"Osteogenic activity of Diabetes induced Rat MSCs on Bioactive ceramics."

A DISSERTATION SUBMITTED

BY

"DINESH KUMAR. K"

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DECLARATION

I, Dinesh Kumar. K, hereby declare that I had personally carried out the work depicted in the dissertation entitled "Osteogenic activity of Diabetes induced Rat MSCs on Bioactive ceramics." under the direct supervision of "**Dr. Annie John, Scientist E, Transmission Electron Microscopy, Laboratory, Division of Implant Biology**, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled "Osteogenic activity of Diabetes induced Rat MSCs on Bioactive ceramics." submitted by **Dinesh Kumar. K** in partial fulfillment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by **him** under my supervision and guidance at Transmission Electron Microscopy Laboratory, Division of Implant Biology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences & Technology (SCTIMST), Thiruvananthapuram-695012.

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The Dissertation

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Abbrevations

TE	Tissue Engineering
DM	Diabetes Mellitus
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
MSC	Mesenchymal Stem Cells
HASi	Silica coated Hydroxy apatite
HA	Hydroxy apatite
XRD	X-ray Diffraction
FTIR	Fourier transform infrared spectroscopy
SEM	Scanning electron microscopy
rBMSC	Rat Bone marrow derived Mesenchymal Stem cells
rADMSC	Rat Adipose derived Mesenchymal Stem cells
BMD	Bone Mineral Density
AGE	Advanced Glycation End products
STZ	Streptozotocin
ALP	Alkaline Phosphatase
ROS	Reactive oxygen species
Runx2	runt related transcription factor domain factor-2
FOXO	Forkhead box O
PDGF-B	Platelet-derived growth factor
Cbfa1	core-binding factor alpha1
RAGE	Receptor for advanced glycation end products
ECM	Extra Cellular matrix
TZDs	Thiazolidinediones
PPARγ	Peroxisome proliferator-activated receptors-γ
ERK	Extracellular signal-regulated kinase
IGF	Insulin-like growth factor-I
IRS-1 and IRS-2	Insulin receptor substrates 1 and 2
MCSF	Macrophage colony stimulating factor
RANKL	Receptor activator of nuclear factor-kB ligand
aP2	adipocyte fatty acid binding protein
IGFBP-1	Insulin Growth factor Bindin Protein-1
PRP	Platelet-rich plasma
rhBMP-2	recombinant human bone morphogenetic protein- 2
GLP-2	glucagon-like peptide 2
DLAS	Division of Laboratory Animal Sciences

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Synopsis

Diabetes mellitus (DM) is a pandemic metabolic disease with substantial morbidity and mortality. With an estimated 50.8 million people living with diabetes, India has the world's largest diabetes population. In Kerala the prevalence of self-reported diabetes was found to be 13.1% and that it was seen to be associated with increasing age [Rajnarayan R Tiwari *et al.,* 2008]. It is reported that diabetes mellitus has profound effects on various systems of the human body, including musculoskeletal abnormalities such as diminished bone formation and retardation of bone healing, making diabetic bone more fragile and prone to fracture. This report has been substantiated by several clinical and animal studies. The available therapeutics are limited for treating diabetic fractures, as they mainly focus on ameliorating the hyperglycemic condition of diabetes, by anti diabetogenic agents. Bone is a dynamic organ, which performs several function, It has its inherent property of natural healing, but under certain conditions such as diabetes, the bone metabolism is altered, where in the osteogenesis is impaired. There are several reports in literature stating that diabetic BMSC (Bone marrow mesenchymal stem cells) have impaired proliferation and differentiation.

To substantiate this and moreover to propose a therapeutic regimen for diabetic associated fracture, a diabetic animal model was developed by the administration of Streptozotocin (STZ) in rats, maintained for a period and then isolated stem cells from two different sources, bone marrow and adipose. Body weight and blood glucose of the experimental animals were monitored throughout the experimental period. Serum calcium and ALP level was measured before and after administration which showed a significant change in the diabetic group compared to control group based on statistical analysis.

Hydroxyapatite and Silica coated hydroxyapatite (HASi - in-house developed ceramic) were used as scaffolds for fabrication of cell-seeded constructs towards bone tissue engineering. Scaffolds were characterized using Environmental scanning electron

microscopy (ESEM), Fourier transform infrared spectroscopy (FTIR) and X-ray Diffraction (XRD). Primary cultures of rBMSC and rADMSC from diabetic and control rats were isolated, expanded and differentiated into the osteogenic lineage. As both the sources showed a similar performance, the rest of the study was done using rBMSC, Population doubling time of rBMSC showed impaired proliferation of diabetic cells, which is clearly evident from statistical analysis, which showed significant difference between the control and diabetic cells ($P = 0.0001$). Cell-seeded scaffolds were fabricated (cells from passage 2 were seeded in the concentration of $1*10^5$ in each scaffold) and maintained for 28 days in culture. Viability, adhesion, proliferation and osteogenic potential were evaluated at the 2^{nd} and 4^{th} week of the culture period. The performance of diabetic MSC on bioactive ceramic is comparable to that of control cells and hence this therapeutic regimen of using diabetic autologous cells may be applicable in clinical situations.

Scope of the study:

Inadequate bone formation resulting in osteopenia and delayed fracture healing is one of the complications in Type 1 diabetes. Mechanisms by which diabetes may affect skeletal mass and strength include obesity, insulin levels, hyperglycemia, and AGEs in bone collagen.

It is clear from several population based cohort studies that diabetic patients are more prone to fracture, and the bone quality is deteriorated in diabetic patients. There are several observations and hypothesis substantiating diabetic induced osteopenia, Some of the studies report that the bone forming precursor stem cell population is affected in diabetic patients due to the accumulate of Advanced end glycation products and its interaction with its receptor, which further down regulate essential gene expression for osteogenesis. Studies have also demonstrated that insulin and estradiol administration have improved the proliferation and differentiation potential of MSCs. An ideal treatment option for fracture healing under diabetic conditions is yet to be materialized.

The idea is to enhance the impaired proliferation and differentiation capability of diabetic Mesenchymal stem cells with the help of an *in house* developed bioactive ceramic scaffold, which has already proven to be osteoconductive, osteoinductive and osteointegrative from previous studies employing goat bone marrow derived MSCs [Manitha B. Nair *et al.,* 2008], there by proposing a novel therapeutic regimen for treating diabetic fractures.

Literature survey reports detrimental effects of DM on bone physiology that emanates from hyperglycemia and its consequences. Considering the limitations of available therapeutic regimen, which mainly focus on anti diabetogenic agents and growth factors with its role in ameliorating the diabetic condition, a therapeutic regimen was

proposed of fabricating *in vitro* tissue constructs comprising of bioactive ceramic as scaffold and mesenchymal stem cells.

This work demonstrated an approach to treat diabetes associated fractures. Here we substantiate the literature reports of impaired proliferation and differentiation of BMSCs, by studying the potential of stem cell in terms of proliferation and differentiation on ceramic scaffolds into the osteogenic lineage. Further research will help to delineate the exact mechanisms by which diabetes affects bone and the most effective approaches to reduce fracture risk.

Chapter 1 - INTRODUCTION

Background Information:

Bone is a dynamic organ that serves the structural function—providing mobility, support, and safeguard for the body, as a reservoir of indispensable minerals and also offers site for muscle attachment enabling locomotion. Bone and its marrow are also in charge of blood cell production and maintenance of calcium and phosphate homeostasis in serum. To act in response to its multi roles of support and regulation of minerals, as well as to renovate any damage to the skeleton, bone is relentlessly changing. Old bone breaks down and new bone is formed incessantly. This requires an elegantly controlled regulatory system that involves specialized cells that communicate with each other. These cells must respond to many different signals, internal and external, mechanical and hormonal, and systemic (distressing the whole body) and local (distressing only a small region of the skeleton). It is not astounding that with so many diverse tasks to execute and so many different factors regulating how the skeleton grows, adapts, and responds to varying demands; there are many ways that these processes can go astray. Any alteration in the health condition of the individual directly or indirectly affects bone homeostasis. Thus the significance of bone becomes obvious in the case of several diseases such as osteoporosis, osteogenesis imperfecta, osteoarthritis, osteomyelitis, and in Diabetes, wherein the homeostasis of bone modeling is affected. Apart from these diseases several other conditions like traumatic injury, orthopedic surgeries (i.e., total joint arthroplasty, spine arthrodesis, implant fixation, etc.) and primary tumor resection lead to or induce bone defects or voids. The clinical and economic impact of treatments of bone defects is staggering.

The association between diabetes and bone health has long been a matter of debate. India has the world's largest diabetes population, it has been estimated that 50.8 million people are living with diabetes in India, followed by China with 43.2 million. In the United States, approximately 8 percent of adults have diabetes and it has been

predicted that there will be a huge increase in the prevalence by 2050. In Kerala the prevalence of self-reported diabetes was found to be 13.1% and that it was seen to be associated with increasing age. Furthermore, the growing diabetes prevalence as a result of aging and obesity epidemic will further contribute to the public health burden of low-trauma fractures. Osteopenia with microvascular complications is another common impediment in patients with diabetes mellitus. These findings emphasize the need for fracture prevention strategies in patients with diabetes.

Both type 1 diabetes and type 2 diabetes have been linked to increased risk of fractures with reports suggesting decreased bone mineral density in type 1 diabetes and increased bone mineral density in type 2 diabetes. It has also been reported that poorly controlled diabetes mellitus leads to neuropathy, vasculopathy and immunopathy, all of which may contribute to osteopathy. While osteoporosis is the root cause of brittle bones and fractures, diabetes has also been implicated in raising the risk for fractures in middle-aged and elderly people. Numerous studies have been published since the early 1980s analyzing the relationship between diabetes and fractures, but study findings were inconsistent. These studies suggest that one in fifteen type 1 diabetic patients could break their hip before the age of 65. According to Mohnsen *et al.,* (2007) as reported in the American Journal of Epidemiology, the association between type of diabetes and hip fracture incidence was stronger for type 1 diabetes than for type 2 diabetes. Type 2 diabetes was weakly associated with fractures at other sites, and most effect estimates were not statistically significant. Hence they conclude that diabetic population, either type 1 or type 2, are more likely than the general population to fracture a hip. These results have significant clinical and public health implications.

However, with regard to orthopaedic and reconstructive surgery, an exemplar shift is taking place from using medical devices and tissue grafts to a tissue engineering (TE) approach that uses biodegradable scaffolds combined with cells or biological molecules to repair and/or regenerate tissues that are currently untreatable. Tissue engineering, has many advantages compared to the conventional treatment options like eliminating re-operations by using biological substitutes, which resolves the

tribulations of implant rejection, transmission of diseases associated with xenografts and allografts, and shortage in autografts, Thus Bone Tissue Engineering is a promising interdisciplinary field that seeks to address the needs of bone regeneration in several disease conditions such as trauma, fracture, osteoporosis, by applying the principles of biology and engineering to the development of viable substitutes that restore and maintain the function of human bone tissues.

The major challenge in bone tissue engineering, includes the use of appropriate matrix materials for scaffolds, control of porosity and pore characteristics of scaffolds, mechanical strength of scaffolds, scaffold degradation properties, and bioactivity (i.e., osteoconductivity, or osteoinductivity if possible) of scaffolds. A variety of biomaterials have been developed as bone substitutes, which includes hydroxyapatite (HA), alumina, zirconia, bioglass, polymers, metal, and organic or inorganic bone substitutes. Among the available bone substitutes Calcium phosphate ceramics have been widely useful as bone substitutes due to their resemblance to the mineral portion of the bone tissue, its biocompatibility, safety, predictability, surplus availability, lower morbidity for the patient and cost effectiveness. These advantages make calcium phosphate ceramics a good choice for orthopedics and reconstructive surgery. Over the years, various modifications has been done on several parameters of the scaffold such as the purity of the starting products, sintering temperature, pH and so on, which has given rise to calcium phosphates with desirable chemical and physical characteristics such as specific surface areas, surface energy, surface charge, roughness and porosity. Pores of desired size can be created with the use of porogens/pore-formers and heat treatment, by this way one can create either Macropores (diameter > 100 μm) or micropores (diameter < 10 μm) depending on the mode of usage. As a consequence of these advances in materials science field, it is possible to have a biomaterial that improves the adhesion, proliferation and differentiation of cells, which leads to better osteoconductivity, bioactivity and mechanical properties with less brittleness. Thus the nature of the chosen biomaterial is a vital factor determining the ultimate success of a tissue-engineered graft.

In this scenario a rational approach is to make use of the desirable properties of more than one material that add strength to the material, and also mimic the natural bone so as to ensure the homing of stem cells, there by minimizing any detrimental characteristics. Commonly bioactive ceramics, particularly hydroxyapatite (HA), which is currently used as an excellent source of bone substitutes, exhibits good osteoconductive and osteointegrative properties because of its similarity in composition and structure to the mineral phase of bone. Although it is biocompatible, it is nevertheless biodegradable, but at a very slow rate. Several comparative studies have demonstrated the efficacy of silica-based bioactive glass (BG) as a better biodegradable and bioactive ceramic than HA. So as to improve the degradability of HA ceramic, an in-house built ceramic was made combining the dual benefits of HA and silica as a single product, which is done by giving a coating of silica over HA (hydroxyapatite) scaffold (HASi), has been developed and used for this study.

Thus a tissue engineering approach to the above said diabetes associated complication in bone healing is the incorporation of autologous Bone marrow mesenchymal stem cells (BMSCs) on to a biocompatible ceramic construct. Thereby, an *in-vitro* tissue construct may be fabricated as a remedy for diabetes induced osteopenia leading to fractures and impaired bone healing.

Review of Literature

Diabetes and osteopenia- an overview and epidemiology:

Diabetes mellitus (DM) is a chronic disease, which occurs either due to the lack of insulin production by the pancreas, or when the body cannot effectively use the insulin produced by pancreas. This leads to an increased concentration of glucose in the blood (hyperglycaemia). Type 1 diabetes (T1DM), previously known as insulin-dependent diabetes mellitus (IDDM) or childhood-onset diabetes is characterized by a lack of insulin production. It is an autoimmune disorder, where in the immune system attacks and destroys the insulin-producing beta cells in the pancreas. But the cause of this is unknown, but several factors such as genetic, and environmental factors, possibly viruses, may be involved in the cause of T1DM. Type 2 diabetes (T2DM) formerly called non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes is caused by the body's ineffective use of insulin. This is the most common form of diabetes; about 90 to 95 percent of people with diabetes have type 2. This form of diabetes is most often associated with older age, obesity, family history of diabetes, previous history of gestational diabetes, physical inactivity, and certain ethnicities. It often results from excess body weight and physical inactivity. [WHO Report 1999]

Diabetes mellitus (DM) is a pandemic metabolic disease with extensive morbidity and mortality and is estimated to affect 171 million persons world wide, a figure that is anticipated to rise to 366 million in 2030 [Wild S *et al.,* 2004]. It has been found out that India has the world's largest diabetes population. The list of top 10 countries which is found to have large proportion of people affected by diabetes, a survey by international diabetes federation is shown in table 1. In Kerala the prevalence of self-reported diabetes was found to be 13.1% and that it was seen to be associated with increasing age [Rajnarayan R Tiwari *et al.,* 2008]. Diabetes mellitus (DM) is hence a chief public health concern, whose impact is increased by the high incidence of co-existing chronic medical conditions among subjects with DM.

Diabetes also has detrimental effects on a range of health outcomes including coronary artery and peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure and blindness which results in increasing disability, reduced life expectancy and enormous health costs for virtually every society. It has been more than 60 years since Drs. Albright and Reifenstein demonstrated that diabetes might be associated with loss of bone mass by describing a reduction in bone mineral density (BMD) in diabetic patients with poor glycemic control. Since that time, it was well recognized that hyperglycemia leads to increased bone resorption and osteopenia. The effect of osteopenia is thought to contribute significantly to the increased risk of fractures, as evidenced by increase in the risk of hip fracture by at least sixfold, as well as increased incidence of long-bone fractures in diabetics. Thus the correlation between osteopenia and type 1 diabetes has become well documented; several lines of evidence have shown that osteopenia is one of the established chronic complications associated with type 1 diabetes mellitus, especially during the first few years following diagnosis. While the effects of type 2 diabetes on bone metabolism have remained less clear. Many experimental animal studies of type 1 diabetes mellitus have demonstrated low bone turnover accompanied by reduced mineral content in Diabetic animal models [Reddy GK *et al.,* 2001], as well as in spontaneous diabetic rodent models [Waud CE *et al.,* 1994]. Whereas in patients with type 2 diabetes mellitus, this issue is contentious, in which there is either decreased, normal, or increased skeletal mass and they have also found out that there is a correlation between parathyroid hormone-related peptide, which plays a compensatory role on the maintenance of calcium homeostasis in type 2 diabetes mellitus [Suzuki K *et al.,* 2000].

Thus literature survey indicates specific alterations in bone metabolism that have been associated with diabetes mellitus, and numerous pathogenic possibilities have been proposed, such as bone microangiopathy in which AGE(Advanced Glycation End products) and its Receptor interaction has been shown to inhibit osteoblast function, insulinopenia, NaCl cotransporter upregulation and down regulation of calcium/polyvalent cation-sensing receptor (CaR) which may be the cause for impaired regulation of mineral metabolism, alterations in growth factors and its

receptor that regulate bone formation and remodeling, and even an intrinsic disorder associated with type 1 diabetes mellitus [Ronaldo B *et al.,* 2003; Kemink SAG *et al.,* 2000; Ward DT *et al.,* 2001]. Even though, several research studies proposed various mechanisms that could be the cause of Diabetes induced osteopenia and its complications, the pathogenesis of diabetic osteopenia is understood only to some extent and it has turned out to be apparent that none of the proposed mechanisms can alone elucidate all the complications of this metabolic disorder on mineral and bone integrity. More insights of research in the cellular and molecular mechanism associated with diabetic osteopenia is needed to understand its pathophysiology which would help to innovate a more effective preventive and therapeutic procedures to cure such disorders.

This section will give a brief review on current knowledge of potential contributing factors of diabetic osteopenia and the underlying molecular mechanism and to propose an ideal approach as a remedy for diabetic fracture healing.

Table 1- Top 10 Countries for estimated numbers of adults with diabetes, 2010 and 2030

Rank	Country/ Territory	2010 (millions)	Country/ Territory	2030 (millions)
1	India	50.8	India	87.0
2	China	43.2	China	62.6
3	US	26.8	US	36.0
4	Russian Federation	9.6	Pakistan	13.8
5	Brazil	7.6	Brazil	12.7
6	Germany	7.5	Indonesia	12.0
7	Pakistan	7.1	Mexico	11.9
8	Japan	7.1	Bangaladesh	10.4
9	Indonesia	7.0	Russian Federation	10.3
10	Mexico	6.8	Egypt	8.6

Adopted from Ramachandran *et al.,* 2010 (Main source- International Diabetes Federation- Diabetes Atlas, Fourth Edition.

Animal Models for Diabetes:

The first diabetic model was created unintentionally in 1880 when von Mering removed the pancreas of a dog and observed subsequent polyuria and polydipsia. Pancreatomy in small animals for manageable, convenient and affordable animal models was then developed.

T1 Diabetic Models:

A range of T1 diabetic rodent models are available, some of the models develop diabetes spontaneously, such as the non-obese diabetic (NOD) mouse and bio

breeding (BB) rat, the two most commonly used animals that spontaneously develop diseases with similarities to human Type 1 diabetes.

Other T1 diabetic models are pharmacologically induced by compounds such as alloxan, streptozotocin (STZ), Vacor, Dithizone, and 8-hydroxyquinone [Rees D *et al.,* 2005]. The streptozotocin-induced diabetes model has been extensively used, making it particularly useful for building upon and comparing results of other studies.

Streptozotocin (STZ, 2-deoxy-2-(3-(methyl-3-nitrosoureido)-D-glucopyranose) is a nitros urea, synthesized by Streptomyces achromogenes with broad-spectrum antibiotic and anti-neoplastic activity [Bono VH, 1976]. It is a powerful alkylating agent that has been shown to interfere with glucose transport [Wang Z *et al.,* 1998], glucokinase function [Zahner D *et al.,* 1990] and induce multiple DNA strand breaks [Bolzan AD *et al.,* 2002]. STZ is used to induce both insulin-dependent and non-insulin-dependent diabetes mellitus (IDDM and NIDDM, respectively). It enters insulin secreting pancreatic β cells through glucose transporter 2 (GLUT2) channels in the plasma membrane and causes DNA damage and finally triggers pancreatic β cell necrosis.

Type 1 diabetes mellitus in humans is characterized by a specific destruction of the pancreatic β cells, commonly associated with immune-mediated damage [Dahlquist G, 1998]. Although the damage may occur silently over many years, at clinical presentation there is little surviving β cell mass and the disorder progresses to absolute insulinopaenia. In some models, especially rats, a single dose of STZ is effective at inducing T1 diabetes. In mice, however, multiple low doses (40 mg/kg) are the most effective at maintaining mouse viability and inducing pancreatic dysfunction in part through immune destruction. This response is similar to what is seen during the onset of T1 diabetes in humans. [D. A. Rees and J. C. Alcolado, 2004]

STZ administration for creating diabetic model has several advantage compared to alloxan. The range of the STZ dose is not as narrow as in the case of alloxan. The frequently used single intravenous dose in adult rats to induce T1DM is between 40

and 60 mg/kg b.w. [Ganda *et al.,* 1976], but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose, but single dose below 40 mg/kg b.w. may be ineffective [Katsumata *et al.,* 1992].

T2DM was induced by a single intraperitoneal injection of streptozotocin (60mg/kg) and nicotinamide (120mg/kg) to rats [Pellegrino *et al.,* 1998].

Table 2- Available animal models for diabetes

Animal models of Type 1 diabetes	Animal models of Type 2 diabetes
NOD (non-obese diabetic) mouse	Ob/Ob mouse—monogenic model of obesity (leptin deficient)
BB (bio breeding) rat	db/db mouse—monogenic model of obesity (leptin resistant)
LETL (Long Evans Tokushima lean) rat	Zucker (fa/fa) rat—monogenic model of obesity (leptin resistant)
New Zealand white rabbit	Israeli sand rat
Keeshond dog	Fat-fed streptozotocin-treated rat
Chinese hamster	
Celebes black ape (Macacca nigra)	
STZ or Alloxan induced rat models	

Adapted from D.A Rees and J.C Alcolado, 2004

Diabetes and impaired Fracture Healing:

One of the major complications of diabetes, is osteopenia coupled with decreased bone mineral density. Krakauer *et al.,* (1995) have suggested that patients with diabetes have reduced bone formation and accumulation during growth, whereas later in life, hyperglycemia leads to amplified bone resorption and osteopenia. The effect of osteopenia is thought to contribute significantly to the increased risk of fractures, as evidenced by increase in the risk of hip fracture by at least sixfold, [Vestergaard P *et al.,* 2005, Forsen L *et al.,* 1999] as well as increased incidence of long-bone fractures in diabetics [Vestergaard P *et al.,* 2005, Davidson TI *et al.,* 1986, Ivers RQ *et al.,* 2001, Nicodemus KK *et al.,* 2001]. Recent reviews report that both

Type 1 and 2 diabetes are associated with increased fracture risk, as evidenced by recent meta-analyses, cohort studies, and suggest that factors other than decreased bone mineral density, such as hyperglycemia or vascular changes, may contribute to the greater risk. [Khazai NB *et al.,* 2009, Strotmeyer ES *et al.*, 2007]

Many reports project association of low BMD in Type 1 Diabetes, and an average or high BMD in Type 2 diabetes. Although, Type 1 but not Type 2 diabetes is associated with decreased bone mineral density, both are associated with an increased possibility for bone fracture [Adami S, 2009]. Thus it is evident from clinical and animal studies that in spite of the lowered or higher BMD in Type 1 and Type 2 diabetes, diabetic bone appears to be fragile and thus it is more prone to undergo fracture. [Verhaeghe J *et al.,* 1997; Schwartz AV *et al.,* 2001].

