –COOH and –OH CNT.

5.6 Influence of CNT on neurogenic differentiation of cMSCs

Fabbro *et al*., (2012) reported that, MWCNT scaffold modulate the elasto-mechanical properties of neuronal fibers. Khraiche *et al*., (2009) showed that, SWCNT induced enhancement of neuronal excitability and accelerate the onset of neuronal excitability and accelerate the onset of neuronal electrical activity. Our neuronal differentiation studies indicated that, after 7 days of neuronal induction of cBM-MSCs , expression of Nestin, MAP-2 and B-TubulinIII was significantly higher in -OH SWCNT scaffold compared to control and –OH MWCNT scaffold. Flowcytometry analysis of B-Tubulin III positive cells after 7 days of neurogenic differentiation showed that cBM- MSCs cultured over –OH SWCNT scaffold possessed more ß-Tubulin positive cells compared to canine MSCs cultured over control and -OH MWCNT scaffold. In this regard, Hu *et al*., (2005) reported that, chemical modification of carbon nanotubes control neurite extension and branching.

5.7 Cytotoxicity of –OH SWCNT and MWCNT on cMSC

Tian *et al*., (2006) tested the cytotoxicity of 5 forms of carbon on human fibroblast cells and noted decreased cell survival and upregulation of apoptotic genes (p16, Bax, p53). Baktur *et al*., (2013) observed the effect of MWCNT collagen scaffold on hMSC and observed no significant change in cell viability. Our cytototoxicity studies namely, Annexin-PI method of evaluating the early apoptotic, late apoptotic and necrotic cells among the 4th and 6th day of culture indicated that, on both days, percentage of early apoptotic cells was significantly higher in SWCNT and MWCNT scaffold compared to control. But late apoptotic cells were significantly lower among scaffold compared to control. Necrotic cells were significantly higher among SWCNT and MWCNT scaffold compared to control. Relative expresion of apoptotic genes on day 4 indicates: Expression

of Bax and Caspase 3 were significantly higher among cMSC cultured over SWCNT scaffold compared to control and MWCNT scaffold. There was no significant difference with regards to Caspase 8 expression among control and scaffold. Expression of Caspase 9 was significantly higher among cMSC cultured over MWCNT scaffold compared to control and SWCNT scaffold. Relative expression of apoptotic genes (Bax, Caspase3,8,9) on day 6 indicated, expression of all four genes were significantly higher among cMSC cultured over SWCNT scaffold compared to control and MWCNT scaffold. Ursini *et al*., (2016) tested the cytotoxicity of pristine, -OH and –COOH functionalized MWCNTs on respiratory alveolar epithelial cells indicated pristine and –COOH MWCNTs induce significant cytotoxicity.


Summary
and
Conclusions

6 Summary and Conclusions

The intersection of two vibrant modern research areas namely, stem cell research and nanotechnology are widely opening the new avenues. Varied application of nanotechnology in stem cell research includes, isolation and sorting, molecular detection, stem cell microenvironment and tissue engineering, tracking and imaging, transfection, delivery vehicle.

Tissue engineering literature is incomplete without the mentioning of stem cells. Tissue engineering uses the nanomaterials as scaffolds and cellular component as substrates for creation of desired organ or tissue. Among the various cells available stem cells are the preferred candidate. Stem cells are the unique cells having the characteristic of self-renewal and differentiation potential. Mesenchymal stem cells (MSCs) are the kind of stem cells which are derived from various mesodermal lineages of body. MSCs are the most preferred stem cells in tissue engineering and regenerative therapy.

Carbon nanotubes are the unique type of nanomaterial which are made up of grapheme. CNTs have excellent mechanical, electrical and surface properties which make them ideal candidates for a wide range of application. CNTs can be functionalized to improve their biocompatibility, to reduce their toxicity.

So with this background we tested the proliferation and differentiation potential of canine MSCs over –OH functionalized CNT scaffolds as well as cytotoxicity effects of CNT to canine MSCs. We summarized and drawn the following conclusion as follows:

6.1 Summary

1. Canine MSCs isolated from bone marrow and were characterized for their cell surface markers immunophenotypically.
2. -OH functionalized SWCNT and MWCNT scaffold prepared and characterized by the scanning electron microscope.
3. Phalloidin staining of actin fibres indicated no such alteration of cellular morphology among control and scaffold.