Bone quality, which refers to micro architectural bone composition and characteristics of bone strength, may be impaired in DM leading to increased fractures. [Hampson G *et al.,* 1998, Inzerillo *et al.,* 2004] Studies report several contributing factors, such as the prolonged expression of cytokines and chemokines [Wetzler C *et al.,* 2000]; the destruction of matrix which causes an imbalance between the accumulation of collagenous and non-collagenous extracellular matrix components and their remodeling by matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). [Lobmann R *et al.,* 2002]; as well as defects in reparative capacity because of diminished production of growth and angiogenic factors, decreased proliferation, and increased apoptosis [Graves DT *et al.,* 2008]. A study of systematic review of published data on the association between diabetes mellitus and fracture shows rates of fractures and the discrepant relationship with BMD in individuals with DM. They have also shown that in both T1DM and T2DM, hip fracture risk was increased; however BMD was increased in T2DM and decreased in T1DM [Mohsen Janghorbani *et al.,* 2007] The risk of various fractures in diabetic population done by various studies are summarized in Table 3. This inconsistent BMD data in the presence of increased fracture rates probably suggests that bone quality may be the critical factor in the etiology of fractures. Diabetes impairs fracture healing of bone, including the mandible, hip, and long bones. [Adami S 2009; Khazai

NB *et al.,* 2009] This fact is supported by several clinical studies, which have reported delayed union or increased healing time in diabetic subjects compared with matched controls. [Cozen L 1972; Loder R 1988 Herskind AM *et al.,* 1992] Studies in streptozotocin-induced diabetic animals also make clear that long-bone fractures in these animals exhibit changes consistent with impaired healing, including smaller calluses with decreased bone and reduced mechanical strength compared with those of controls. [Gooch HL *et al.,* 2000; Macey L *et al.,* 1989] Moreover some studies in which insulin administration is shown to correct the deficits in fracture repair in STZ induced DM animals. From these results it is evident that the effect of decreased bone formation is the result of diabetes and not a side effect of streptozotocin on bone [Hough S *et al.,* 1981; Funk JR *et al.,* 2000]

Healing long bones in diabetic animals also exhibit reduced strength - a finding supported by delayed recovery of structural and material strength by at least 1 week in the healing femurs of diabetic rats when compared with those of normoglycemic controls [Funk JR *et al.,* 2000]. In diabetic animal models, decreased biomechanical strength in femurs and tibias of approximately 20% was reported. [Hou J *et al.,* 1991; Reddy G *et al.,* 2001]. Other studies demonstrate a two fold reduction in callus mechanical strength and size with decreased bone formation, decreased proliferation and differentiation of osteoblastic cells during fracture healing in spontaneously or streptozotocin-induced diabetic animals compared with those of matched controls. [Gooch HL *et al.,* 2000; Beam H *et al.,* 2002; Gandhi A *et al.,* 2005; Kayal RA *et al.,* 2007]. Our understanding of how BMD, bone strength, and other risk factors contribute to the higher rate of fracture in those with diabetes, though still limited, has advanced over the past several years.

Besides other complications caused by diabetes mellitus on several organ systems such as neurovascular, ocular and renal system, it affects bone physiology as it is evident by the increased literature source reporting osteopenia and osteoporosis in DM. Osteoporosis and several other DM complications such as visual impairment and gait imbalance, also add up its contribution to this condition indirectly, by increasing the risk of falls, fragility and low-impact fractures. Apart from its direct interference

with osteoblast function and bone formation, DM also induces lipid accumulation in the marrow of long bones, thereby leading to the expansion of marrow cavity and thinning of cortical envelope. The osteoblast to adipocyte shift might also reduce the number of differentiated osteoblasts available for bone formation. [Kannikar Wongdee and Narattaphol Charoenphandhu, 2011] Thus all of these underlying complications deteriorate bone quality and strength and increase susceptibility to fracture.

Table 3- Cohort Studies that shows the Association Between Diabetes Mellitus and Fracture Risk (Adapted from Mohsen Janghorbani *et al.*, 2007)

Study and year.	**Country**	**Sex**	**Average follow-up period (years)**	**Age (years) at enrollment**	**Study population**	**Fracture site and no. of cases**
Heath *et al.*, 1980.	United States	Both	Unknown	<103 (median, 61)	Rochester, Minnesota: 500 men and 486 women with medically recorded type 1 or type 2 DM. Comparison group: 986 men and women without DM	Vertebra: 26 Proximal humerus: 16 Distal forearm: 39 Proximal femur: 48

Meyer *et al.*, 1993	Norway	Both	10.9	35 - 49	Norwegian Prospective Study: 118 women and 180 men with self-reported DM (type unknown) Comparison group: 23,850 women and 24,333 men without DM	Hip Women: 136 Men: 57
Ivers *et al.*, 2001	Australia	Both	5	≥ 49	Blue Mountains Eye Study: 216 men and women with self-reported type 2 DM Comparison group: 3,438 men and women without DM	All fractures: 251 Hip: 59 Distal forearm: 53 Proximal humerus: 26 Ankle: 36
Ottenbacher *et al.*, 2002	United States	Both	7	>65	Hispanic portion of the Established Populations . Elderly: 291 men and 399 women with self-reported DM (type unknown) Comparison group: 922 men and 1,272 women without DM	Hip: 110

Miao *et al.*, 2005	Sweden	Both	9.9	20.7	Population-based cohort study: 12,551 men and 12,054 women hospitalized for type 1 DM Comparison group: register of total population.	Hip: 121
de Liefde *et al.*, 2005	The Netherlands	Both	6.8	≥ 55	Rotterdam Study: 309 men and 483 women with type 2 DM defined by oral glucose tolerance testing and/or medication history Comparison group: 2,382 men and 3,481 women without DM	Any non-vertebral: 771 Hip: 215 Wrist: 204

Holmberg *et al.*, 2006	Sweden	Both	Men: 19 Women: 15	Men: mean = 44 Women: mean = 48	Malmö Preventive Project: 166 women and 276 men with type 2 DM (not defined) Comparison group: 22,444 men and 10,902 women without DM	Any fracture Women: 1,257 Men: 1,278 Forearm Women: 600 Men: 315 Vertebral Women: 138 Men: 156 Proximal humerus Women: 146 Men: 115 Ankle Women: 217 Men: 250 Hip Women 135 Men: 163
Ahmed *et al.*, 2006	Norway	Both	6	25 - 98	Tromsø Study: 52 men and 29 women with self-reported type 1 DM and 175 men and 198 women with self-reported type 2 DM Comparison group: 12,639 men and 14,065 women without DM	Any nonvertebral Men: 446 Women: 803 Hip Men: 72 Women: 177

Diabetes and impaired osteogenesis:

Diabetes decreases the formation of bone, there by causing impaired fracture healing. [Jehle PM *et al.,* 1998; Kemink SA *et al.,* 2000; Pietschmann P *et al.,* 1988; Cakatay U *et al.,* 1998]. It was also reported that osteocalcin, which is a non-collagenous protein is solely expressed in bone during the later differentiation stage of osteoblast and play a major role in the regulation of matrix mineralization. Thus osteocalcin serves as a marker of bone formation and its expression is found to be decreased in diabetes. [Thrailkill KM *et al.,* 2005]. A well-known marker of bone formation is bone alkaline phosphatase (ALP), which is produced by osteoblasts and is required for osteoid formation and matrix mineralization (DeLaurier *et al.,* 2002).The isoenzyme of alkaline phosphatase found in bone is a tetrameric glycoprotein localized on the cell membrane of osteoblasts. As it is involved in the mineralization of bone, it is considered as the classic marker for increased osteoblastic activity. In a clinical study of Type 1 diabetes patients, serum alkaline phosphatase (ALP) and osteocalcin levels were significantly lower compared to those of the control subjects and this suggested reduced bone formation in DM. [Jehle PM *et al.,* 1998; Pietschmann P *et al.,* 1988]. Other clinical studies also support this view as their investigations shows decreased bone formation in some diabetic patients [Kemink SA *et al.,* 2000; Pietschmann P *et al.,* 1988]. Streptozotocin-induced diabetic mice and nonobese diabetic mice that spontaneously develop Type 1 diabetes exhibit loss of trabecular bone, increased bone marrow adiposity, and decreased osteocalcin mRNA levels in the tibia [Botolin S *et al.,* 2007]. *In vitro* studies using MSC isolated from a rat model of type I diabetes supports this theory as osteoblastic colonies from mesenchymal stem cells have been shown to decrease in number and size. From their studies it is evident that MSCs become exhausted during diabetes and lose their differentiation potential leading to a net loss of Trabecular bone. Therefore direct effects on MSC may be responsible for some of the orthopaedic effects associated with diabetes. [Stolzing A *et al.,* 2010]. Clinical studies report reduced serum level of insulin-like growth factor (IGF-I), dysregulated insulin-like growth factor binding proteins (IGF-BPs). Under normal conditions Osteoblasts synthesize IGF-I and IGF-II which have mitogenic effects on bone cells, thus it acts as a key regulator of bone cell function. From the clinical study

results it is evident that diabetes disturbs the IGF system. By which they suggest that this imbalance between IGF system components and lack of endogenous proinsulin may contribute to the lowered BMD in Type 1 DM.

Diabetes also has effects on the formation and function of osteoblasts. A study in spontaneously diabetic animal models showed that poorly compensated diabetic metabolic state in DM is the cause for impaired fracture healing. They evaluated fracture healing at 2, 4, and 6 weeks, which revealed decreased mineralization, apposition, and timing of mineralization in diabetic rats with poor glucose control. [Follak N *et al.,* 2005] On a cellular level, it has been shown that hyperglycemia can induce insulin resistance on osteoblasts, which affects the transport and function of glucose transporter 1. [Lv J *et al.,* 2009] Formation of advanced glycation end products and the activation of its receptors, has been found to inhibit osteoblast function and there by impairs bone formation. [Santana RB *et al.,* 2003] All together, these studies suggested that diabetes decreases the anabolic aspect of fracture healing by affecting osteoblasts in terms of formation, function, and bone deposition. Although it is known that diabetes affects bone, the exact mechanism is not yet clear.

One of the possible underlying processes is high glucose, which, in addition to advanced glycation end products, has been linked to inhibition of osteoblast function and reduced formation of a mineralized matrix *in vitro*. [Ogawa N, *et al.,* 2007] Treatment of rat bone marrow stromal cells with high glucose levels reduces proliferation, ALP activity, and the number of bone nodules formed *in vitro*. They have also shown that insulin and estradiol are able to control the deleterious effect of high concentrations of glucose on BMSC-derived osteoblast proliferation and function. [Gopalakrishnan V *et al.,* 2006]

One of the major molecular changes in diabetes is the formation of reactive oxygen species (ROS) as a result of high glucose and/or insulin insufficiency or resistance. High levels of ROS have been linked to many diabetic complications. [Hamada Y *et al.,* 2009] In osteoblasts, ROS has been reported to inhibit differentiation of osteoblast via extracellular signal-regulated kinase (ERK) and ERK-dependent NF-kappaB

activation, which inhibits osteoblast differentiation, This is further proved by the reduction of differentiation markers, including ALP, Type I collagen, colony-forming units, osteoprogenitor formation, and nuclear phosphorylation of Runx2. [Bai XC *et al.,* 2004] Taken together, these studies suggest that high glucose levels are associated with inhibited osteoblast formation or function.

On the other hand, the relative absence of insulin rather than the hyperglycemic effects alone may contribute to alterations in bone caused by diabetes. [Gandhi A *et al.,* 2005; Thrailkill KM *et al.,* 2005] It has been reported that diabetes affect bone through inadequate expression of genes regulating osteoblast differentiation, such as Cbfa1 (core-binding factor alpha1) /Runx2 (runt domain factor-2) and Dlx5 (drosophila distal-less gene), as reported from multiple low-dose streptozotocin induced diabetes in animal models. [Lu H *et al.,* 2003] Another proposed mechanism operating at the transcriptional level is that increase in oxidative stress induces Forkhead box O (FOXO) tra.nscription factor, which antagonizes Wnt signaling, an essential stimulus for osteoblastogenesis and thereby decreases bone formation. [Almeida M *et al.,* 2007). Dysfunction in endothelial progenitor cells is also one of the proposed mechanisms for diabetic complications. [Loomans CJ *et al.,* 2004] This hypothesis was supported by the study which shows that adenoviral mediated platelet-derived growth factor (PDGF-B) gene transfer improves endothelial progenitor cell recruitment and neovascularization in diabetic wound healing. [Keswani SG *et al.,* 2004] Recently, it has been reported that blood flow and microvascular density were reduced in bone marrow isolated from Type 1 diabetic mice, suggesting that microangiopathy might impinge on the integrity of diabetic bone marrow. [Oikawa A *et al.,* 2010] Together, these studies suggest that vascular changes in diabetic subjects may impact healing complications.

Role of Advanced Glycation end products in diabetic osteopenia.

AGEs (Advanced glycation end products) are formed as a result of nonenzymatic reaction between reducing sugars and oxidized lipids with amine residues of proteins.

They act directly to induce cross-linking of long-lived proteins resulting in alteration of vascular structure and function. In diabetes, AGE accumulation occurs as a result of chronic hyperglycemia and impaired renal function because kidney is the major site of AGE Clearance. [Goh SY & Cooper ME 2008] Advanced glycation end products are formed and accumulate progressively in diabetes mellitus, these accumulated advanced glycation end products (AGEs) in bone collagen is thought to contribute to the decline in bone strength for a given BMD [Goh SY & Cooper ME 2008]. The major targets of AGE accumulation are the structural components of the connective tissue matrix because of their slow turnover rate these extracellular matrix (ECM) proteins are susceptible to AGE modification. Accumulation of AGEs alter collagen function by the formation of intermolecular and intramolecular crosslinks with collagen as a result of the glycation which causes structural alterations, leading to increased stiffness and resistance to proteolytic digestion and thereby alter the function of bone. Thus AGEs such as pentosidine accumulation in bone reduces the mechanical property of bone, in particular they make the collagen fibers brittle, there by making the bone more fragile, thus prone to fracture.

AGEs not only affect the extra cellular matrix of bone but also the progenitor cells that forms bone. Studies have demonstrated that AGEs and the receptor for advanced glycation end products (RAGE) have detrimental effects on osteoblasts, the cells responsible for bone formation, and enhance osteoclasts, which are the cells responsible for bone resorption. Among the diverse known receptors for AGEs, the receptor for AGEs (RAGE) is expressed by a variety of different cell types including the bone forming osteoblast cells. AGE-RAGE interactions result in the generation of reactive oxygen species and in NF-κB activation in endothelial and smooth muscle cells, which could in turn contribute to the tissue damage and metabolic imbalances seen in diabetic complications [Kislinger T, *et al.,* 1999; Lalla E *et al.,* 1999]. Studies using knock out mice for RAGE gene highlights that RAGE deletion results in increased bone mineral density due to decreased osteoclast function under physiological conditions, there is an imbalance in bone formation and remodeling. In diabetes, although oxidant stress responses are essential to eliminate pathogenic periodontal pathogens, ongoing AGE/RAGE-mediated cellular activation affects

periodontium, with a concomitant increase in the generation of proinflammatory cytokines and tissue-destructive matrix metalloproteinases, which leads to the destruction of alveolar bone [Lalla E *et al.,* 2000]

Vitamin B6 acts not only as a coenzyme of lysyl oxidase, which aids in the proper collagen cross linkage, but also as an inhibitor of AGEs formation. Several clinical studies support this fact, as there were reports of lower concentration of serum pyridoxal (Vitamin B6) in patients with Diabetes compared to normal healthy controls, this is obvious as there will be more consumption of Vitamin B6 during the upregulation of gluconeogenesis. Thus the deficiency of Vitamin B6, may provoke an impaired lysyl oxidase controlled enzymatic crosslinking, there by making it more brittle and the accumulation of AGEs in collagen inducing reactive oxygen species. A study by Katayama *et al.,* (1996) demonstrates that AGE modification of type I collagen might alter its ability to enhance the expression of the osteoblast phenotype. In particular they noted that AGE-modified collagen impairs osteoblastic cell function, especially the process of maturation [Katayama Y *et al.,* 1996]. On the other hand its receptor RAGE is thought to enhance bone resorption by contributing to osteoclast maturation and function. [Zhou Z *et al.,* 2006].

Moreover AGEs accumulation may induce oxidative stress. Studies highlight that persistent hyperglycemia can lead to oxidative stress caused by a variety of mechanisms, which includes increased polyol pathway flux, activation of protein kinase C isoforms, d mitochondrial overproduction of superoxide and glucose autoxidation, which causes accelerated glycation reaction, with the formation of glucose-derived advanced glycosylation end products (AGEs), which in turn catalyze lipid peroxidation. Other circulating factors that are elevated in diabetes, such as free fatty acids and leptin, also contribute to this condition by increasing ROS generation. It is now widely accepted that ROS can cause severe damage to DNA, proteins, and lipids. There is also evidence that in chronic diabetes mellitus, hyperglycemia causes increased production or ineffective scavenging of reactive oxygen species, which play a critical role in the impairment of antioxidant defense mechanisms by altering

antioxidant enzymes, impairing glutathione metabolism, and lowering concentrations of glutathione and vitamins C, E, A and D [Rahimi *et al.,* 2005].

Persistent hyperglycemia increases polyol pathway flux, as well as ROS generation rates and AGEs formation, leading to increased GSH oxidation [Oyama *et al.,* 2006]. Thus from these studies it is clear that the underlying mechanisms of diabetic complications is a diabetes-induced increase in oxidative stress, because reactive oxygen species (ROS) are increased under diabetic conditions and are known to induce cellular dysfunction in a wide variety of cell types including bone forming precursor cells. Moreover it has been found out that AGEs enhance osteoclast-induced bone resorption in cultured mouse unfractionated bone cells. Furthermore, several studies report that AGEs-RAGE interactions induce human mesenchymal stem cell apoptosis, there by preventing its differentiation into cartilage, and bone. Thus, there is a correlation between increased oxidative stress and lower antioxidant defense, which has an important role in the pathogenesis of diabetic osteopenia [Ding *et al.,* 2006].

In vitro studies with bone metabolism, shows that oxidative stress inhibits osteoblastic differentiation and induces osteoblast apoptosis [Hamada Y *et al.,* 2009]. Thus any loss of osteoblastic activity increases osteoclastic activity or osteocyte death by necrosis or apoptosis. This phenomenon could lead to osteoporosis, characteristic of lower bone mineral density (BMD), and render bone weaker and more likely to fracture [Janssens *et al.,* 2005].

Thiazolidinediones and impaired osteogenesis in Type 2 Diabetes:

The thiazolidinediones (TZDs) are a class of medications used in the treatment of diabetes mellitus type 2. They act by binding to PPARs (peroxisome proliferator-activated receptors), a group of receptor molecules inside the cell nucleus, in particular PPARγ (gamma). The ligands for these receptors are free fatty acids (FFAs) and eicosanoids. Once activated, the receptor migrates to the DNA, activating

transcription of a number of specific genes. There is abundant evidence from *in vitro, in vivo* and observational research trials, which suggests that TZDs increase fracture risk. A study by Christian Meier *et al.,* elucidated the association between long-term use of TZDs and fractures. They have reported that approximately 2 to 3 fold increase in hip and other nonvertebral fractures in patients with Type 2 diabetes mellitus who take TZD [Christian Meier *et al.,* 2008]. TZDs exert their effect in part by improving muscle and adipose tissue insulin sensitivity [Kahn CR *et al.,* 2000].

Mesenchymal Stem cells can give rise to multiple lineages such as osteoblasts, Chondrocytes and adipocytes. TZDs stimulate adipocyte differentiation, generating smaller more insulin sensitive adipocytes [Okuno A *et al.,* 1998]. TZDs appear to promote adipocyte differentiation at the expense of osteoblast formation [Rzonca SO *et al.,* 2004; Akune T *et al.,* 2004]. Rzonca *et al.,* demonstrated that there is a 3-fold increase in the number of adipocytes and concomitant decrease in osteoblast surface and activity in rosiglitazone administered mice with the aid of histomorphometric analysis of the trabecular bone of the proximal tibia [Rzonca SO *et al.,* 2004]. Similarly Akune *et al.,* demonstrated that PPAR-γ haploinsufficiency in mice leads to an increase in bone mass by stimulating osteoblastogenesis from bone marrow progenitors [Akune T *et al.,* 2004]. Other hypotheses have been suggested as well to explain the interplay between TZDs and osteoblasts. Lecka-Czernik et al. published *in vitro* and *in vivo* data demonstrating that activation of PPAR-γ suppressed insulin-like growth factor-1 expression in bone, which had deleterious effects on bone formation [Lecka-Czernik B *et al.,* 2007]. Thus these studies make clear that TZD has direct impact on impaired bone formation in Type 2 diabetes and these facts emphasize that TZD should be prescribed cautiously since they are used in a population that is already at a greater risk for fractures.

Possible mechanism of bone deterioration in diabetic osteopenia:

From several studies it is well documented that insulin is essential for osteoblast function, as well as for chondrogenesis and collagen synthesis [Phornphutkul C, *et al.,* 2006]. Animal models authenticate a direct anabolic effect of insulin on bone. A low

bone turn over rate which is manifested by decreased serum levels of osteocalcin, low osteoblast number, scant osteoid tissue and low mineral apposition rates, have been reported in streptozotocin induced diabetic rats [Goodman WG *et al.,* 1984]. Further experimental data reveals that the anabolic effects of insulin on the skeleton maybe mediated through the IGF-1 pathway. Insulin is found to inhibit IGFBP-1 gene expression in liver and in human osteoblasts in culture. In diabetes which is characterized by insulinopenia (T1DM) or insulin resistance (T2DM), local bone IGFBP-1 increases reducing the availability of IGF-1 to stimulate bone acquisition [Conover CA, *et al.,* 1996].

Microangiopathy at the bone tissue was also highlighted as a possible reason for diabetic osteopenia. It was shown that insulin and insulin like growth factors (IGF-1, IGF-2) have an influence on bone metabolism itself and other growth factors, cytokines and hormones may determine changes in diabetic bone metabolism. Insulin receptor substrates (IRS-1 and IRS-2) are essential for intracellular signaling by insulin and insulin-like growth factor-I (IGF-I), which are the anabolic regulators of bone metabolism. In a knock out animal study mice lacking the IRS-2 gene (IRS-$2^{-/-}$ mice) developed normally, but they exhibited osteopenia with decreased bone formation and increased bone resorption. It is concluded that IRS-2 deficiency in osteoblasts causes osteopenia through impaired anabolic function and enhanced supporting ability of osteoclastogenesis. Thus they propose that IRS-2 is needed to maintain the predominance of bone formation over bone resorption, whereas IRS-1 maintains bone turnover, thus the integration of these two signalings causes a potent bone anabolic action by insulin and IGF-I [Akin T *et al.,* 2002].

Recent findings suggest that leptin is involved in the regulation of osteoblast function and bone mass, which is of special interest in diabetes mellitus type 2. The clinical relevance of osteoporosis or osteopenia is determined by the increased risk for insufficiency fractures. Several studies using diabetic animal models highlight the importance of the IGF-1 pathway and the skeleton. Knock out mouse lacking the insulin receptor substrate shows a low bone turnover state with impaired osteoblast function [Akune T *et al.,* 2002], thus it is predictable from these results that defective

insulin production and IGF-1 signaling may cause drastic effects in bone physiology. This is also true in the case of streptozocin-induced diabetic mouse, which shows reduced expression of IGF-1, IGF-1 receptors and insulin receptors with reduced bone growth. Some studies suggest that skeletal growth retardation in type I diabetes may be associated with reduced expression of the GLUT4 and IGF-I receptor in the bone growth center. [Maor G & Karnieli E. 1999] Moreover decreased IGF-1 expression is found to affect trabecular bones more than the cortical bones. When systemic levels of IGF-1 are lower, trabecular bone is more affected than cortical bone [Jiang J *et al.,* 2006].

The association between low IGF-1 and impaired osteoblast function is seen clinically in individuals with T1DM and low-turnover osteopenia. Thus insulin therapy may enhance bone health in T1DM, which is demonstrated by a 7 years intensive insulin therapy study on T1DM patients, who showed improvement in metabolic control, increase in body mass index and decrease in resorption parameters and they conclude that insulin contribute to the stabilization of bone mass in type I DM but the presence of retinopathy is a critical factor in the progression of diabetic osteopenia [Campos Pastor MM *et al.,* 2000]. A better understanding of the effect of insulin on the behavior of bone cells and their signals (Wnt/betacatenin, and the RANK-L/OPG pathways) could help out to devise pharmacologic treatments for diabetes induced osteopenia and osteoporosis.

Furthermore diabetes mellitus (DM) increases osteoclast function but decreases osteoblast function, thereby leading to accelerated bone loss, osteopenia and osteoporosis. DM/hyperglycemia induces production of macrophage colony stimulating factor (MCSF), tumor necrosis factor (TNF)-a and receptor activator of nuclear factor-kB ligand (RANKL), all of which are osteoblast-derived activators of osteoclast proliferation and differentiation. Moreover, DM/hyperglycemia suppresses osteoblast proliferation and function, in part, by decreasing runt related transcription factor (Runx)-2, osteocalcin and osteopontin expressions.

Adipogenic differentiation of mesenchymal stem cells is favored in diabetic condition, which is indicated by the overexpression of adipocyte differentiation markers, including peroxisome proliferator-activated receptor (PPAR)-γ, adipocyte fatty acid binding protein (aP2), adipsin and resistin. A decrease in neovascularization may further aggravate bone loss. Bone quality is also reduced as a result of advanced glycation end products (AGE) production, which may eventually result in low impact or fragility fractures. All these factors are depicted in table 4, which shows the possible underlying pathology in diabetes induced osteopenia and osteoporosis.

Table 4- Factors contributing to decreased bone quality in Diabetes Mellitus.

Osteoblast function	Osteoclast function	Bone microangiopathy	Mesenchymal cell differentiation	Bone strength
↓ Runx2 (runt domain factor-2), core-binding factor alpha1 (Cbfa1) & human homolog of the drosophila distal-less (Dlx5) gene expression.	↑ Osteoclast proliferation	↓ platelet-derived growth factor (PDGF-B)	↑ Adipocyte differentiation.	↑AGEs (advanced glycation end products)
↓ Osteocalcin expression	↑ TNF (Tumor Necrosis Factor)-α	↓ proliferation of endothelial progenitor cells (EPCs)	↑ Bone marrow fat deposition.	↓ Type I collagen
↓ Osteonectin expression	↑ Macrophage Colony stimulating	↑ Oxidative stress- Hypoxic microenvironment	↑ peroxisome proliferator-activated receptor (PPAR)-γ,	↓ Bone rigidity

	factor (MCSF)			
↓ Osteoblast proliferation	↑ Receptor activator of nuclear factor-κ B ligand (RANKL)	↓ basic fibroblast growth factor (bFGF)	↑ adipocyte fatty acid binding protein (aP2)	↓ Yield moment
↑ IGFBP-1 (Insulin Growth factor Binding Protein-1) and ↓ IGF (Insulin Growth Factor)	-	↓ Neo vascularisation	↑ Adipsin and resistin	↓ Ultimate moment
↓ Insulin receptor substrates (IRS-1 and IRS-2)	-	-	↓ Osteoblast differentiation	↓ Yield stress
↑ reactive oxygen species (ROS), decreases Osteoblast differentiation.	-	-	↑ oxidative stress induces Forkhead box O (FOXO) transcription, factor, an antagonize of Wnt signaling thus decreased bone formation.	↓ Energy to fracture

↓- Decreased , ↑- Increased

Available therapeutics and its limitations:

It is obvious from the literature survey that detrimental effects of DM on bone physiology emanate from hyperglycemia and its consequences. Therefore effective glycemic control may restore this clinical condition. Various antidiabetogenic agents are available in the market, among which recombinant insulin therapy is still a promising option for treating diabetic osteopenia and associated fracture treatment.

An *in vitro* study conducted by Gopalakrishnan V *et al.,* have demonstrated that under high concentrations of glucose, bone marrow mesenchymal stem cells (BMSC; progenitors of both osteoblasts and adipocytes) show impaired proliferation, ALP activity, and also the number of nodules formed, the area stained for collagen were greatly reduced. Whereas insulin treatment alone was able to increase [3H]-thymidine uptake or ALP activity, when the cells were treated with both insulin and estradiol it results in the increase in the number of mineralized nodules and the area stained for collagen and mineralization [Gopalakrishnan V *et al.,* 2006]. Moreover, *in vivo* animal studies where in treatment of diabetic animals with subcutaneous, controlled-release of insulin implants had been shown to normalize glucose homeostasis, which in turn resulted in normalization of fracture healing [Beam HA *et al.,* 2002; Follak N *et al.,* 2005]. So from these studies it is clear that insulin indirectly benefits bone by reducing the harmful effects of chronic hyperglycemia. Besides lowering plasma glucose levels and promoting anabolic bone function such as enhancing osteoblast proliferation and osteogenesis, insulin also boost up the production of proteoglycans, the components of the gel-like extracellular matrix of cartilage, in the articular cartilage of streptozotocin-induced DM mice, this study suggest that insulin might also protect against osteoarthritis in overweighed Type 2 DM patients [Cai L *et al.,* 2002].

Other antidiabetic drugs that was found to enhance osteogenesis either through direct actions on osteoblasts or BMSC or by reducing adipogenesis include Metformin, glimepiride etc. Metformin administration in STZ induced diabetic rats shows positive effects on osteoblast differentiation and function, such as upregulation of Runx2 and osteocalcin protein expression mediated by Runx2/Cbfa1 and AMPK activation, as well as increases in alkaline phosphatase activity, type 1 collagen synthesis and bone calcium accumulation. Similarly glimepiride was also found to enhance the proliferation and differentiation of osteoblasts which leads to the activation of several key signaling molecules including insulin receptor substrate–1/2, PI3K/Akt, and endothelial nitric oxide synthase there by positively regulating osteogenesis under diabetic conditions.

Besides synthetic drugs, certain herbal preparations, such as cinnamon bark extract from the plant Cinnamomi Cassiae, have been found to be a promising anti diabetic agents which when administered in animal models show increase in serum insulin levels and also improves insulin sensitivity in adipose tissue by increasing serum adiponectin levels as well as upregulating PPAR-α and -γ mRNA expression. This antihyperglycemic and antihyperlipidemic action of Cinnamon was found to be regulated by the PPAR-medicated glucose and lipid metabolism. Thus by reducing fat accumulation in bone marrow, cinnamon extract helps indirectly facilitating bone formation under diabetic conditions [Kim SH and Choung SY, 2010].

Other additional benefits of using insulin and antihyperglycemic drugs, is the alleviation of microangiopathy and restoration of microcirculation in diabetic bone. As discussed early some medications of the class thiazolidinediones antidiabetic drugs, such as rosiglitazone, cause negative effect in bone metabolism, making the condition worse, so one has to be precautious in using such drugs, especially in diabetic population, who already have a threat of fracture risk.

Some studies shows that the impaired fracture healing in diabetes was associated with decreased levels of basic fibroblast growth factor. Their results clearly demonstrate that bFGF is expressed during the early stage of fracture repair, and that the impaired fracture-repairing ability in diabetic rats is coupled with reduced expression of bFGF at the fracture site. A single application of bFGF right away after fracture not only facilitates the repair process in normal rats, but also recovers the impaired repairing ability in diabetic rats. There by they postulate that local application of bFGF may aid bone union in patients with diabetes [Kawaguchi H *et al.,* 1994].

As it is evident from various studies that diabetes impairs fracture healing, several attempts have been investigated by various researchers to enhance the healing process in diabetes. Platelet-rich plasma (PRP) was investigated as a potential agent for improving diabetic fracture repair because PRP contains high levels of mitogenic growth factors. The percutaneous delivery of platelet rich plasma (PRP) was used in a study to investigate the use of PRP as a concentrated source of critical early growth

factors on bone healing in the diabetic BB Wistar femur fracture model. They found out that PRP delivery at the fracture site normalized the early (cellular proliferation and chondrogenesis) parameters and also improves the late (mechanical strength) parameters of diabetic fracture healing [Gandhi A *et al.*, 2006].

A recent study by Azad *et al.*, reported that recombinant human bone morphogenetic protein- 2 (rhBMP-2) enhanced bone formation radiographically and histologically in spontaneously diabetic BB Wistar rats, possibly through an increase in angiogenesis. Mechanically, fracture calluses from diabetic rats treated with rhBMP-2 showed better torque to failure and torsional rigidity values. From these results they conclude that the negative effects of DM osseous healing is ameliorated with the application of the rhBMP-2-collagen carrier and demonstrates the potential clinical role of this adjunct in the clinical arena [Azad V *et al.*, 2009].

Platelet derived growth factor was also one of the factors investigated in early diabetic fracture callus compared with that of healthy controls. In a study it has been elucidated that decreased cell proliferation rates associated with diabetic fracture healing are consistent with decreased platelet derived growth factor levels, by which they suggest a causal relationship, and concluded that diabetes is affecting the early phase of fracture healing by inhibiting cell proliferation through decreasing expression of platelet derived growth factor [Tyndal WA *et al.*, 2003]. It has been reported that PDGF was decreased at the mRNA level and protein levels in spontaneously diabetic animals [Tyndal WA *et al.*, 2003]. Al-Zube *et al.*, investigated the effect of direct application of recombinant human PDGF-BB (rhPDGF-BB) to femur fracture sites in diabetic BB Wistar rats. Their results indicate that rhPDGF-BB treatment ameliorates the effects of diabetes on fracture healing by promoting early cellular proliferation that ultimately leads to more bone formation. Thus they conclude that local application of rhPDGF-BB may be a new therapeutic approach to treat diabetes-impaired fracture healing. From these sources it is clear that several therapeutic approaches can be developed to overcome the negative consequences of diabetes on fracture healing. But one has to sort out which acts as a best source of treatment, without any side effects.

From a research point of view it will be important to clarify at the cellular level the impact of hyperglycemia and insulin resistance deficiency on bone homeostasis. Appropriate animal models will explain the underlying pathology. The role of neurogenic influences such as ghrelin, leptin, and GLP-2 (glucagon-like peptide 2) on bone homeostasis in both T1DM and T2DM needs to be studied. From a clinical perspective it will be critical to tackle the mushrooming epidemic of obesity that predisposes to both T2 DM osteoporosis. The various co-morbid conditions that accompany diabetes mellitus such as cardiovascular disease, obesity, hypertension, renal impairment, and neuropathy need to be considered as potential risk factors for diabetes associated osteopenia, fracture and osteoporosis. Perhaps these co morbidities will affect the choice of treatment for fractures indirectly. Because of the importance of bone quality, new biomechanical and imaging techniques that assess bone quality will be needed. Finally physicians need to choose appropriate medical therapy based upon the underlying pathophysiology. In view of the low bone turnover state in T1DM with reduced osteoblastic function, anabolic agents would appear to be a logical choice. The potent bisphosphonates have also been used successfully in treating glucocorticoid [Sol Epstein and Derek Le Roith 2008].

Bone tissue engineering as a therapeutic regimen to diabetic fractures:

In this scenario, a novel therapeutic regimen may favor diabetic bone healing. Bone Tissue Engineering is a promising interdisciplinary field that seeks to address the needs of bone substitutes to replace or regenerate the lost bone under severe conditions like fracture, trauma or injury. It utilizes the principles of both biology and engineering to the development of viable substitutes that restore and maintain the function of human bone tissues. This form of therapy differs from typical drug therapy or permanent implants in that the engineered bone becomes integrated within the patient, affording a potentially permanent and specific cure of the disease state. It involves the approach of mimicking the natural milieu by placing the cells and growth factors in synthetic scaffolds that act as temporary ECMs. The basic concept behind

tissue engineering is to utilize the body's natural biological response to tissue damage in combination with engineering principles. As the role of cell signaling and subsequent functionality in tissue engineering emerges with greater clarity, tissue engineers are developing multifunctional bioactive scaffolds. Thus an ideal synthetic scaffold must be capable of presenting a physiochemical biomimetic environment and also biodegradable when native tissue integrates and actively promotes desirable and prevents undesirable physiological responses, respectively.

To address these biomimetic requirements specifically, a synthetic bone scaffold must provide temporary mechanical support to the affected area. It should act as a substrate for osteoid deposition, moreover it should contain a porous architecture to permit vascularization and bone in-growth, thus encouraging bone cell migration into the scaffold. Besides this it should support and promote osteogenic differentiation in the non-osseous, synthetic scaffold (osteoinduction), there by enhancing cellular activity towards scaffold-host tissue integration (osseointegration). It should also degrade in a controlled manner to aid load transfer to developing bone. The degradation products should be non-toxic, it should also not incite an active chronic inflammatory response. Finally it should withstand sterilization without loosing its bioactivity, and deliver bioactive molecules or drugs in a controlled manner (if needed) to accelerate healing and prevent pathology.

Thus the development of ideal scaffold is the major challenge in bone tissue engineering field. Among the available biomaterials, Bioactive ceramics are used as an excellent source of bone substitutes over the past three decades, because of their similarity in structure to the mineral phase of bone and have been shown to be osteoconductive depicting direct bone deposition on ceramic surfaces. Although hydroxyapatite exhibits good osteoconductive and osteointegrative properties, the success is impaired by poor resorption properties. Several comparative studies have demonstrated the efficacy of silica in stimulating bone cell function and its presence easily facilitates bone formation and in growth, and ultimately bone bonding *in vivo* [So K *et al.,* 2006; Hing K A *et al.,* 2006]. Silica-based bioactive glass plays an important role in the surface bioactivity by the exchange of ions at the glass tissue

interface which results in the formation of carbonated HA layer, similar to the mineral phase of bone. Investigations on tissue regeneration and repair of goat segmental femur defect by Manitha B. Nair *et al.,* using Silica coated hydroxyapatite, showed positive results which proves that this novel material has good osteointegration and osteoconduction, as observed in bare and tissue-engineered HASi implants. The performance of tissue-engineered HASi was better and faster, evident by the lamellar bone organization of newly formed bone throughout the defect together with the degradation of the material [Manitha B. Nair *et al.,* 2008]. These findings indicated that the bioactivity of HA endowed with a silica-containing coating has definitely influenced the cellular activity, projecting HASi as a suitable candidate material for bone regenerative therapy. This indigenous silica-based bioactive ceramic with dual advantages of both Si-O and HA, comprised of a porous HA ceramic coated with calcium silicate, HA, and Tricalcium phosphate (HASi).

Mesenchymal stem cells (MSCs) residing in the bone marrow stromal fraction, serves as a stem cell pool supporting hematopoeisis and osteogenesis. MSCs were first described as bone-forming progenitors from the stromal fraction of rats by Friedenstein *et al.,* [Friedenstein *et al.,* 1966]. Due to their plastic adherent nature, they are easily isolated and are expandable in culture, and it is relatively easy to obtain a sufficient number of cells for cell therapy. MSCs also have been shown to differentiate into multilineage cell types [Pittenger MF *et al.,* 1999; Jackson L *et al.,* 2007] and to secrete various cytokines, including bFGF and VEGF [Kinnaird T *et al.,* 2004]. Through these actions, transplantation of MSCs has been experimentally reported to be a promising strategy for the treatment of several diseases. From the literature survey it is clear that diabetic MSC shows impaired proliferation and differentiation potential and tend to undergo apoptosis there by leading to reduced number of MSCs in bone marrow, there by affecting the bone health in diabetes [Stolzing *et al.,* 2010]. Some attempts have been made to enhance MSC proliferation and differentiation by hormone administration such as Insulin and estradiol and found to be successful [Gopalakrishnan V *et al.,* 2006].

To date there are no studies using biomaterial to improve the impaired growth of MSCs in diabetic condition. It is understood that BMSC have its own limitations such as being an invasive painful procedure accompanied by a risk of infection. Therefore an alternative source such as adipose, which has its own advantage of high yield and easily obtainable with minimal discomfort, may serve as a better alternative. To propose an appropriate regimen for diabetic fracture healing, MSCs from two different sources such as bone marrow and adipose were used along with the bioactive ceramics to find a better option for treatment. Thus we hypothesize that this novel approach of bone tissue engineering may serve as a good regimen for impaired fracture healing under diabetic condition.

Hypodissertation

Mesenchymal stem cells under the influence of pathologic systemic conditions would exhibit significant differences that will affect their selection and role in tissue engineering applications.

Autologous diabetic Mesenchymal stem cells (MSCs) on a biocompatible ceramic construct such as the Silica coated hydroxyapatite for a therapeutic approach towards bone fracture healing under systemic diabetic conditions.

Objectives

1. Development of diabetic animal model.
2. Evaluation of diabetic animal model by estimation of blood glucose, body weight and serum analysis.
3. Characterisation of bioactive ceramic scaffold.
4. Isolation and expansion of Mesenchymal stem cells (Diabetic versus Control).
5. Evaluating the differentiation potential of rBMSC and rADMSC into osteogenic lineages (Diabetic versus Control)
6. Fabrication of cell-seeded scaffolds.
7. Characterization of cell-seeded scaffold by testing adhesion, viability, proliferation and osteogenic differentiation.

Chapter 2 – Materials and methods

1. DEVELOPMENT OF DIABETIC ANIMAL MODEL

1.1 Animal model:

Wistar male rats (3 months old) were obtained from the Division of Laboratory Animal Sciences, BMT Wing, SCTIMST (DLAS). All animal procedures were conducted following the guidelines/approval of CPCSEA and Institutional Animal Ethics Committee-IAEC.

1.2 Diabetic induction:

Seven male Wistar rats, with an average weight of 240 g were selected for the study. Animals were divided into two groups – test & control. In the test group, for diabetic induction, rats were administered intraperitoneally with single dose of streptozotocin (45 mg/kg body weight) (Sigma–Aldrich) dissolved in 0.1 M cold citrate buffer (pH-4.5) after overnight fasting, whereas the control group received citrate buffer alone (vehicle alone). Animals were fed after 30 minutes of administration. The experimental animals were maintained for a period of 10 weeks.

1.3 DIABETIC MODEL EVALUATION

1.3.1 Evaluation of Blood Glucose:

Blood glucose values of both the control and test rats were measured using Glucometer (One touch Horizon- China) throughout the experiment. Rats were considered diabetic if the blood glucose (measured using glucometer) value was higher than 140mg/dL, 72 hours after the injection.

1.3.2 Evaluation of BodyWeight:

The weight of the animals (both control and test) was regularly monitored to observe the increase or decrease in their weight due to the induction procedure.

1.3.3 Biochemical Analysis of Serum:

1.3.3.1 Serum collection:

Control and diabetic rats were anesthetized with ether and 1ml Blood was obtained via orbital sinus puncture, at two different period, one before and the other after diabetic induction (i.e. at the time of euthanasia). Blood serum was prepared from each sample by centrifugation for 10 min at 2000 rpm at room temperature. Serum was stored at -80°C until the analysis.

1.3.3.2 Calcium estimation:

Calcium level was estimated in serum using Calcium biochemical kit (Agappe Diagnostics), comprising of calcium dye reagent – R1 (Diethylamine), Calcium base reagent- R2 (o-Cresolphtalein Complex, 8-Hydroxyquinoline) and Calcium standard solution (10mg/dL)

Principle of the Method

Calcium with o-cresolphtalein complex, at alkaline pH, yields a red colored complex, whose intensity is proportional to the calcium concentration.

Ca^{++} + o-Cresolphtalein ---- OH^{+}⇨ Colored complex

Assay:

Working reagent is prepared from reagent 1 and reagent 2 in the ratio 1:1 (Mixture is stable for 14 days at 2-8^0 C). To 10µl of serum sample 1000 µl of working reagent was added, mixed and incubated for 5 minutes at room temperature. Similarly

Calcium standard (10µl) was added to 1000 µl of working reagent, mixed and incubated for 5 minutes at room temperature. The absorbance of the samples and standard was measured at 570nm using Hidex-Chameleon spectrophotometer. Calcium concentration in the sample was calculated as follows:
(Absorbance of Sample/ Absorbance of standard) x 10 = mg/dL calcium in the sample.

1.3.3.3 ALP estimation:

ALP level in serum was estimated using ALP biochemical kit (Agappe Diagnostics), which consists of Reagent 1- (R1) Diethanolamine buffer-1mmol/L and Magnesium Chloride- 05mmol/L and Reagent 2- R2 (P-Nitrophenyl phosphate)

Principle of the Method:
The estimation of ALP is according to the following reaction.

Para nitro phenyl Phosphate + H_2O -ALP----→ p-nitrophenol + Inorganic phosphate.