4. Cell surface area significantly enhanced over SWCNT scaffold. This is mainly attributed to the formation of filopodia(microspikes).
5. Colony forming ability, which indicated cultures containing subpopulation of cells capable of generating new colonies from single cell, was lower among both scaffolds.
6. MTT assay indicated less proliferation of cMSCs over scaffold compared to control.
7. Relative expression of OCN, OPN and flowcytometric analysis of OCN positive cells were higher among cMSCs differentiated into osteocytes over MWCNT scaffold.
8. Relative expression of COL.2A, Aggrecan, SOX-9 and flowcytometric analysis of Aggrecan positive cells were higher among cMSCs differentiated into chondrocytes over MWCNT scaffold.
9. Relative expression of Nestin, MAP-2, B-Tubulin III and flowcytometric analysis of B-Tubulin III positive cells were higher among cMSCs differentiated into neurons over SWCNT scaffold.
10. Annexin-PI method of evaluating cytotoxicity indicates total apoptotic and necrotic cells were higher among scaffold compared to control.
11. Relative expression of apoptotic related genes (Bax, Caspase3, Caspase8 and Caspase9) were higher among scaffold compared to control.

6.2 Conclusion

1. Canine MSC proliferation was decreased over the –OH SWCNT and MWCNT scaffold whereas cell surface area enhanced among the scaffold.
2. Osteogenic and chondrogenic differentiation was enhanced over the –OH MWCNT scaffold compared to –OH SWCNT scaffold and control.
3. Neurogenic differentiation was enhanced over the –OH SWCNT scaffold compared to -OH MWCNT and control.
4. -OH SWCNT and MWCNT scaffold posses cytotoxic effect which is at tolerable level which is evident by 80% viable cells.
5. -OH SWCNT and MWCNT scaffold promotes the differentiation and lowers the proliferation of canine MSC.
6. Due to lack of nanoroughness in control, canine MSCs experienced least stress as well as lesser cytotoxicity, hence proliferation was better.


Mini Abstract

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Nanotechnology is an emerging and rapidly developing field of modern science. This area of study entails the application of various industrial sectors, such as electronics, energy production, chemical engineering, and diverse fields of basic science and biomedicine. During the past decade, nanotechnology has been efficiently integrated into biomedical research, providing new methods for cell imaging, gene and small-molecule delivery, and scaffold design for tissue engineering purposes. Tissue engineering is a clinically driven field and has emerged as a potential alternative to organ transplantation. The cornerstone of successful tissue engineering rests upon two essential elements: cells and scaffolds. Recently, it was found that stem cells have unique capabilities of self-renewal and multilineage differentiation to serve as a versatile cell source, while nanomaterials have lately emerged as promising candidates in producing scaffolds able to better mimic the nanostructure in natural extracellular matrix and to efficiently replace defective tissues. In this regard, inorder to check the applicability of CNT in canine regenerative therapy, we had designed experiment to check the proliferation and differentiation ability of cMSCs over –OH functionalized SWCNT and MWCNT scaffold. Cytotoxicity studies were also undertaken. For the experiment fresh cells were isolated from canine bone marrow and characterized morphologically as well as immunophenotypically for surface markers. Proliferative ability of cMSCs over the scaffold was less but cell surface area was enhanced. Differentiation into osteoctes and chondrocytes was enhanced over –OH MWCNT scaffold whereas differentiation into neurons was enhanced over –OH SWCNT scaffold, CNTs posses cytotoxic effect but is at tolerable level as evident by 80% viable cells. So with these we can conclude that CNTs promotes the differentiation of cMSCs into osteocytes, chondrocytes and neurons with tolerable cytotoxicity. There is a wide research scope to determine the underlying molecular mechanisms responsible for this differentiation. Thus, CNTs can be used as scaffolds to promote the differentiation of cMSCs.