Assay:
Working reagent was prepared by adding 10 ml of Reagent 1 to the vial containing R2, mixed well (Reconstituted reagent is stable for 7 days at 2-8° C). To 20µl of serum sample, 1000 µl of working reagent was added, mixed and incubated for 1 minute at room temperature. The absorbance was measured at 405 nm Hidex-Chameleon spectrophotometer. The ALP activity was measured using

ALP activity (U/L) = OD/min * 2750.

1.4 Histology:

The pancreas collected from the diabetic group and control group were fixed in 10% neutral buffered formalin in Sorensen's phosphate buffer. Subsequently the samples were processed, first they were dehydrated in ascending grades of ethanol (30%, 50%, 70%, 90% and 100% acetone) for 20 min each; then infiltrated in xylene I (10 min)

and xylene II (10 min); embedded in paraffin wax for 1h (2 changes). The processed samples were then embedded in wax and thin sections (5μm) were made using microtome (Leica RM2255). The sections were then kept at 37°C overnight and then stained.

1.4.1 Hematoxylin and Eosin staining:

The sections were deparaffinised first with xylene (3 changes, 10 min each); then rehydrated in descending grade of ethanol series (90%, 80% and 70% for 5 min) followed by washing in tap water for 3 min. The sections were then stained with Harris's Hematoxylin for 15 min followed by washing in tap water for 3 min and then differentiated in 1% acid alcohol for 30 sec (2-4 fast dip), rinsed in tap water and blued with 0.2% ammonia water for 2 sec. It was then rinsed again with tap water for 5 min, counterstained with 1% eosin for 4 min; dehydrated in ethanol (70% for 5 min, 100% ethanol for 5 min - 2 changes). Finally the sections were cleared in xylene (3 changes, 10 min each) and were then mounted in DPX and viewed under Light microscope (Leica DM 6000).

1.5 Transmission Electron Microscope

A portion of pancreas from control and diabetic animals was grossed into small pieces and was fixed in 3% Glutaraldehyde. The samples were then stained with 1% Osmium tetroxide , dehydrated in ascending grades of acetone, infiltrated and embedded in epoxy resin (Epon 812) at 60 °C for 2-3 days. Ultra thin sections (60-90 nm thick) were cut using ultramicrotome (LKB) and sections were collected onto grids. Dry grids were stained with uranyl acetate and lead citrate and observed under Transmission electron microscope (Hitachi H-7650) at an accelerating voltage of 80Kv.

2. Material:

The bioactive ceramic Hydroxyapatite (HA) and HA-Si (Silica Coated Hydroxyapatite) were the materials of choice for the fabrication of tissue engineered construct for *in-vitro* studies.

2.1 Sterilization:

HA and HA-Si blocks gifted from Bioceramics Lab, SCTIMST, were polished down to discs with 5mm diameter and 1-2mm thickness. The discs were then washed in distilled water and subjected to ultrasonic cleaning (Cole Parmer) 10 minutes each twice, for the complete removal of fine powders that adhered over the surface. Subsequently, the materials were sterilized by autoclaving (121°C, 15psi pressure for 15minutes) prior to the *in vitro* study.

2.2 Material Characterization:

2.2.1 Environmental Scanning Electron Microscopy (ESEM):

Surface microstructure, topography, and porosity of the materials were observed by ESEM (FEI Quanta 200).The scaffolds were processed first and then gold coated (E101-Hitachi) in an ion sputter unit and viewed.

2.2.2 X-Ray Diffraction (XRD):

X- ray diffraction was performed to assess the phase purity and crystallinity of the sintered HA and HA-Si.. The materials were scanned between 20° and 40° 2 θ at at a rate of 2° per minute under a step size of 0.02° using Cu-Kα1 radiation at a voltage of 40 kV and a current strength of 30 mA (Siemens D-5005 X-ray

Diffractometer, Germany). Materials were identified by comparing the data with the JCPDS files (Joint Committee on Powder Diffraction Standard).

2.2.3 Fourier Transform Infrared Spectroscopy (FT-IR):

Fourier Transform Infrared Spectroscopy analysis was conducted on Thermo Nicolet 5700 spectrometer, U.S.A. and the spectra were collected in diffuse reflectance (DRIFT) mode to determine the functional groups. HA and HASi were powdered and mixed with optical grade KBr powder. The spectrum were recorded at a resolution of 4 cm^{-1} and scanned between of 400 to 4000 cm^{-1} wave number range and average scan of 200. Pure KBr was used as the background.

3. Isolation and Expansion of mesenchymal stem cells (MSC's):

3.1 Growth and Differentiation Media for cell culture procedures:

3.1.1 Medium used for Cell culture:

Medium used for isolation and expansion of Rat BMSC culture was Dulbecco's Modified Eagles Medium (DMEM) Low Glucose (LG) (Invitrogen) supplemented with 15% Fetal bovine serum (FBS) (Invitrogen) and 1% antibiotics (anti- anti mix, Invitrogen). Medium used for isolation and expansion of Rat ADMSC culture was Dulbecco's Modified Eagles Medium (DMEM) High Glucose (HG) (Invitrogen) supplemented with 10% Fetal bovine serum (FBS) (Invitrogen)and 1% antibiotics (anti- anti mix, Invitrogen).

3.1.2 Osteogenic Induction Medium:

Medium used for osteogenic differentiation is DMEM HG (Invitrogen) supplemented with 15%FBS (Invitrogen) and 1% antibiotics (Invitrogen). The working concentration of the osteogenic inducers in the medium are

- 10mM β-glycerophosphate (Sigma Chemicals),
- 10^{-8} M dexamethasone (Sigma Chemicals),
- 0.05mg/ml L-ascorbic acid (Sigma Chemicals).

The medium was then filtered with 0.22μm filter. (Millex, Millipore) and used.

3.2 Isolation of Rat Bone marrow derived Mesenchymal stem Cells (rBMSC):

The experimental animals and control animals were euthanized by cervical dislocation at the end of study period. The animals were clipped to remove the fur and 70% alcohol was sprayed over the leg area, under sterile conditions, both femur and tibiae from each rat were excised. The limbs were stored in ice in PBS with 2% antibiotics (anti anti mix Invitrogen) until isolation. Each hind limb is bisected by cutting through the knee joint. After cleaning, the bones were stored in PBS with 1% antibiotics (anti anti mix Invitrogen), the proximal ends of the bones were cut and a 21-gauge needle, was inserted into the shaft of the BM, and DMEM-LG supplemented with 15% FBS and 1% antibiotics (anti anti mix Invitrogen) was flushed through the shaft. Marrow plug suspension which were collected was dispersed by pipetting and centrifuged (Hettich germany) at 2,500 rpm for 10 minutes at 14° C. Supernatant was discarded, and the cell pellet was resuspended in the medium. The isolated cells were seeded onto plastic tissue culture flasks (Nunc) and incubated at 37° C in a humidified atmosphere (Hereaus) containing 5% CO_2 for 3 days. On the third day, red blood cells and other nonadherent cells were removed and fresh medium was added to allow further growth and become confluent.

3.3 Expansion of rBMSC in Culture:

The adherent cells grown to 80% confluency were defined as passage zero (P0)

cells. The P0 MSCs were washed with phosphate-buffered saline (PBS) and detached by incubating with 0.25% Trypsin-EDTA solution (Invitrogen/GIBCO) for 5 min at 37° C. Fresh medium was added to inactivate the action of trypsin. The cells were centrifuged at 2,500 rpm for 10 min, supernatant was discarded and the pellet was resuspended in 1ml complete medium and counted manually in duplicate using the Neubar counting chamber. Then the cells were plated as P1 in 75 cm^2 flasks (Nunc) at densities of $1*10^6$ cell/flask. Complete medium was replaced once in 3 days. The cells were maintained and further expanded into consequent passages as previously mentioned.

3.3.1 Determination of population doubling time and number of rBMSC:

To evaluate the proliferative potential of both control and diabetic rBMSC, as literature reports impaired proliferative capacity of diabetic rBMSC, to substantiate this hypothesis, both control and diabetic rBMSC were seeded with a cell concentration of 1x 10^5 cells on to 6 well plates in triplicates, When cells attained 90% confluency they were trypsinised, pelleted, and the cell suspension was stained with tryphan blue and manually counted using the hemocytometer. The same concentration (1x 10^5) of cells was re-seeded for the next passage. The number of population doubling time (PDT) was determined using the following formula (according to Stolzing *et.al.,* 2008)

$PDN = \log (N/N_0) \times 3.31$

$PDT = CT/PDN$

Where, N is the no of cells at the end of growth period, N_0 the initial no of cells, CT-time of cultivation between passages, PDN- Population doublings numbers.

3.4 Isolation of Rat Adipose derived Mesenchymal stem Cells (rADMSC):

Fat tissue was isolated from the experimental and control rats, at the end of the experiment. Subcutaneous fat which is located between the two scapulas at the dorsal part of the animal was collected under sterile conditions into cold Phosphate Buffer

Saline (PBS) and was washed with fresh PBS containing 1% antibiotics (Invitrogen). Collected adipose tissue was cleaned, minced well and digested with 0.2% collagenase Type I (Gibco, Invitrogen). Tissue was digested at 37°C for one hour, the digest was filtered using a nylon mesh membrane filter (Millipore). Filtrate was collected and cells were pelleted down at 15°C, 2500 rpm, 10 min (Hettich Germany). Pellet was re-suspended in fresh medium and cells were plated in 5ml of DMEM - HG, 10% FBS and 1% Antibiotics in a 25cm^2 flask (Nunc) and maintained in a CO_2 incubator (Hereaus) at 37°C with 5% CO_2. Medium was changed after 24 hours to remove non adherent fat cells.

3.5 Expansion of rADMSC in Culture:

The adherent cells grown to 80% confluency were defined as passage zero (P0) cells. The P0 MSCs were washed with phosphate-buffered saline (PBS) and detached by incubating with 0.25% Trypsin-EDTA solution (Invitrogen/GIBCO) for 5 min at 37° C. Complete medium was added to inactivate the action of trypsin. The cells were centrifuged at 2500 rpm for 10 min, resuspended in 1ml complete medium (DMEM-HG) and counted manually in duplicate using the Neubar counting chamber. Then the cells were plated as P1 in 75 cm^2 flasks (Nunc) at densities of 1*10^6 cell/flask. Complete medium was replaced once in 3 days.

4. Differentiation potential.of rADMSC and rBMSC:

4.1 Induction of rBMSC and rADMSC to osteogenic lineage:

rBMSC's and rADMSC's (P3) from both control and diabetic rats were trypsinized and 1*10^5 cells were seeded on to the cover slips (Blue star) to study their osteogenic potential. After 24h the medium was renewed and cells were induced to osteogenic lineage by maintaining the cells in osteogenic medium for a period of 28 days. The cell seeded coverslips were then fixed in 3.7% paraformaldehyde in Sorrensen's phosphate buffer for further studies. For confirming the osteogenic differentiation the

cell seeded coverslips were stained for Alizarin red and Vonkossa staining to detect mineralization nodules.

4.2 Evaluation of Osteogenic Potential:

4.2.1 Alizarin Red Staining:

The cell-seeded coverslips were washed with PBS (3 changes- 10 minutes each) and stained with 1% Alizarin red (Sigma Chemicals) to determine calcium deposition. Mounted with DPX and viewed under Light Microscope (Leica DM 6000)

4.2.2 Von Kossa staining:

The cell seeded coverslips were washed with distilled water and stained with 5% silver nitrate (Merck India) in distilled water and exposed to UV light for 5 minutes. The cells are then washed with distilled water, air dried and viewed under Light Microscope (Leica DM 6000)

5. Fabrication of cell seeded construct:

The autoclaved material is used for the *in vitro* study. The materials were placed for conditioning in DMEM HG for 2-3 hours. rBMSC and rADMSC of Passage 2 was used for culturing on HA and HASi were trypsinized with 0.25% trypsin-EDTA for 5 min, centrifuged at 1500 rpm for 5 min and the pellet was suspended in the respective medium mentioned above (section 2.2.4). 1×10^5 cells / material were calculated as described in the section 2.2.2. These cells were seeded on all sides of conditioned HA and HASi (materials incubated in the medium for 24 h prior to cell seeding) placed in 48 well TCPS plates under static condition (Nunc) and cultivated in DMEM containing 2% FCS, 10 U/ml penicillin and 100 µg/ml streptomycin for 24h in order to facilitate initial cell adhesion. After one day of cultivation, cells were induced for osteogenic differentiation by the addition of 10^{-7} M dexamethasone (Sigma-Aldrich, Taufkirchen, Germany), 3.5 mM β-glycerophosphate (Sigma), and 50 µM L-ascorbic

acid-2-phosphate (Sigma) to the medium. The osteogenic medium change was done once in three days.

6. Evaluation of cell seeded tissue construct:

Cell adhesion as well as viability of the adhered cells on HA and HA-Si scaffolds was studied using Environmental Scanning Electron Microscopy (ESEM) (FEI Quanta 200) and Confocal Laser Scanning Microscopy (cLSM).

6.1 Cell Adhesion:

The adhesion of rBMSC and rADMSC on HA and HA-Si was evaluated using Environmental Scanning Electron Microscope (ESEM) (FEI Quanta 200). 1x 10^5 cells were seeded on the scaffolds, cultured for 28 days and fixed in 1% gluteraldehyde and proceeded for SEM analysis. The scaffolds were treated with gradients (30%, 50%, 70%, 80%, 90%, 100%) of ethanol (Merck) rinsed in distilled water and critically point dried (HCP2-Hitachi) to remove moisture, and coated with gold in an ion sputter unit (E101-Hitachi). Samples were viewed using ESEM (FEI Quanta 200).

6.2 Cell viability:

6.2.1 Live Dead Assay

The viability of rBMSC on fabricated scaffolds after 14 days were qualitatively determined by cLSM (Carl Zeiss LSM 510 Meta). The rBMSC seeded scaffolds after 14 days in culture were washed in PBS and stained with Acridine orange (1:100 dilution in PBS) and ethidium bromide (1:100 dilution in PBS) and incubated for 15 minutes in dark. It was then washed again with PBS and imaged using cLSM at an excitation and emission wavelength of 480/526 and 518/605 for Acridine orange and ethidium bromide respectively. The excitation was carried out with Argon 2 laser.

6.2.2 Lactate Dehydrogenase assay:

The cell viability was determined quantitatively through the total activity of lactate dehydrogenase (LDH) in the cell lysates using LDH reaction buffer (Cytotox96 kit, Promega, USA).

Principle:

LDH is measured in an enzymatic reaction that occurs in two steps NAD+ is reduced to NADH/H+ by the LDH catalysed conversion of lactate to pyruvate (2) The catalyst (diaphorase) transfers H/H+ of NADH/H+ to the tetrazolium salt INT which is reduced to formazan. This leads to colour change from pale yellow to red.

Assay:

The viability of Rat BMSC on 2^{nd} week and 4^{th} week in culture was measured. For this, the fabricated cell-seeded materials (n=3) were washed with PBS twice and kept in -80°C until analysis. Frozen cell samples were thawed for 20 min on ice and lysed with 1% Triton X-100 (300 µl) for 50 min with sonication for 10 min. An aliquot of each cell lysate (50µl) was mixed with LDH substrate (50µl) at room temperature and the enzymatic reaction was stopped after 30 min with 0.1 M acetic acid (50µl). The absorbance was read at 492 nm (HIDEX Chameleon). The absorbance (OD values) was correlated with cell viability using a calibration line constructed from the cell suspension with increasing concentrations of cell numbers.

6.3 Cell proliferation:

6.3.1 Picogreen assay:

The proliferation of rBMSC of both control and diabetic cells on 2^{nd} week and 4^{th} week in culture was determined using Picogreen® dsDNA Quantitation reagent (Molecular probes).

Principle:

PicoGreen dsDNA Quantitation Reagent is an ultra-sensitive fluorescent nucleic acid stain for quantitating double-stranded DNA (dsDNA). PicoGreen is a fluorochrome that selectively binds dsDNA and has characteristics similar to that of SYBR-Green I.

It has an excitation maximum at 480 nm (lesser peaks in the short-wave UV range) and an emission peak at 520 nm. When bound to dsDNA, fluorescence enhancement of PicoGreen is exceptionally high; little background occurs since the unbound dye has virtually no fluorescence. PicoGreen is very stable to photo-bleaching, allowing longer exposure times and assay flexibility.

Assay:

For this, the fabricated cell-seeded materials (n=3) were washed with PBS twice and kept in -80°C until analysis. Frozen cell samples were thawed for 20 min on ice and lysed with 1% Triton X-100 (300 µl) for 50 min with sonication for 10 min. The lysate (10µl) was then mixed with picogreen in Tris-EDTA buffer (190µl) for 5 min and the intensity of fluorescence was measured with a multifunction microplate reader (HIDEX Chameleon) at an excitation and emission wavelength of 485 / 535 nm. Relative fluorescence units were correlated with cell number using a calibration line constructed from cell suspensions with increasing concentrations of cell numbers.

6.4 Osteogenic differentiation:

The ALP activity of rBMSC of both control and diabetic seeded on scaffolds (HA and HA-Si) at the end of 2 weeks, was determined with ELF-97 endogenous phosphatase detection kit (Invitrogen).

Principle:

The ELF® 97 Endogenous Phosphatase Kit is used to detect phosphatase activity with the ELF® 97 phosphatase substrate that upon hydrolysis produces a bright and photostable yellow-green fluorescent precipitate at the site of enzymatic activity. This fluorescent precipitate has several unique spectral characteristics, including an extremely large Stokes shift, 180 nm that makes it easily distinguishable from endogenous fluorescence.

Assay:

The cell seeded scaffolds cultured for 2 weeks were washed with PBS, after removing the medium and fixed in 3.7% paraformaldehyde in Sorensen's phosphate buffer for 20 min, the cells were washed with PBS; permeabilised with 0.2% Triton X-100 in PBS and blocked with 3% BSA in PBS for 30 min. The cytoskeletal actin

filaments were stained with FITC-phalloidin (1: 100 in PBS) (Sigma chemicals) for 30 min. The cells were then stained with ELF-97 phosphatase substrate (component A was diluted 20 fold with component B (buffer) in detection kit) for 5 min and visualized through Hoechst filter using the fluorescent microscope (Leica DM 6000).

7. Statistical analysis:

The datas are expressed as mean and standard deviation, the p value was calculated based on student t test. The statistical analysis was done using graph pad software.

Chapter 3 - Results and Discussion

1 Diabetic Model Development:

1.1 Evaluation of blood Glucose and body weight:

The experimental rats were administered intra peritoneal with a single dose of streptozotocin (STZ 45mg/kg body weight) in ice-cold 0.1 M sodium citrate buffer (pH 4.2) and the control animals received only the vehicle buffer. A comparative pictorial representation of the control and diabetic rat groups are shown in Figure 1 and 2. Before and after induction, the animals were examined for change in blood glucose and body weight and the data are represented in Table 5.1 and 5.2 respectively. The blood glucose level was significantly increased compared to the control rats and the body weight was decreased compared to the control rats which clearly manifest the disease condition. Significant weight loss and elevated blood glucose was observed in Diabetic rats compared with buffer injected control animals throughout all time points in the experiment. The significant change in blood glucose and body weight of the experimental animals is represented graphically in Figure 3 and 4 respectively. This significant weight loss in diabetic animals may be due to the decreased accessibility of glucose and amino acids to cells, creating a shortage of substrates for cellular biosynthesis and affecting related cellular metabolism. In this energy-deficient state, intracellular proteins are selectively sacrificed to restore ATP production by oxidating amino acids in the Krebs cycle. [D Voet and JC Voet, Biochemistry 2005].

1.2. Biochemical Analysis of Serum:

Serum Calcium and ALP level were also estimated before and after induction in both control and diabetic animals.

Figure 1- Control animal group

Figure 2- Diabetic animal group

Animal Label	Before Induction	3rd Day After Induction	4th week- After Induction	6th week- After Induction	10th Week-After Induction
C1	100	111	106	99	96
C2	97	98	97	96	95
C3	107	104	99	94	95
Mean	101.33	104.33	100.67	96.33	95.33
SD	5.13	6.51	4.73	2.52	0.58
D1	112	417	439	478	498
D2	148	390	397	405	435
D3	139	407	413	415	420
D4	115	434	446	455	477
Mean	128.5	412	423.75	438.25	457.5
SD	17.75	18.42	22.79	34.19	36.21

Table 5.1- Glucose levels in the Diabetes-induced Wistar rat model/Control animals. (C1, C2, C3 are Control animal ID; D1, D2, D3, D4 are Diabetic animal ID)

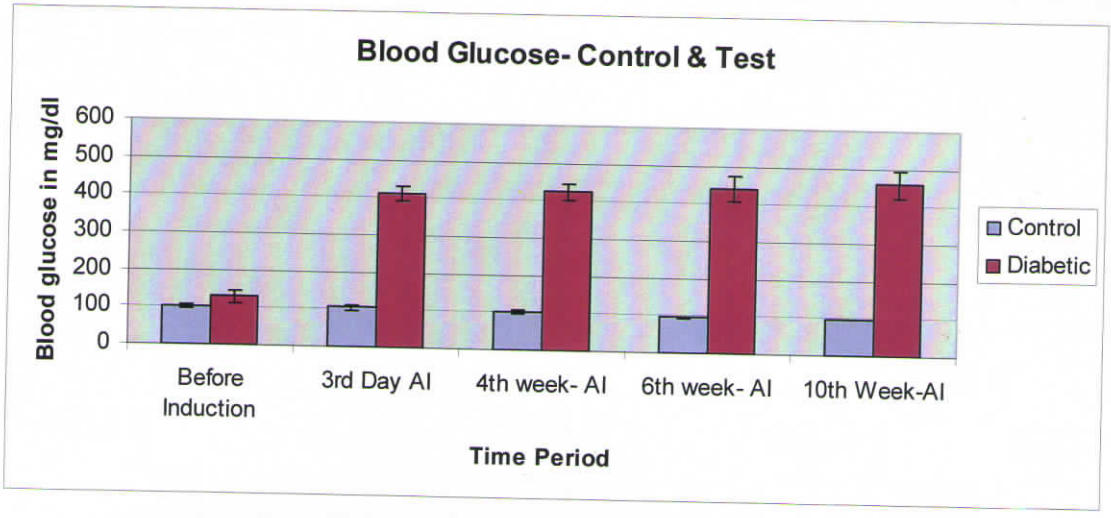


Figure 3- Blood glucose level of Control and Diabetic animal groups.

Animal Label	Before Induction	4th week- After Induction	6th week- After Induction	10th Week- After Induction
C1	253	275	320	340
C2	229	254	329	356
C3	266	289	376	398
Mean	249.33	272.67	341.67	364.67
SD	18.77	17.62	30.07	29.96
D1	242	239	221	199
D2	228	245	225	211
D3	233	248	227	208
D4	231	232	214	191
Mean	233.5	241	221.75	202.25
SD	6.03	7.07	5.74	9.07

Table 5.2- Body weight in the Diabetes-induced Wistar rat model/Control animals. (C1, C2, C3 are Control animal ID; D1, D2, D3, D4 are Diabetic animal ID)

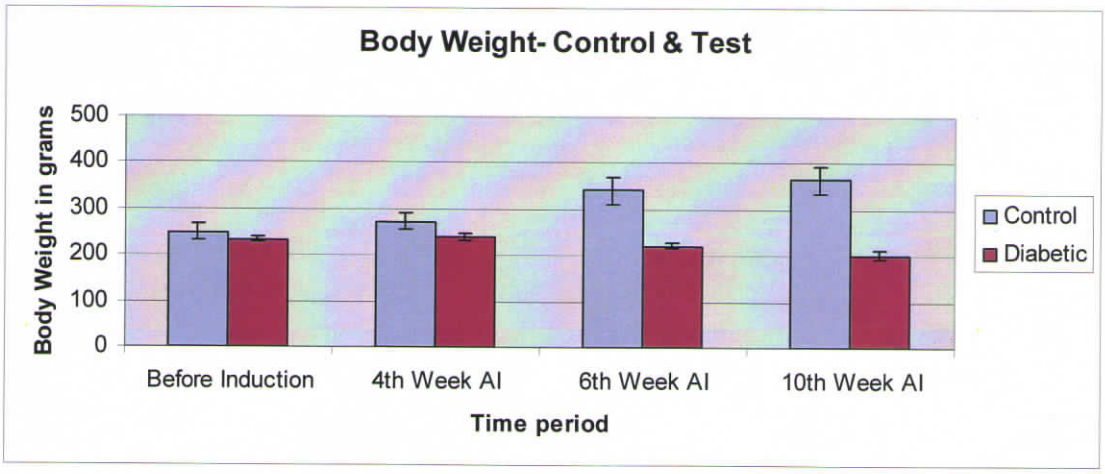


Figure 4 – Body weight of control and diabetic animal groups.

Animal ID	Calcium Concentration (mg/dL)-BI	Calcium Concentration (mg/dL)- AI
C1	9.52	9.01
C2	8.99	8.69
C3	9.57	8.88
Mean	9.36	8.86
SD	0.32	0.16
D1	8.76	10.32
D2	9.47	10.05
D3	8.97	10.45
D4	9.02	10.11
Mean	9.05	10.23
SD	0.29	0.18

Table 6- Serum Calcium levels in the Diabetes-induced Wistar rat model/Control animals.
(C1, C2, C3 are Control animal ID; D1, D2, D3, D4 are Diabetic animal ID)

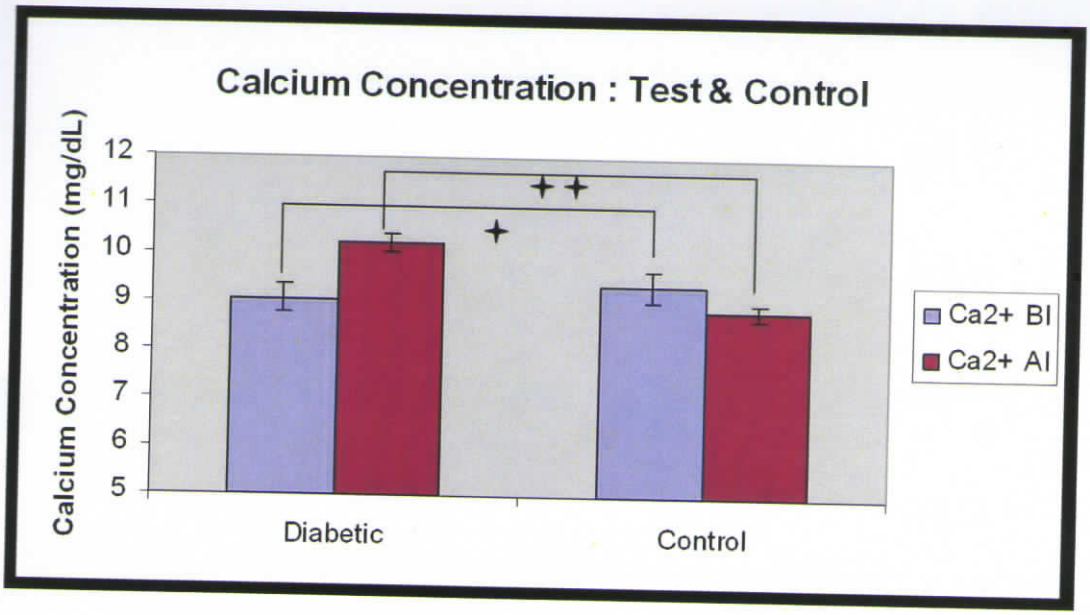


Figure 5 – Ca level of experimental animals (BI- Before Induction and AI- After Induction) (P Value – 0.0002, shows significant change between control and diabetic animal group after induction)

Animal ID	ALP Before Induction(U/L)	ALP After Induction (U/L)
C1	355.36	255.75
C2	339.16	287.37
C3	363.68	260.79
Mean	352.73	267.97
SD	12.47	16.99
D1	339.77	778.71
D2	337.41	690.47
D3	325.11	760.61
D4	332.06	754.87
Mean	333.59	746.17
SD	6.51	38.49

Table 7- Serum ALP level in the Diabetes-induced Wistar rat model/control animals (C1, C2, C3 are Control animal ID; D1, D2, D3, D4 are Diabetic animal ID)

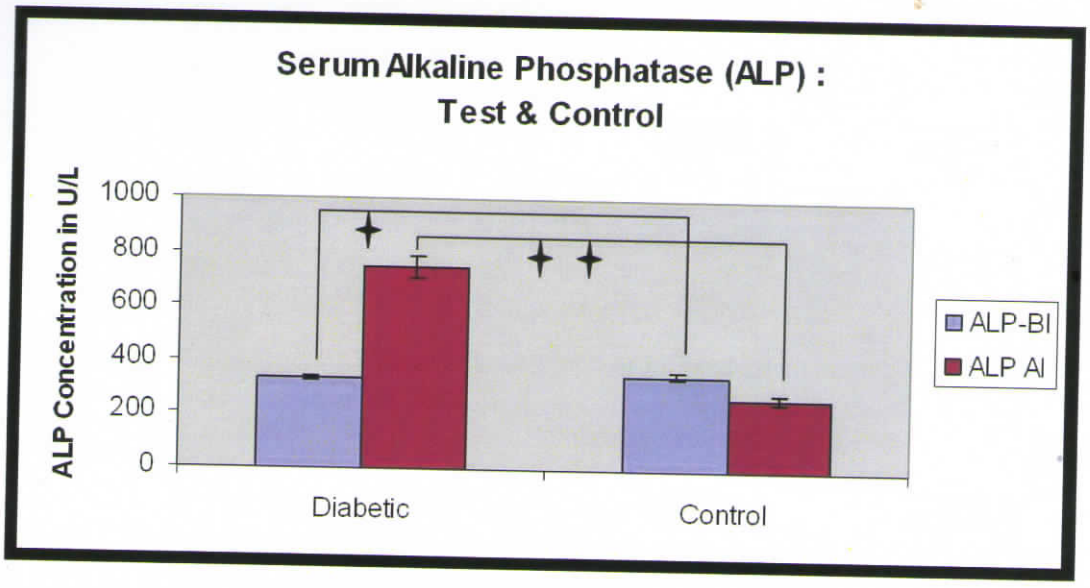


Figure 6 – ALP level of experimental animals (Before Induction and After Induction) (P Value – 0.0001, shows significant change between control and diabetic animal group after induction)

1.21 Calcium:

Serum calcium level was estimated using the Agappe estimation kit at regular intervals. Statistical analysis (t-test) of change in calcium level using graph pad software and the data obtained is represented in Table 6. The graph shown in Figure 5 shows significant difference in the diabetic group after induction (P Value- 0.002) and there was no significant change in calcium level before induction (P Value- 0.2514) compared to the control group. This data substantiate the change in serum calcium level as reported by Satoshi Uchiyama *et al., i*n diabetic animals. [Satoshi Uchiyama *et al.,* 2003].

1.22 ALP:

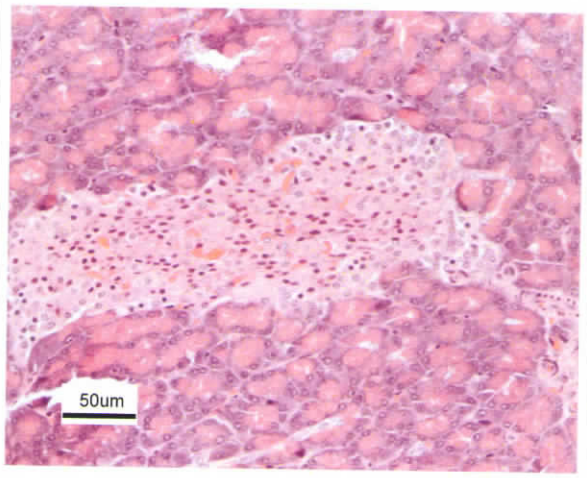
The Agappe ALP estimation kit was used to evaluate the change in ALP level in serum at different time periods. Change in alkaline phosphatase level was calculated by t-test using graph ad software, significant difference was observed in the diabetic group before and after induction and is represented in Table 7. The graph generated in Figure 6 using this data is quiet controversy to clinical data which shows decreased serum ALP level [Jehle PM *et al.,* 1998; Pietschmann P *et al.,* 1988]. This increase in serum ALP level may be due to initial proliferation of MSCs to compensate the bone loss as reported by Stolzing *et al.,* (2010). More over it has been reported that young rats have a high alkaline phosphatase activity, which decreases with time, the plateau for activity is present by 3 months in males [Disease of the Wistar rat, Mary J. Tucker, 1997]. Thus diabetic induction in animals with streptozotocin also resulted in the characteristic development of diabetic symptoms such as polydisia, polyphagia and polyuria. The change in serum ALP in diabetic animal group after induction is statistically significant (P = 0.0001) compared to that of the buffer injected control animal group.

1.3. Histology:

Hematoxylin and Eosin staining:

The H& E stained sections of the normal and diabetic pancreas were viewed under light microscope and is represented in Figure 7.1 and Figure 7.2. Cells of the pancreas were present in their normal proportions in the histology of control rats represented in

Pancreas Histology of Control and Diabetic animals.



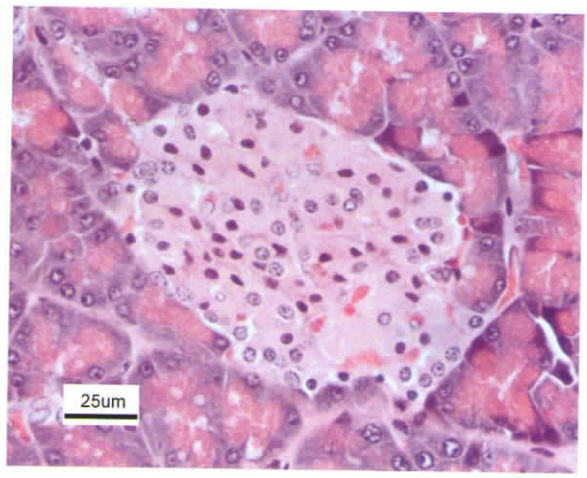
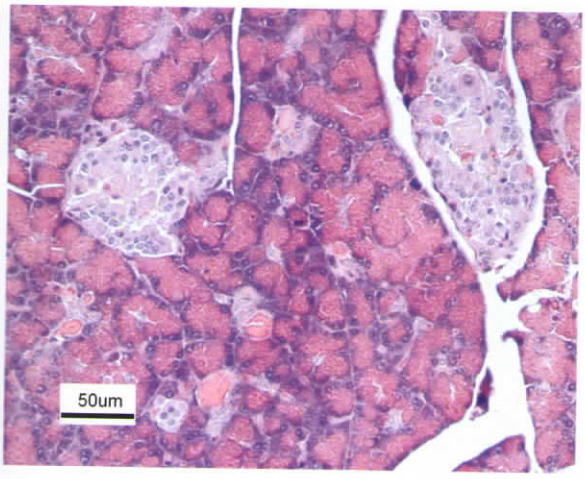


Figure 7.1 Control rat pancreas-histology



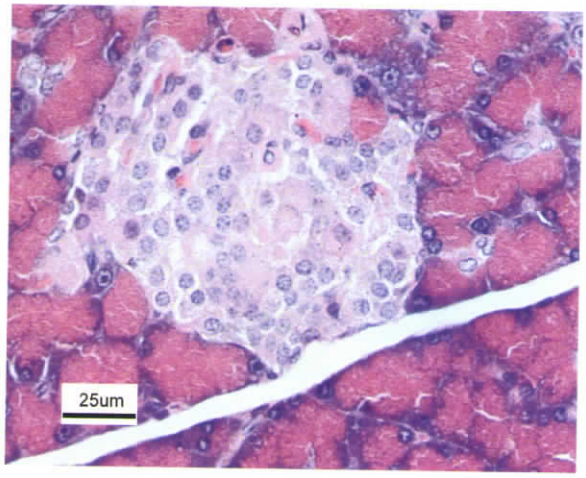


Figure 7.2 Diabetic rat pancreas-histology

Figure 7.1. The acinar cells which stained strongly are arranged in lobules with prominent nuclei. Endocrine Islets of Langerhans are the lighter staining areas, the islet cells are seen embedded within the acinar cells and surrounded by a fine capsule. Small intralobular ducts are scattered throughout. In the histology of diabetic pancreas, represented in Figure 7.2 the acinar cells were seen to be normal, but the islets were seen shrinked (insulinitis).. In this study, Streptozotocin, which selectively destroy β cells of the islet was used to induce type 1 diabetes mellitus. Insulinitis and loss of β cells were observed - a characteristic feature of type 1 DM. Insulinitis is evidenced by heavy lymphocytic infiltration in and around the islet, which is clearly seen in the histology of diabetic pancreas. This is commonly seen in islets containing residual β cells and it supports the possibility of a specific, immunologically mediated destruction of β cells as the cause of type 1 DM, [Anderson, 1986]. Depletion of β cells which results in insulin deficiency with a consequential hyperglycaemia. The histological observation thus substantiate that the induction of diabetes in rats were effective, thereby rendering the animal hyperglycemic.

1.4 TEM

Transmission electron micrographs of the ultra thin section of control and diabetic pancreas are depicted in figure 8 a and b. The normal pancreas showed large secretory granules depicting high synthetic activity while the diabetic pancreas showed smaller secretory granules.

2 Material Characterization:

2.1 Scanning Electron Microscopy:

Images depicted an elaborate porous interconnected architecture (50 – 500 microns pore sizes) ideal for the infiltration of cells into the internal voids of the ceramic. (Fig 9 (a) - HA & Fig 9 (b) - HASi) respectively.

2.2 XRD:

X-ray diffraction pattern exhibited crystallinity with hydroxyapatite as the major phase in all the materials. The outer coating layer in HASi showed peaks for calcium

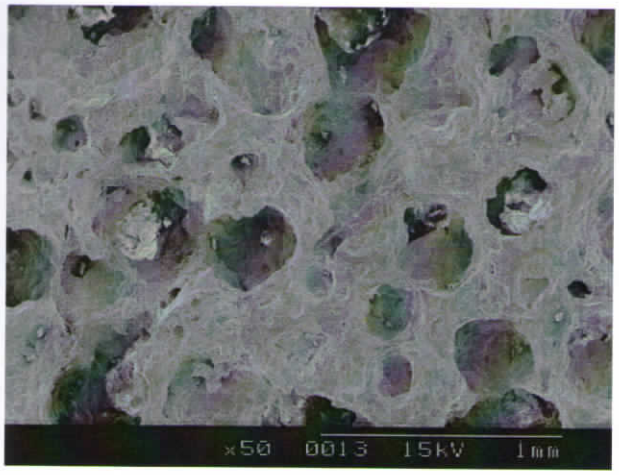


Fig. 9 (a) SEM image of HA

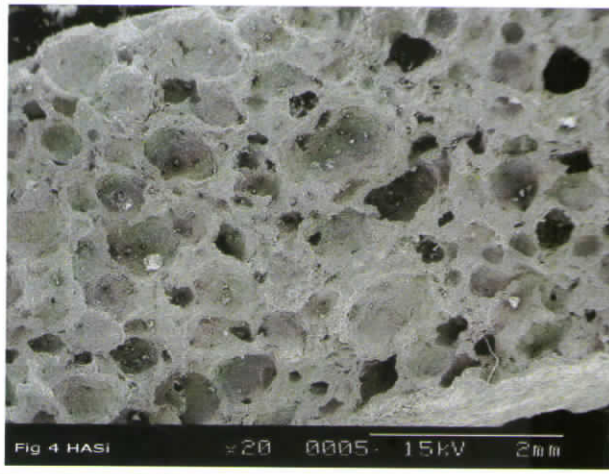


Fig. 9(b) SEM image of HASi

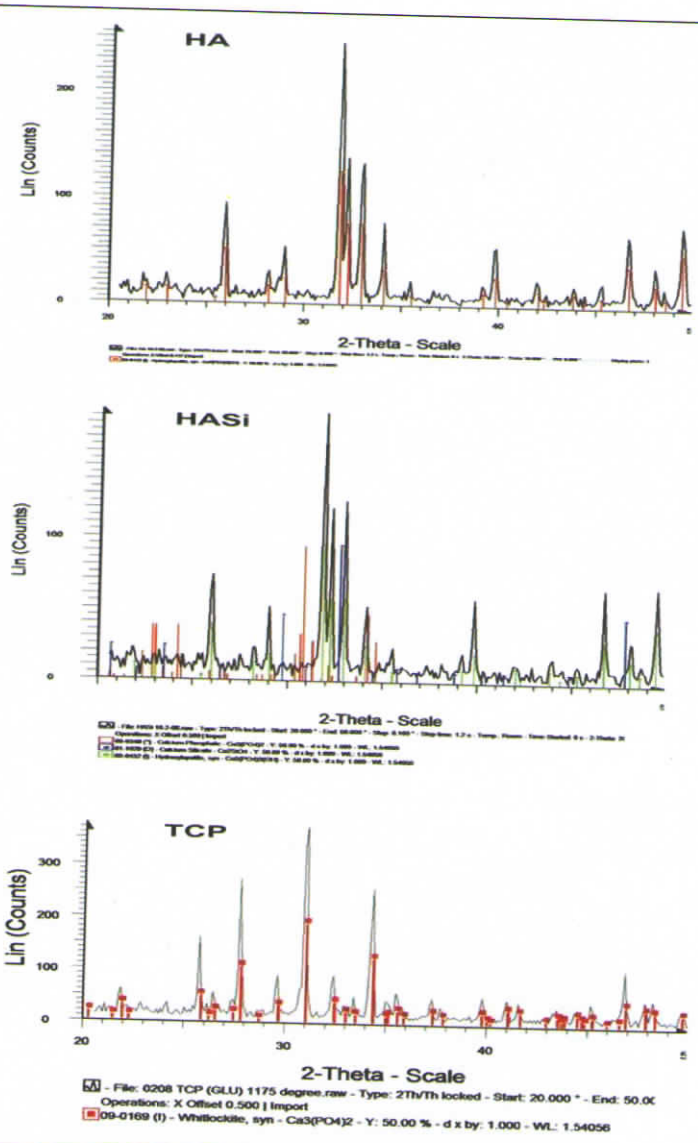


Fig. 10 – XRD of HA, HASi and TCP

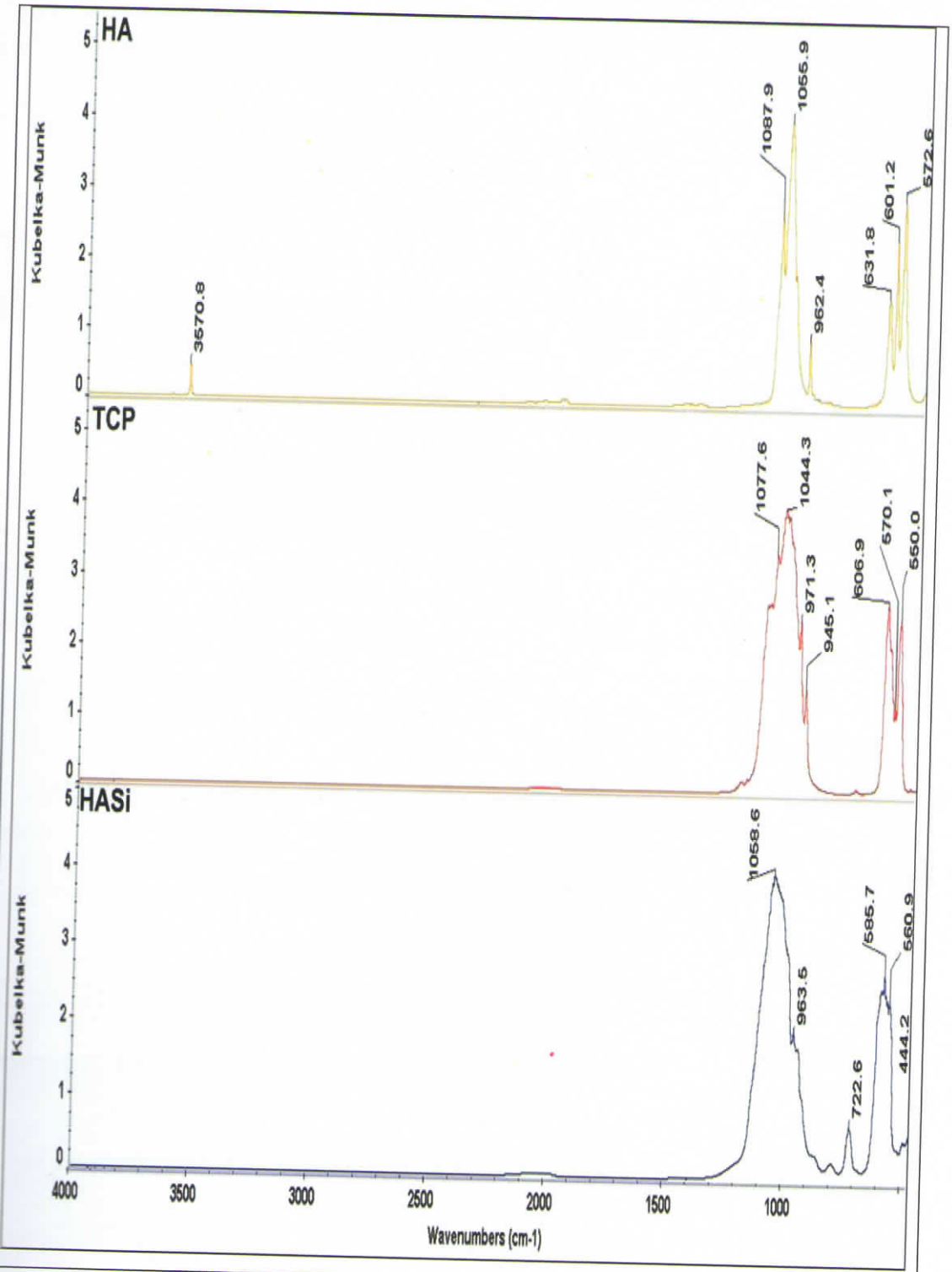


Fig. 11 – FTIR of HA HASi and TCP

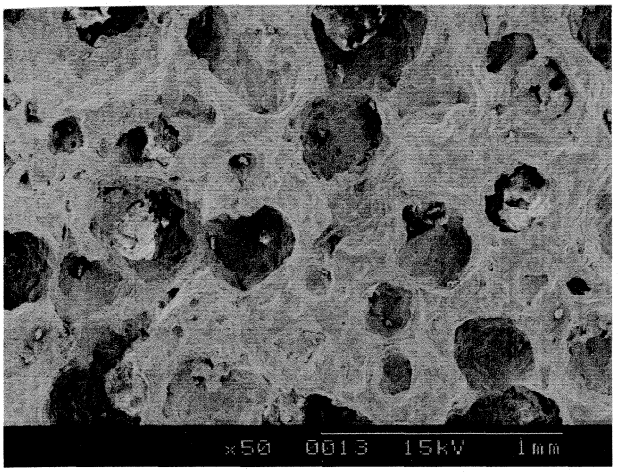


Fig. 9 (a) SEM image of HA

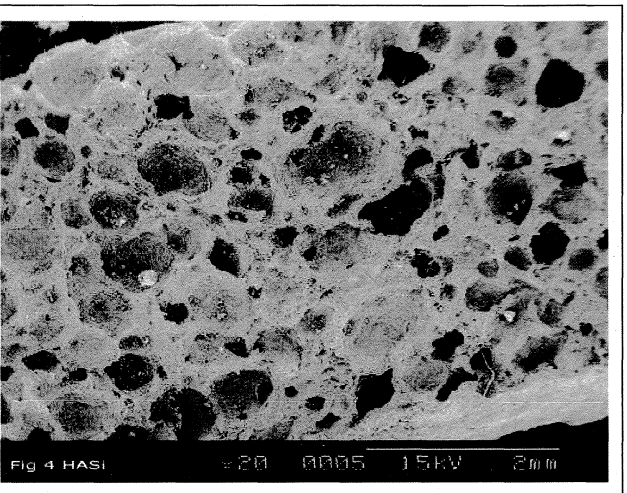


Fig. 9(b) SEM image of HASi

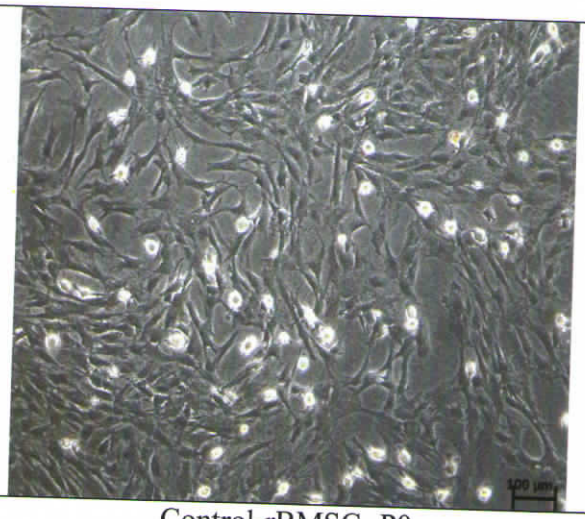
silicate, tricalcium phosphate and hydroxyapatite. Results show the presence of triphasic ceramic elements within the system (Fig 10).