लघु सारांश

४ लघु सारांश

नैनोटेक्नोलॉजी आधुनिक विज्ञान की एक उभरती हुई और तेजी से विकसित क्षेत्र है। अध्ययन का यह क्षेत्र विभिन्न औघोगिक क्षेत्रो के उपयोग पर जोर देता है, जैसे की इलेक्टानिक्स, उर्जा उत्पादन, केमिकल इंजीनियनिंग और बुनियादी विज्ञान और वायोमेडिसिन के विविध क्षेत्रो के रूप मे पिछले एक दशक के दौरान नैनोटेक्नोलजी को कुशलता से जैव चिकित्सा अनुसंधान सेल इमेजिग के लिए नए तरीके को उपलब्ध कराने, जीन और छोटे अणुवितरण और ऊतक इंजीनियरिग प्रयोजनो के लिए स्केफोल्ड डिजाइन में एकीकृत किया गया। ऊतक इंजीनियनिग एक चिकित्सकीय प्रेरित क्षेत्र हैं और अंग प्रत्यारोपण के लिए एक संभावित विकल्प के रूप में उभरा है। सफल ऊतक इंजीनियनिग की आधारशिला दो आवश्यक तत्वो पर टिकी हुई है : कोशिकाओं ओर स्केफोल्ड। हाल ही में यह पाया गया है कि स्टेम कोशिकाऐ एक बहुमुखी सेल स्रोत के रूप में सेवा करने के लिए, आत्म नव. ीनीकरण और मल्टीलाइनेस भेदभाव परिवर्तन की अद्वितीय क्षमता रखती है। जबकि हाल ही में नेनोमेटिरियल्स बेहतर प्राकृतिक बाहय मैट्रिक्स में नेनोस्ट्रक्चर की नकल करने के लिए कुशलता से दोषपूर्ण ऊतको को बदलने में सक्षम स्केफोल्ड के उत्पादन के रूप में होनहार उम्मीदवार की तरह उभरा है। इस संबध में कुत्ते की पुनर्योजी चिकित्सा में सीएनटी की प्रयोज्यता की जांच के लिए हमने एमएससी के प्रसार परिवर्तन की क्षमता ओएच कियाशीली एसडब्लूसीएनटी ओर एमडब्लूसीएनटी स्केफोल्ड पर देखने के लिए प्रयोग डिजाइन किया था। कोशिका आविषता का अध्ययन भी किया गया था। प्रयोग के लिए कुत्ते की अस्थि मज्जा से ताजी कोशिकाऐं अलग की गयी और सतह मार्करो के लिए आकृति विज्ञान के साथ ही इम्यूनोफीनोटिपिकल को चिन्हित किया गया था। स्केफोल्ड के ऊपर सीएमएससी प्रसार क्षमता कम थी लेकिन कोशिका की सतह क्षेत्र बढ़ गया था। अस्थयणु और उपास्थणु में परिवर्तन ओएच एमडब्लूसीटीएनटी स्केफोल्ड पर बढ़ गया था जबकि न्यूरॉन्स में परिवर्तन ओएच एसडब्लूसीटीटी स्केफोन्ड पर बढ़ गया था, सीएनटी साइटोटोक्सिक प्रभाव को धारण करता हे लेकिन व्यवहार्य कोशिकाओं स्पष्ट एक संतोषजनक स्तर पर है। तो इनके साथ हम यह निष्कर्ष निकाल सकते है कि सीएनटीएस अस्थयणु, उपास्थयणु और सहनीय साइटोटोक्सिसिटी के साथ न्यूरॉन्स में सीएमएससी के परिवर्तन को बढ़ावा देता है इस परिवर्तन के लिए जिम्मेदार अतर्निहित आणविक तंत्र निर्धारित करने के लिए एक व्यापक अनुसंधान गुंजाइश है। यही कारण है कि सीएनटीएस अस्थि मज्जा के परिवर्तन को बढ़ावा देने के लिए स्केफोल्ड के रूप में इस्तेमाल किया जा सकता है।


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Appendix

1. Composition of DMEM (15%) media	**-100ml.**
FBS	-15ml.
L-Glutamine	-1ml.
Antibiotics (Penicillin(100U/ml+Amphotericin0.25µg/ml)	-1ml.
DMEM	-83ml.

2. Composition of MSC freezing media	**-10ml.**
DMEM	-5ml.
FBS	-4ml.
DMSO	-1ml.

3. Composition of Phosphate Buffer Saline(PBS)	**-1000ml.**
NaCl	-8g.
KCl	-0.2g.
Na2HPO4	-1.44g.
KH2PO4	-0.24g.
MilliQ water	-1000ml.

4. Composition of Osteogenic differentiation media

DMEM	
FBS	-10%.
Antibiotics (Penicillin(100U/ml+Amphotericin0.25µg/ml)	
Dexamethasone	-0.1µM.
L-Ascorbic acid	-0.25mM.
ß-Glycerophosphate	-10mM.

5. Composition of Chondrogenic differentiation media

DMEM	
FBS	-10%.
Antibiotics (Penicillin(100U/ml+Amphotericin0.25µg/ml)	
Dexamethasone	-100nM.
L-Ascorbic acid	-1mM.
Sodium pyruvate	-1mM.
TGF $ß_1$	-10ng/ml.
ITS	-1%.

6. Composition of Pre-induction (Neuronal) media

DMEM	
FBS	-20%.
L-Glutamine	
Antibiotics	
(Penicillin(100U/ml+Amphotericin0.25µg/ml)	
Beta Mercapto Ethanol(ßME)	-1mM.

7. Composition of Induction (Neuronal) media

DMEM	
L-Glutamine	
Antibiotics	
(Penicillin(100U/ml+Amphotericin0.25µg/ml)	
Beta Mercapto Ethanol(ßME)	-2mM.

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