2.3 FTIR:

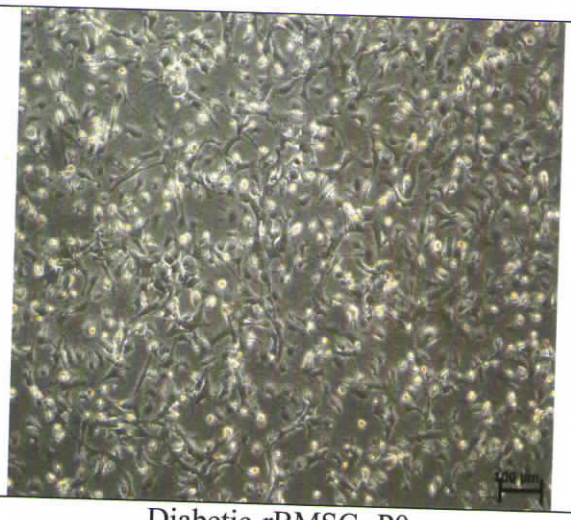
The FTIR spectra (Figure 11) obtained showed the characteristic absorption band corresponding to a hydroxyapatite phase. The bands at 3570.8 cm^{-1} can be attributed to hydroxyl groups, whereas bands at 1190–976 cm^{-1} and 660– 520 cm^{-1} correspond to phosphate groups. In HASi, the peak at 722.6 cm^{-1} corresponds to silica.

3 Isolation and Expansion of mesenchymal stem cells (MSC's):

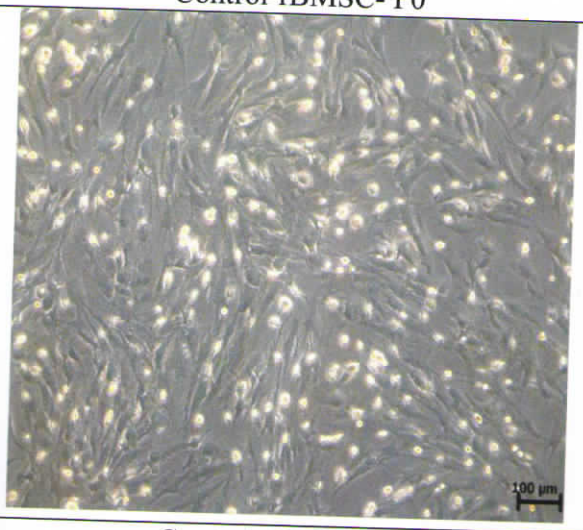
At the end of the experimental period, the animals (both Diabetic and control) were euthanized, femur and tibia was atriculated for rBMSC isolation and fat from subcutaneous fat pad of the animal were dissected out for rADMSC isolation. All the procedures were carried out based on Institutional Animal Ethics Committee (IAEC) Mesenchymal stem cells, or MSCs, are multipotent stem cells that have been isolated from multiple tissue sites. Among them, bone marrow (BM) and adipose are commonly used for therapeutic applications as they are easily accessible and expandable in culture. In the current study MSCs have been isolated from two different source, from both diabetic and control animals to evaluate its potential to differentiate and proliferate *in vitro.* Both rBMSC and rADMSC were isolated by means of their physical propensity to adhere to the plastic substrate of the cell culture plate. The non-adherent cells were removed during the first medium change after 12-24 hours. The control cells became confluent by 7 days in culture, whereas the diabetic rBMSCs became confluent by 11 days from the day of isolation. Similarly the control rADMSC became confluent by 5-6 days from the day of isolation, whereas the diabetic rADMSC became confluent by 8-10 days from the day of isolation. Phase contrast images of both control and diabetic rBMSCs and rADMSC are shown in figure 12 and figure 13 respectively.



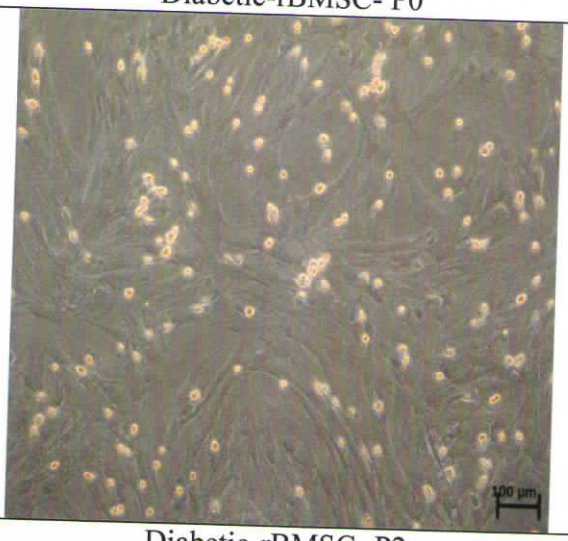
Control-rBMSC- P0



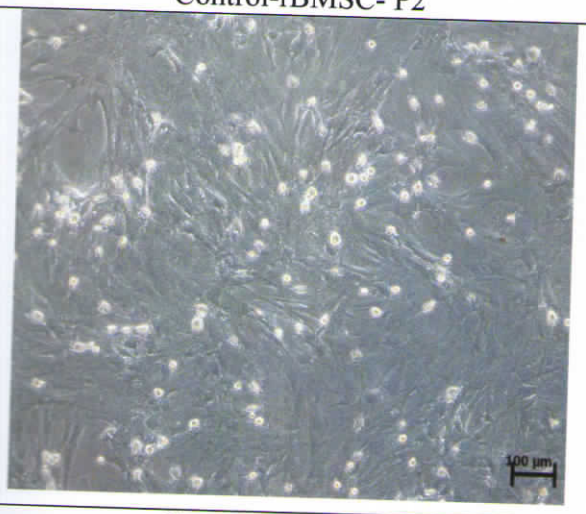
Diabetic-rBMSC- P0



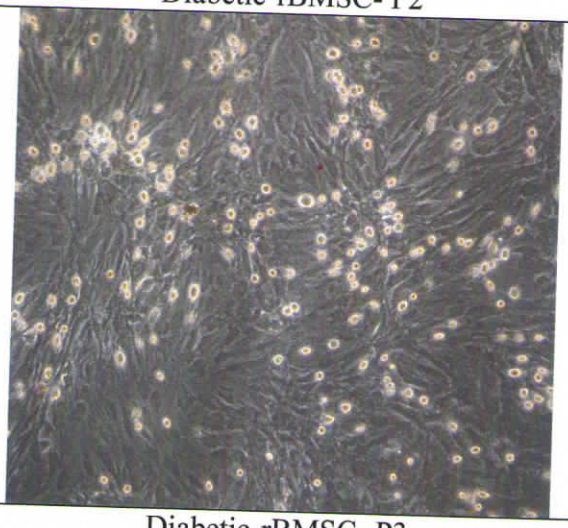
Control-rBMSC- P2



Diabetic-rBMSC- P2

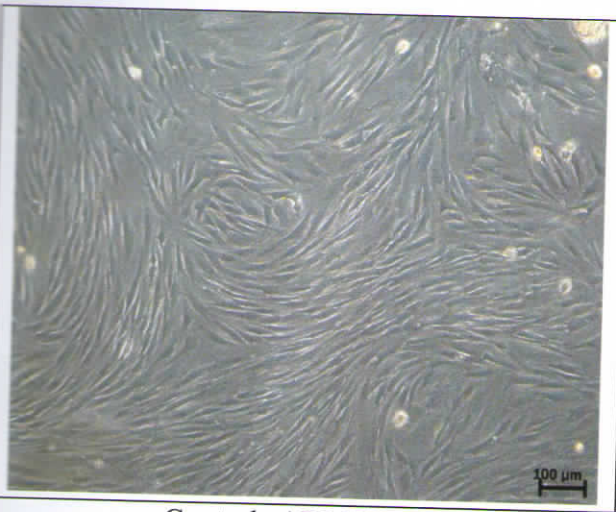


Control-rBMSC- P3

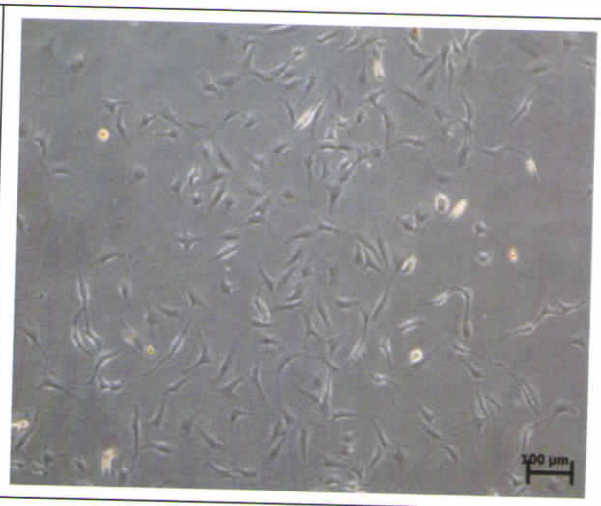


Diabetic-rBMSC- P3

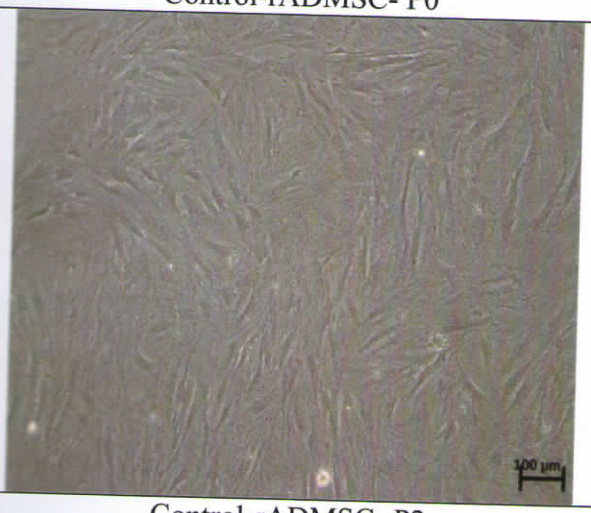
Figure 12- rat Bone marrow derived mesenchymal stem cells P0- isolation flasks, P2 used for invitro studies and P3 used for differentiation studies



Control-rADMSC- P0

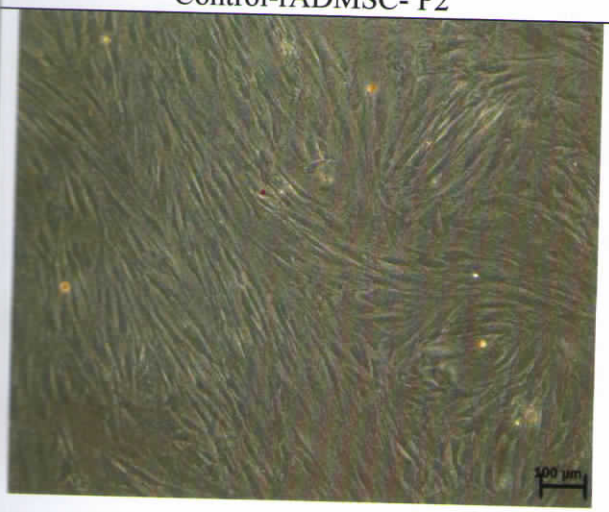


Diabetic-rADMSC- P0

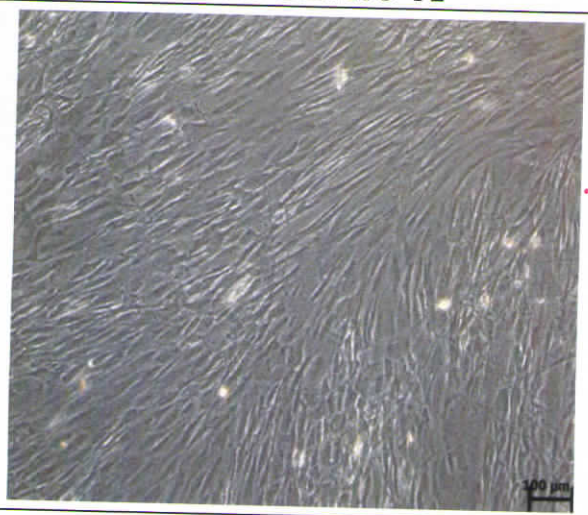


Control-rADMSC- P2

Diabetic-rADMSC- P2



Control-rADMSC- P3

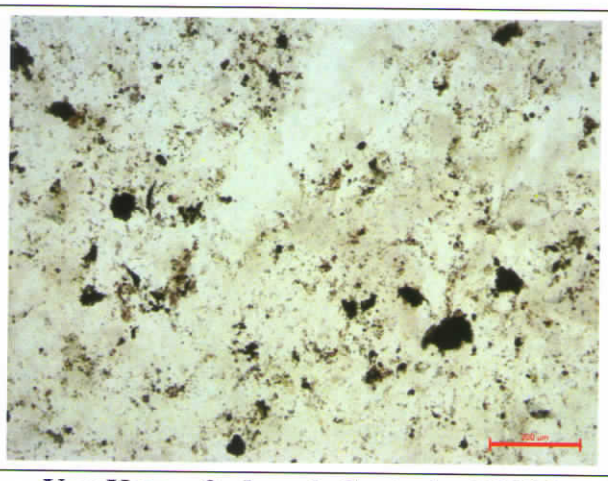


Diabetic-rADMSC- P3

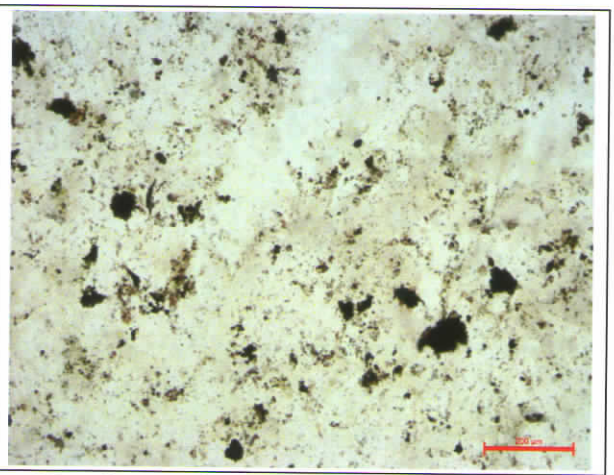
Figure 13- rat Bone marrow derived mesenchymal stem cells P0- isolation flasks, P2 used for i*n vitro* studies and P3 used for differentiation studies

4 Differentiation of rBMSCs and rADMSCs (both control and diabetic) to the Osteogenic Lineage:

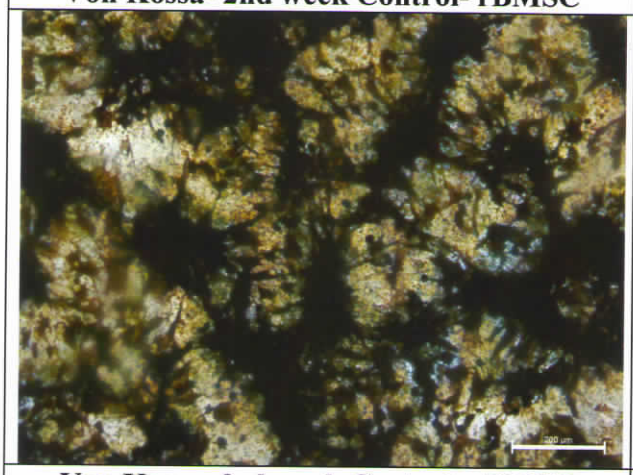
Both rBMSC and rADMSC cells were grown on cover slips and induced to osteogenic lineage with osteogenic medium maintained for 28 days. The fixed cell seeded coverslips were thereafter stained with Alizarin red and Von kossa. Micrographs of differentiated rBMSCs stained for mineralization nodules by Von Kossa and Alizarin red staining was shown in figure 14 and 15 respectively. Similarly figure 16 and 17 represents micrographs of Von Kossa and Alizarin red staining of differentiated rADMSCs. The addition of osteogenic supplements to the culture-expanded MSCs can lead to the differentiation of cells into osteogenic lineage *in vitro,* as visualized by the formation of collagen-rich matrix, increased expression of non-collagenous proteins like ALP, osteocalcin and finally by the formation of mineralized matrix [Kern S *et al.,* 2006]. Deposition of abundant mineralized matrix comprising of calcium and phosphate can be microscopically visualized by Alizarin red and Von kossa staining. Calcium deposition was stained orange-red in Alizarin staining whereas the Von kossa depicted black phosphate deposits in the matrix. The stain works by a precipitation reaction in which silver ions react with phosphate in the presence of acidic material. revealed similar mineralization pattern *in vitro* indicating effective differentiation compared to the rADMSC cells. Cells in non-osteogenic medium were stained after specific period as control samples (Figure 18). Finally photochemical degradation of silver phosphate to silver under UV illumination will result in black colouration [Meloan SN, Puchtler H, 1985], which is observed in both the control and diabetic cells.



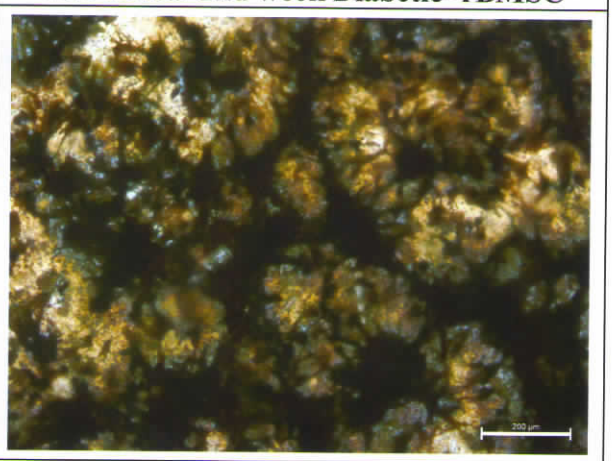
Von Kossa- 2nd week Control- rBMSC



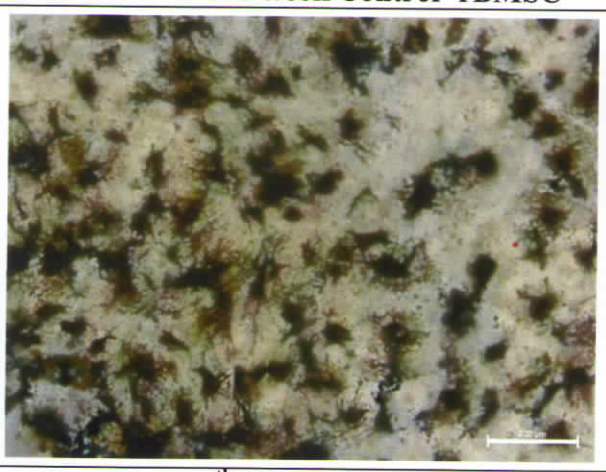
Von Kossa- 2nd week Diabetic- rBMSC



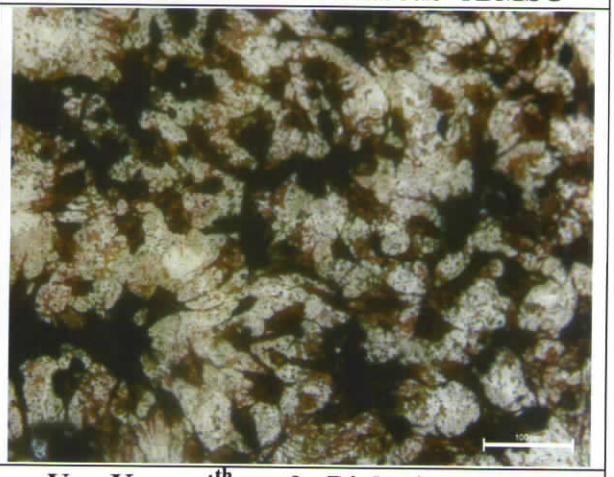
Von Kossa- 3rd week Control- rBMSC



Von Kossa- 3rd week Diabetic- rBMSC

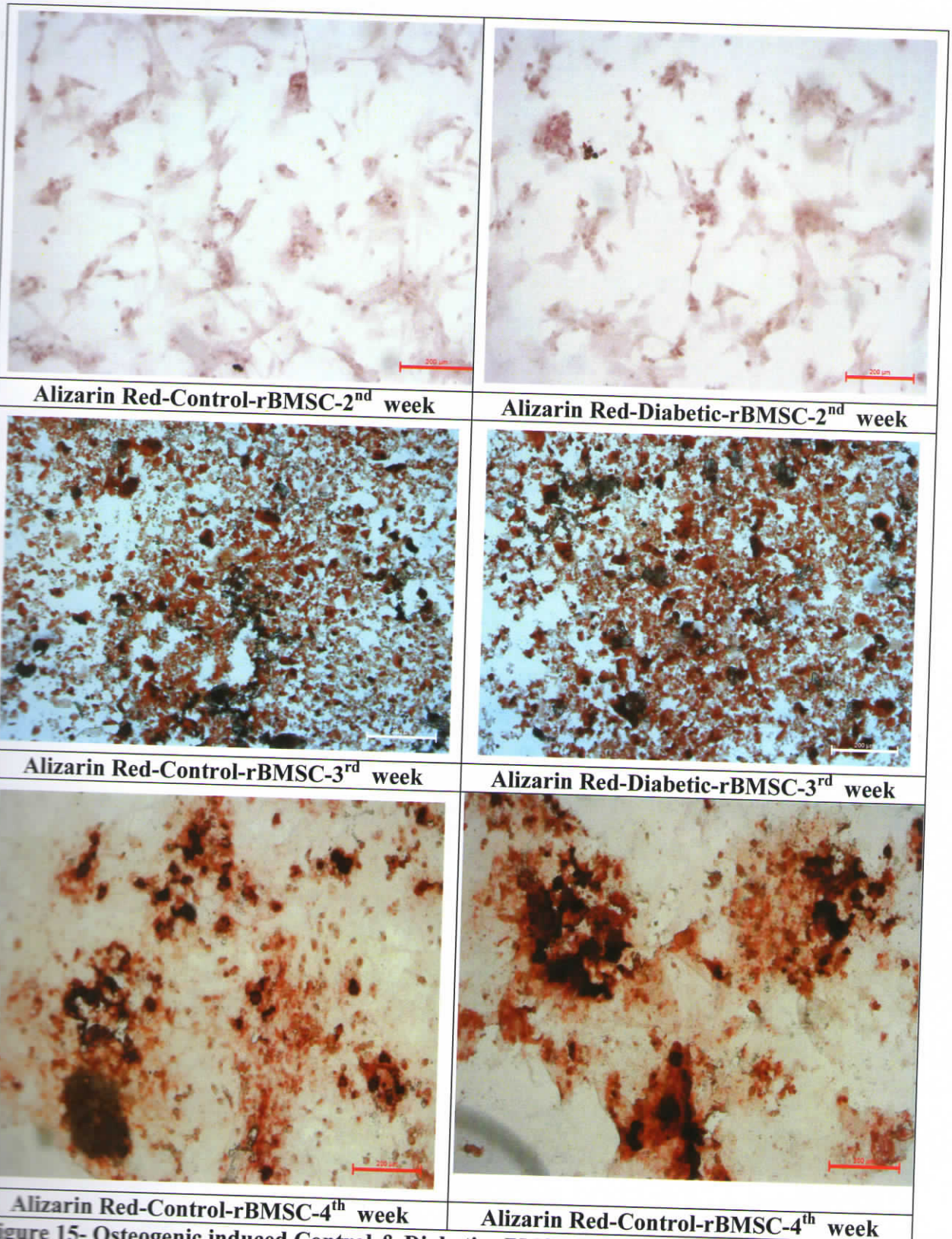


Von Kossa-4th week- Control-rBMSC



Von Kossa-4th week- Diabetic-rBMSC

Figure 14- Osteogenic induced Control and Diabetic rBMSC, stained for mineralized nodules (Von Kossa staining)



Alizarin Red-Control-rBMSC-2nd week

Alizarin Red-Diabetic-rBMSC-2nd week

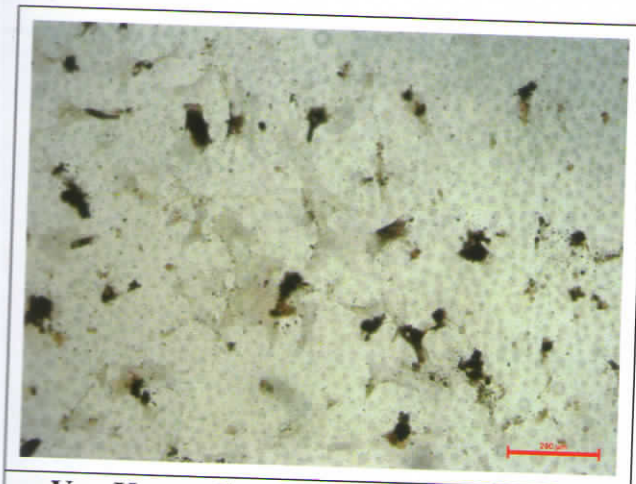
Alizarin Red-Control-rBMSC-3rd week

Alizarin Red-Diabetic-rBMSC-3rd week

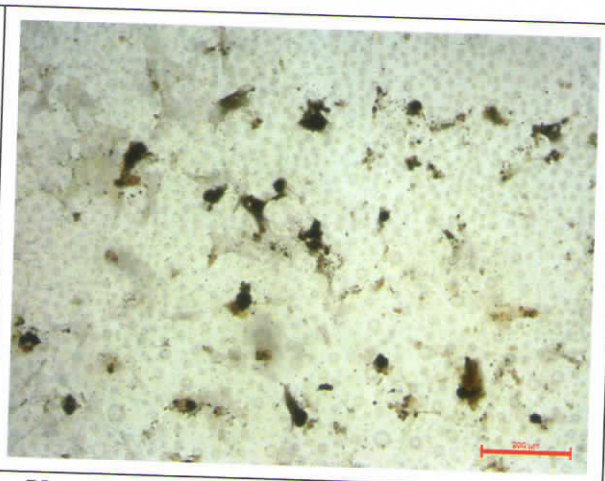
Alizarin Red-Control-rBMSC-4th week

Alizarin Red-Control-rBMSC-4th week

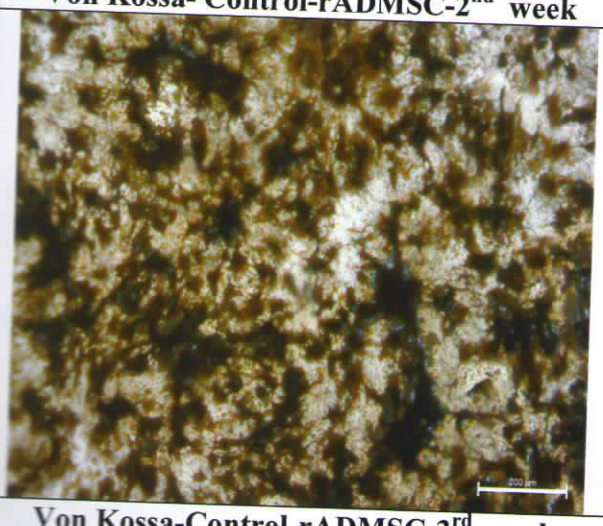
Figure 15- Osteogenic induced Control & Diabetic rBMSC, stained for mineralized nodules (Alizarin red staining)



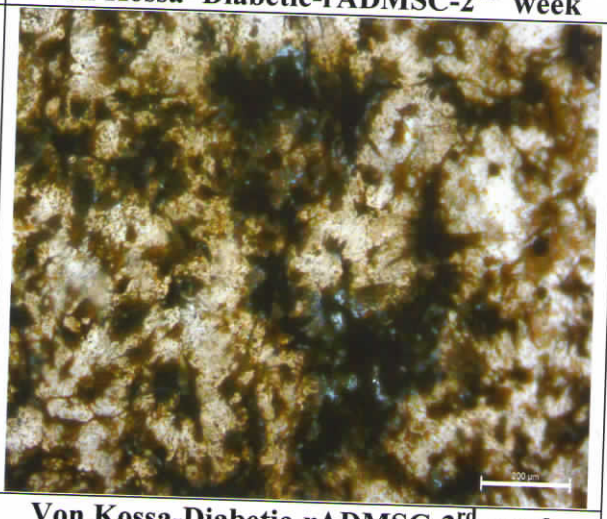
Von Kossa- Control-rADMSC-2nd week



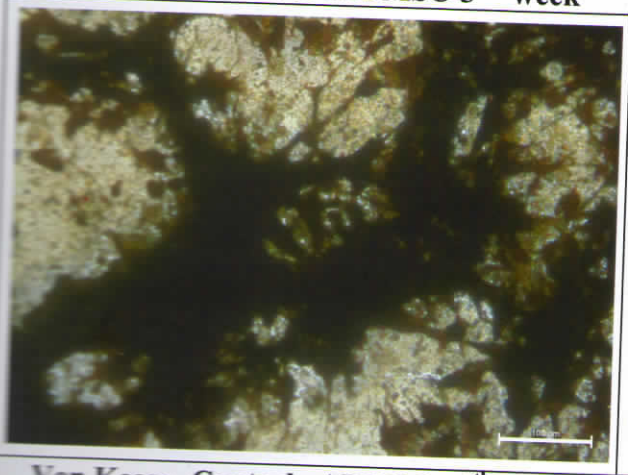
Von Kossa- Diabetic-rADMSC-2nd week



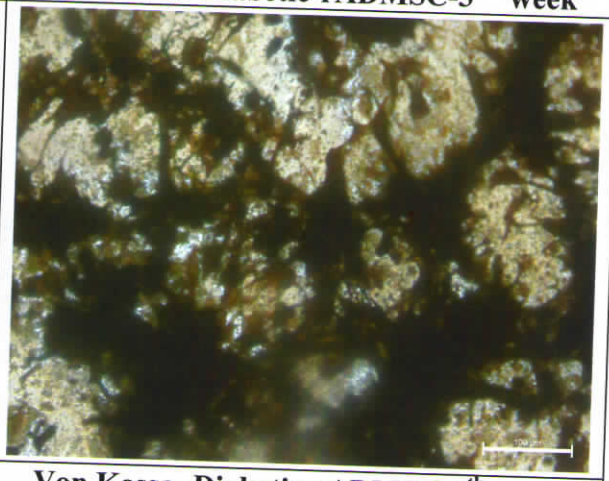
Von Kossa-Control-rADMSC-3rd week



Von Kossa-Diabetic-rADMSC-3rd week

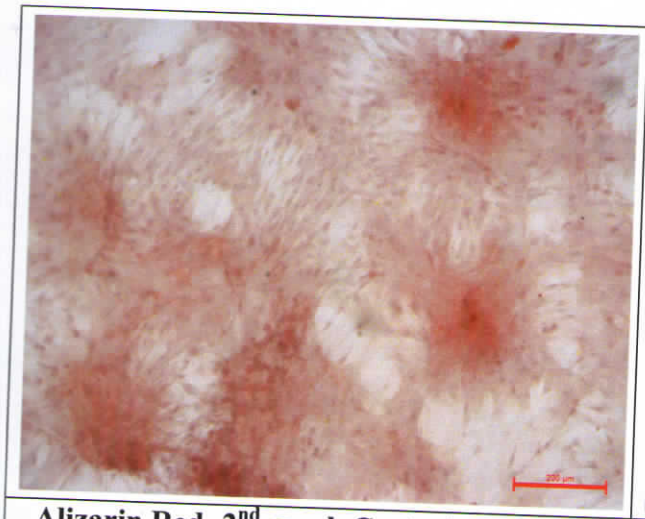


Von Kossa- Control-rADMSC- 4th week

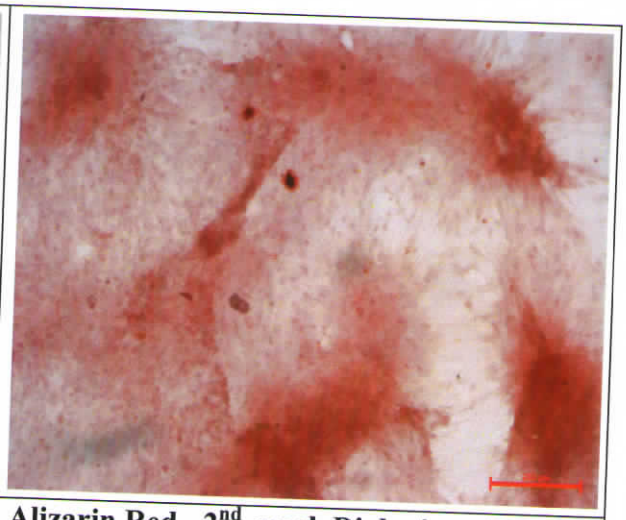


Von Kossa- Diabetic-rADMSC-4th week

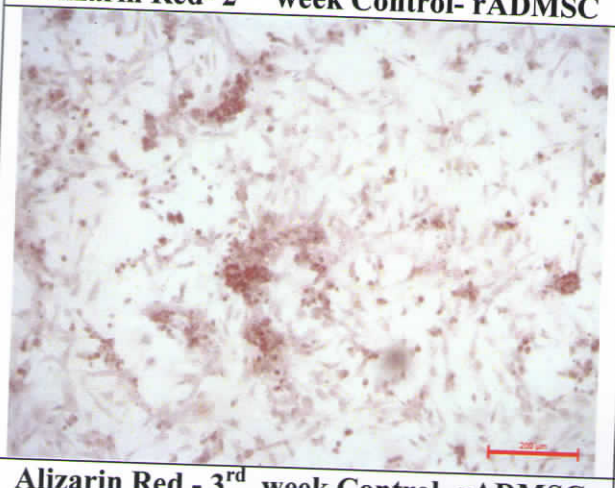
Figure 16- Osteogenic induced Control and Diabetic rADMSC, stained for mineralized nodules (Von Kossa staining)



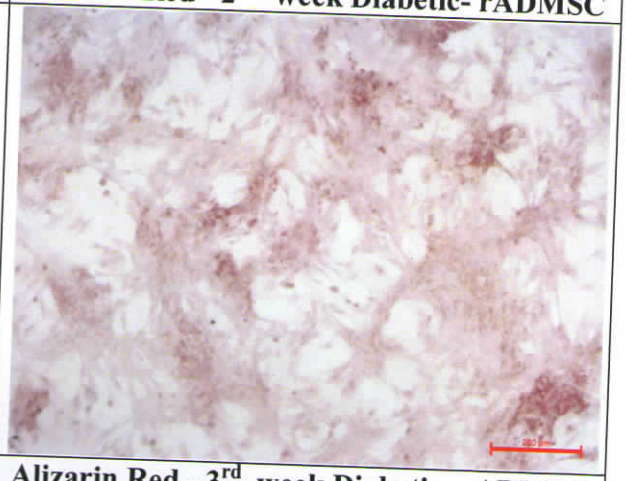
Alizarin Red- 2nd week Control- rADMSC



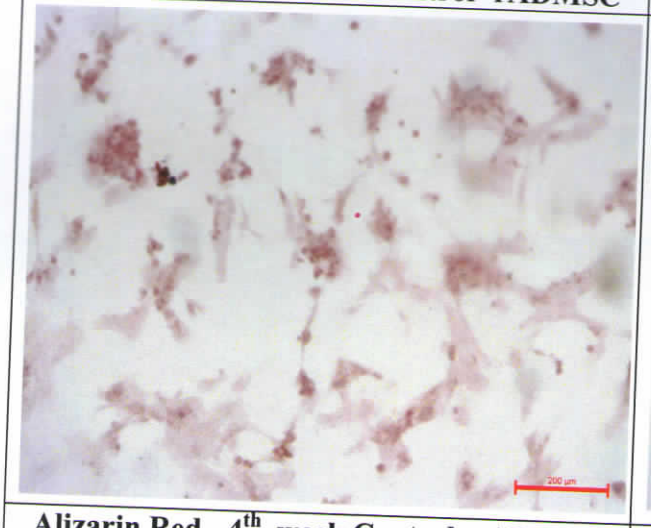
Alizarin Red - 2nd week Diabetic- rADMSC



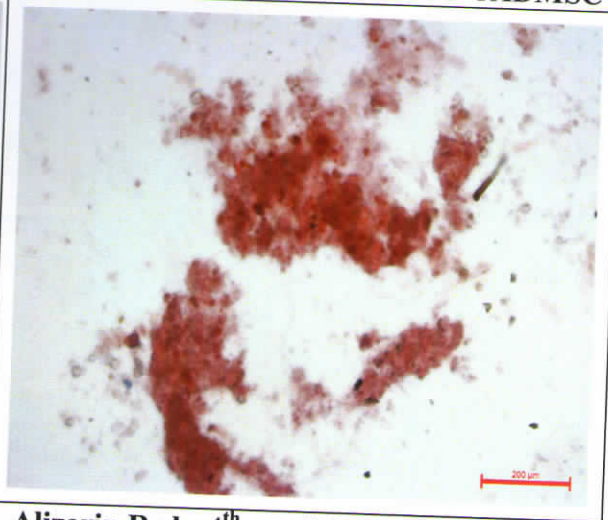
Alizarin Red - 3rd week Control- rADMSC



Alizarin Red - 3rd week Diabetic- rADMSC

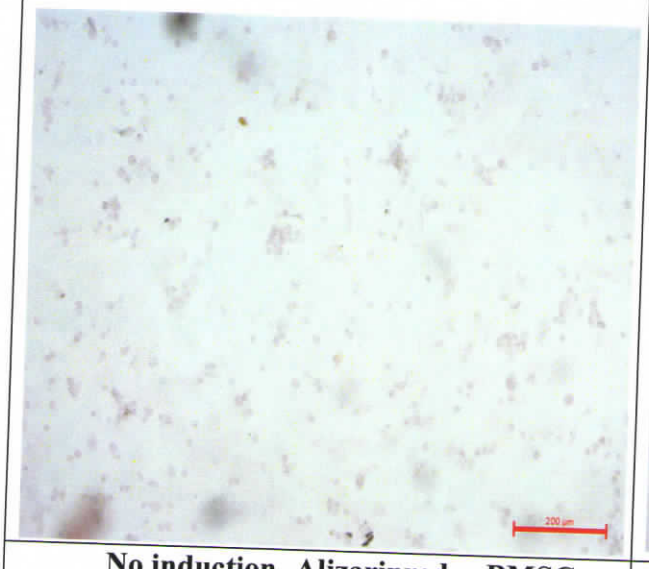


Alizarin Red - 4th week Control- rADMSC

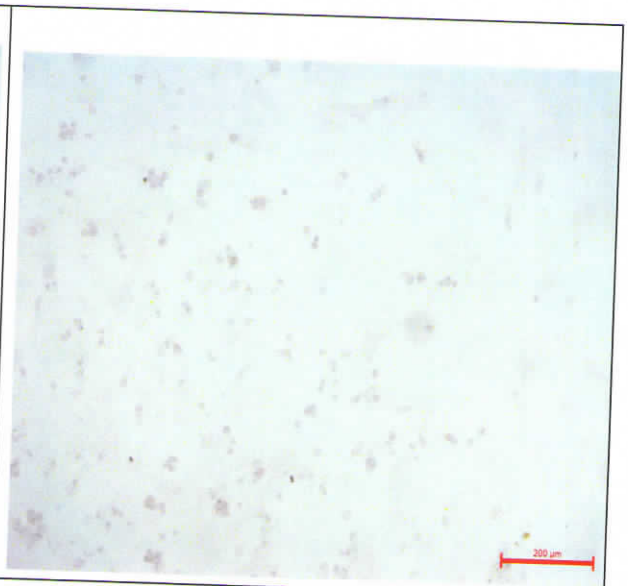


Alizarin Red - 4th week Control- rADMSC

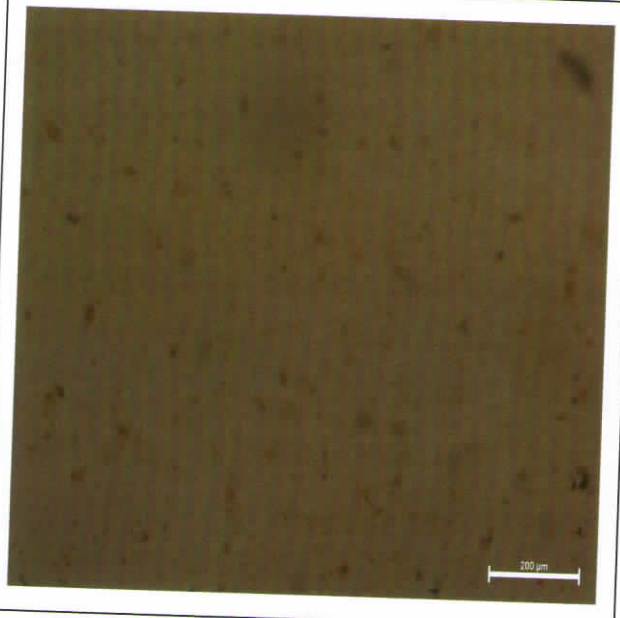
Figure 17- Osteogenic induced Control and Diabetic rADMSC, stained for mineralized nodules (Alizarin Red staining)



No induction- Alizarinred –rBMSC



No induction-Alizarin-rADMSC



No induction -Von kossa - rBMSC

No induction - Von Kossa - rBMSC

Figure 18- Cells without osteo induction-Stained for Alizarin red and Von Kossa

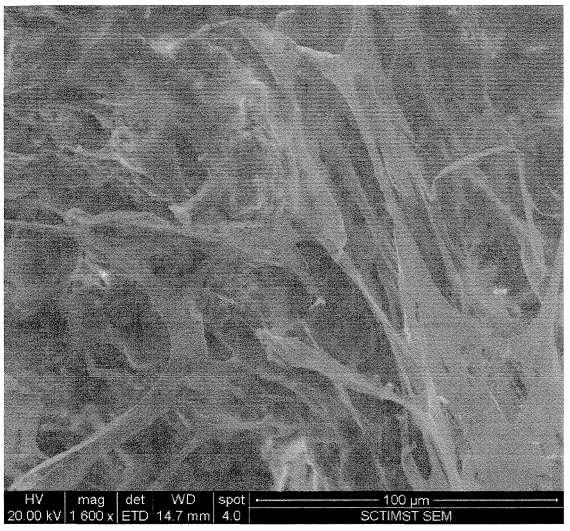
5 Evaluation of the cell seeded construct:

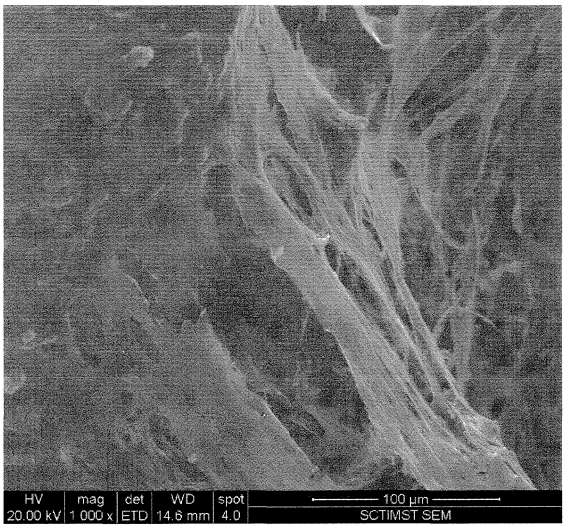
5.1 Cytocompatability:

5.1.1 Cell adhesion of rBMSC and rADMSC (both control and diabetic):

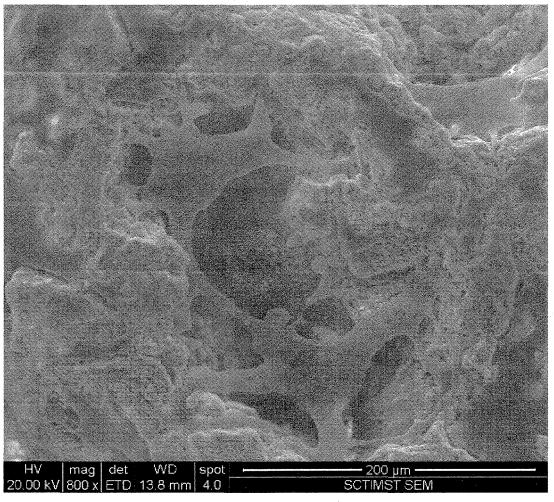
The cells (rBMSC and rADMSC (both control and diabetic) seeded scaffolds (both HA and HASi), were evaluated after 28 days in culture using Environmental Scanning Electron Microscope (ESEM) (FEI Quanta 200). ESEM images of fabricated cell construct, Control and diabetic rADMSCs on HASi was shown in figure 19; Control and diabetic rADMSCs on HA was shown in figure 20; Control and diabetic rBMSCs on HASi was shown in figure 21; Control and diabetic rBMSCs on HA was shown in figure 22 respectively. Adhesion of cells on materials is very essential as it influences their ability to proliferate and differentiate. The constructs were designed as a three-dimensional scaffold promoting controlled ingrowth and maturation of cells. The adhesion of cells on material depends on the initial adsorption of serum proteins like vitronectin, fibronectin, collagen or laminin. These ECM molecules arbitrate functional receptor mediated and signal transmitting cell adhesion on conventional biomaterial. [Anselme K 2000] These molecules can be adsorbed on the material surface from the surrounding environment, such as cell culture media *in vitro* or body fluids *in vivo.* A number of recent studies have shown that the binding of integrins to extracellular matrix proteins results in the activation of signaling pathways including those which result in the tyrosine phosphorylation of focal adhesion kinase (FAK) in several cell types and particularly in osteoblasts [Hildebrand *et al.,* 1995]. Thus activation of FAK is thought to play a critical role in control of adhesion-dependent cell survival and proliferation. Synthetic calcium phosphate or hydroxyapatite (HA) has been shown to be quite similar to the natural component of bone. The surface chemistry of silica-based materials is acquiescent to very sturdy irreversible adsorption of serum proteins which is attributed to high negative-surface-charge density generated by surface silinol (Si-OH) which in turn binds to various functional groups of a protein.[Lobel *et al.,* 1997]

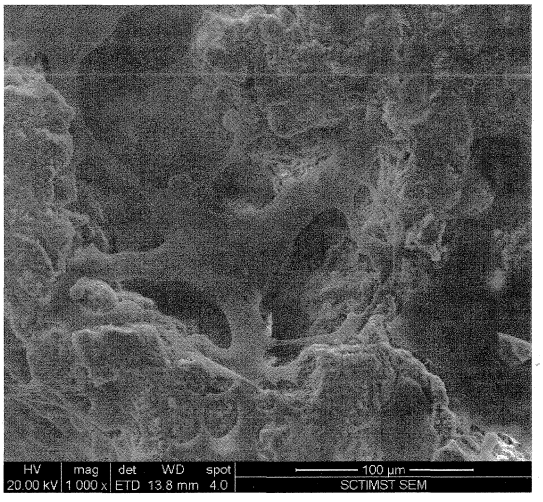
Scanning electron micrographs of cell seeded scaffolds, revealed that both diabetic and control rBMSCs as well as rADMSCs adhered and expanded over HA and HASi





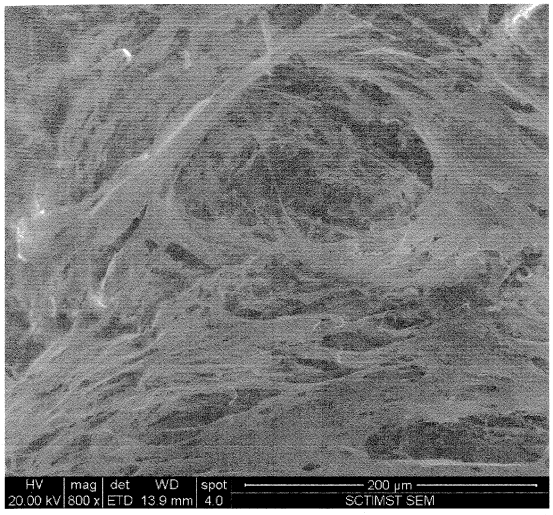
HASi-C1-rADMSC-4th week

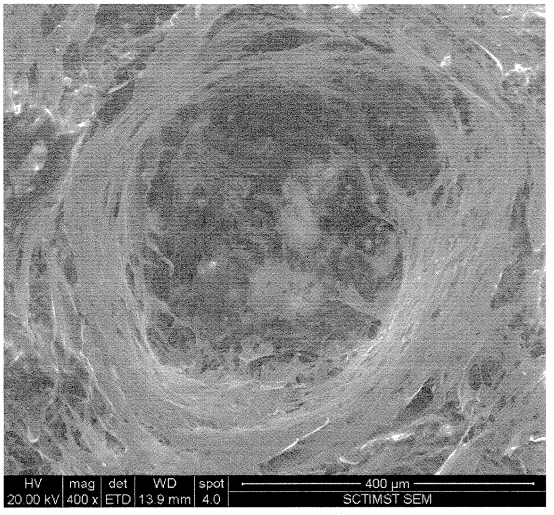




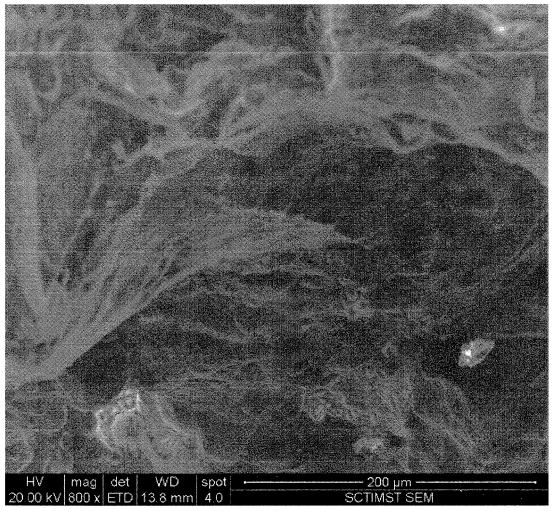
HASi-D1-rADMSC-4th week

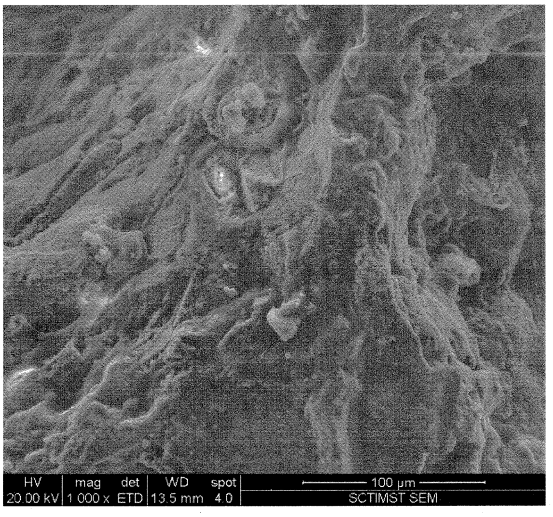
Figure 19- ESEM of cell-seeded scaffolds C1- Control cells and D1 diabetic cells, rADMSC refers to rat Adipose derived Mesenchymal stem cells, and HASi refers to Silica coated hydroxy apatite scaffold





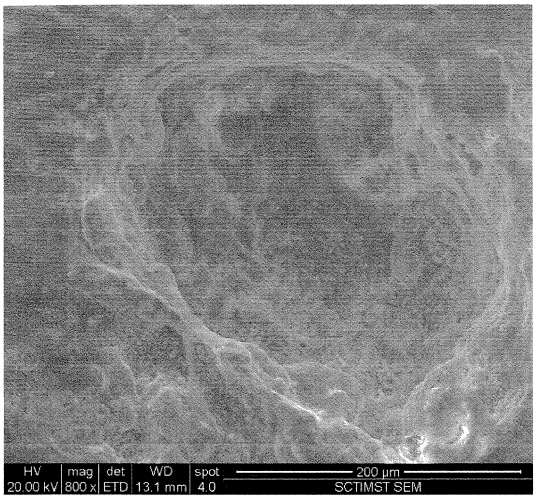
HA-C1-rADMSC-4th week

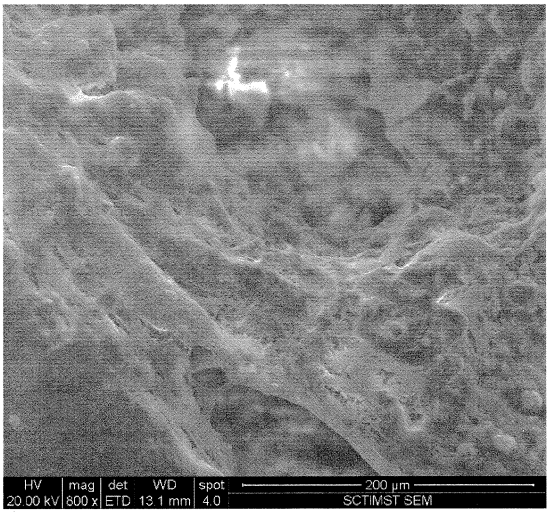




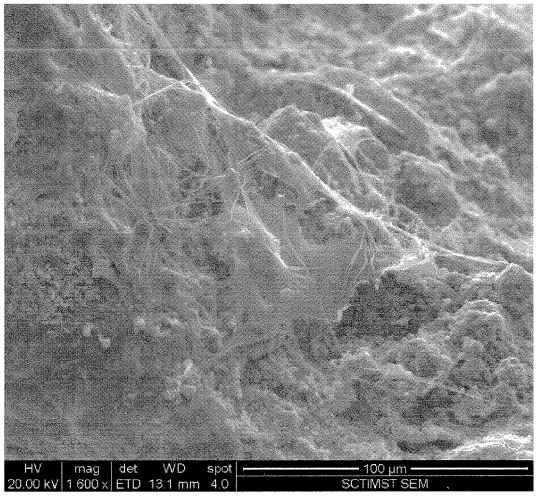
HA-D1-rADMSC-4th week

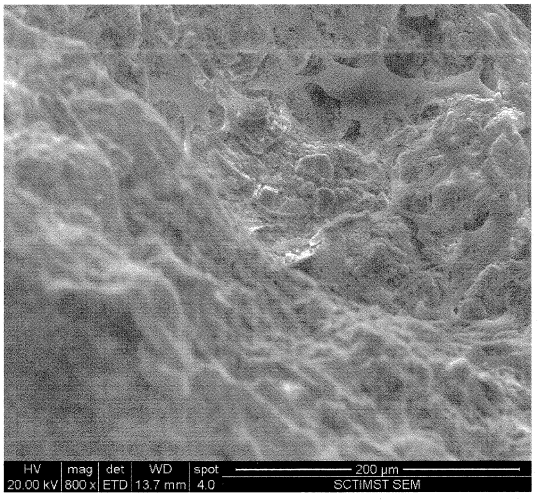
Figure 20- ESEM of cell seeded scaffolds C1- Control cells and D1 diabetic cells, rADMSC refers to rat Adipose derived Mesenchymal stem cells, and HA refers to hydroxy apatite scaffold





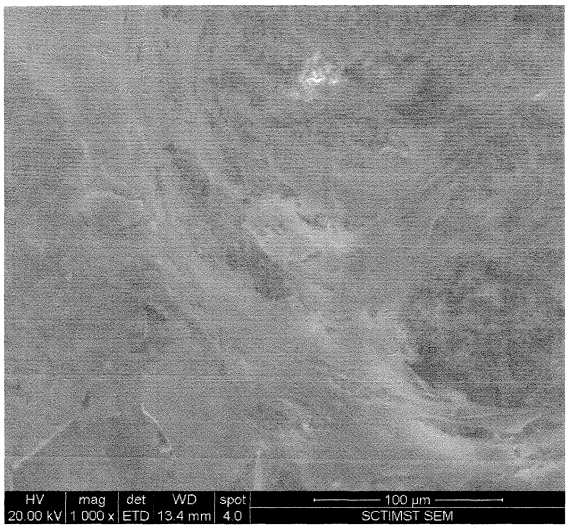
HASi-C1-rBMSC-4th week

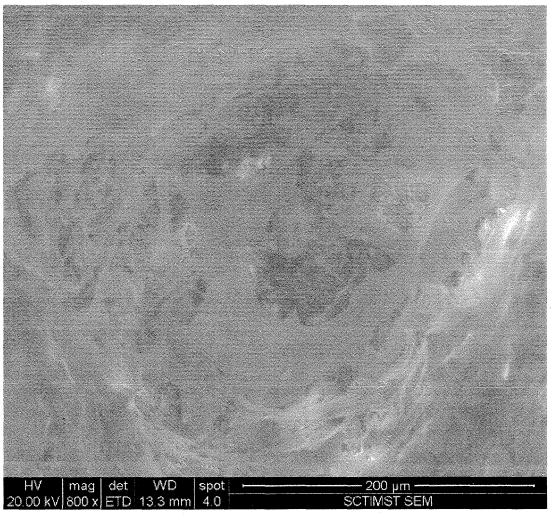




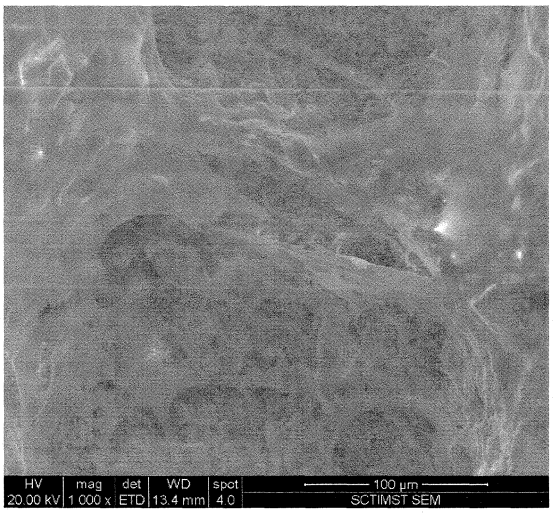
HASi-D1-rBMSC-4th week

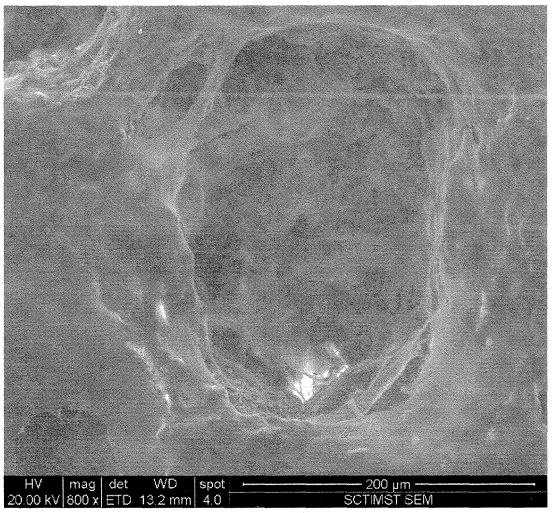
Figure 21- ESEM of cell-seeded scaffolds C1- Control cells and D1 diabetic cells, rBMSC refers to rat Bone marrow derived Mesenchymal stem cells, and HASi refers to Silica coated hydroxy apatite scaffold





HA-C1-rBMSC-4th week





HA-D1-rBMSC-4th week

Figure 22- ESEM of cell seeded scaffolds C1- Control cells and D1 diabetic cells, rBMSC refers to rat Bone marrow derived Mesenchymal stem cells, and HA refers to hydroxy apatite scaffold

scaffolds. Cellular adhesion follows a pattern with initial spherical cells further flattened and later well stretched out so that the nucleus becomes prominent [Malik *et al.,* 1992]. In our study also the cells evolved to form a sheet-like canopy over the entire surface of the scaffolds within 28 days of culture. As per the earlier reports if the pores are occluded the diffusion of the nutrients will be limited towards the interior of the scaffold [Salgado *et al.,* 2004]. This active cell growth and expansion without any pore occlusion affirmed the favorable cell friendly nature of the scaffold.

Qualitative comparison of control and diabetic cell populations from Bone marrow/Adipose tissue revealed no relative difference. Interpreting the literature survey indicates that the bone marrow microenvironment faces the brunt of stress induced (AGE / RAGE accumulation) under diabetic conditions. Hence assessing bone marrow derived stem cells would provide clear picture of the effect of a systemic pathology on the vital nature of the cells. .

6. Determination of Population doubling time of rBMSC:

Extensive subcultivation can alter the growth kinetics as well as the phenotype of cells [Bruder SP *et al.,* 1997], The purpose of this study was to determine the proliferative potential of rBMSC of both control and diabetic cells and the effect of serial passaging on the proliferation potential of both control and diabetic rBMSCs. Cells derived from each passage were assayed for their kinetics of growth. The results substantiate that diabetic rBMSCs possessed impaired expansion and differentiation potential compared to that of the control rBMSC. Control rBMSCs in passages 1 to 6 reached confluence on day 4-5 in 6 well Tissue culture plates (TCPs) and 25 cm^2 TCPs flasks. In later consecutive passages, the time required for the expansion of cells increased resulting in less number of cells on day 4 compared to that of the previous passages, indicating a decline in growth rate. Whereas diabetic rBMSCs showed impaired proliferation compared to that of the control rBMSCs from the beginning, it took about 7 days to become confluent in 6 well Tissue culture plates (TCPs) and 25 cm^2 TCPs flasks. Data of population doubling no and time are shown in table 8 and 9

Passage No	Population doubling number							
Control rBMSCs	C1	C2	C3	C4	C5	C6	Mean	SD
P1	1.48	1.43	1.37	1.67	1.72	1.84	1.59	0.18
P2	1.54	1.50	1.56	1.76	1.86	1.88	1.68	0.17
P3	1.65	1.69	1.72	1.94	1.94	1.96	1.82	0.14
P4	1.80	1.76	1.76	1.96	2.03	2.07	1.90	0.14
P5	1.96	1.80	1.72	2.06	2.08	2.13	1.96	0.16
P6	1.80	1.72	1.63	1.99	1.97	1.96	1.85	0.15
P7	1.60	1.58	1.53	1.67	1.72	1.86	1.66	0.11
P8	1.53	1.43	1.48	1.48	1.53	1.45	1.48	0.04
P9	1.37	1.35	1.20	1.26	1.37	1.32	1.31	0.06
Diabetic rBMSCs	D1	D2	D3	D4	D5	D6	Mean	SD
P1	0.92	1.05	1.08	1.30	1.25	1.19	1.13	0.14
P2	1.10	1.17	1.23	1.23	1.47	1.48	1.28	0.15
P3	1.36	1.40	1.40	1.31	1.43	1.40	1.38	0.04
P4	1.26	1.37	1.35	1.20	1.26	1.43	1.31	0.08
P5	1.14	1.20	1.07	1.07	1.20	1.32	1.17	0.09
P6	1.00	1.07	1.00	1.07	1.13	1.23	1.08	0.08
P7	0.92	0.84	0.99	0.76	0.84	0.92	0.88	0.08
P8	0.76	0.58	0.67	0.48	0.63	0.68	0.63	0.09
P9	0.58	0.48	0.38	0.37	0.48	0.58	0.48	0.09

Table 8 - Population doubling no of rBMSC, (Control rBMSCs- C1, C2, C3 are triplicates of single control animal. Similarly C4, C5 and C6 are triplicates of single control animal; Diabetic rBMSCs- D1, D2, D3 are triplicates of single diabetic animal. Similarly D4, D5, D6 are triplicates of single diabetic animal).

Passage No	Population doubling time							
Control rBMSCs	C1	C2	C3	C4	C5	C6	Mean	SD
P1	3.38	3.50	3.64	3.00	2.90	2.72	3.19	0.36
P2	3.25	3.34	3.21	2.84	2.69	2.67	3.00	0.30
P3	3.02	2.95	2.90	2.58	2.58	2.58	2.77	0.20
P4	2.78	2.84	2.84	2.56	2.47	2.47	2.66	0.17
P5	2.56	2.78	2.91	2.42	2.40	2.40	2.58	0.21
P6	2.78	2.91	3.07	2.51	2.53	2.53	2.72	0.23
P7	3.12	3.17	3.27	3.00	2.91	2.91	3.06	0.14
P8	3.27	3.50	3.38	3.38	3.27	3.27	3.35	0.09
P9	3.64	3.72	4.18	3.97	3.64	3.64	3.80	0.22
Diabetic rBMSCs	D1	D2	D3	D4	D5	D6	Mean	SD
P1	7.57	6.61	6.48	5.36	5.59	5.88	6.25	0.81
P2	6.36	5.97	5.70	5.67	4.76	4.73	5.53	0.65
P3	5.12	5.00	5.00	5.34	4.90	5.00	5.06	0.15
P4	5.56	5.10	5.20	5.85	5.56	4.90	5.36	0.35
P5	6.18	5.85	6.56	6.56	5.85	5.31	6.05	0.48
P6	7.03	6.56	7.03	6.56	6.18	5.70	6.51	0.51
P7	7.59	8.28	7.03	9.18	8.28	7.59	7.99	0.75
P8	9.18	12.01	10.36	14.47	11.11	10.36	11.25	1.83
P9	12.01	14.47	18.56	18.56	14.47	12.01	15.01	2.95

Table 9 - Population doubling time of rBMSCs (Control rBMSCs- C1, C2, C3 are triplicates of single control animal. Similarly C4, C5 and C6 are triplicates of single control animal; Diabetic rBMSCs- D1, D2, D3 are triplicates of single diabetic animal. Similarly D4, D5, D6 are triplicates of single diabetic animal)

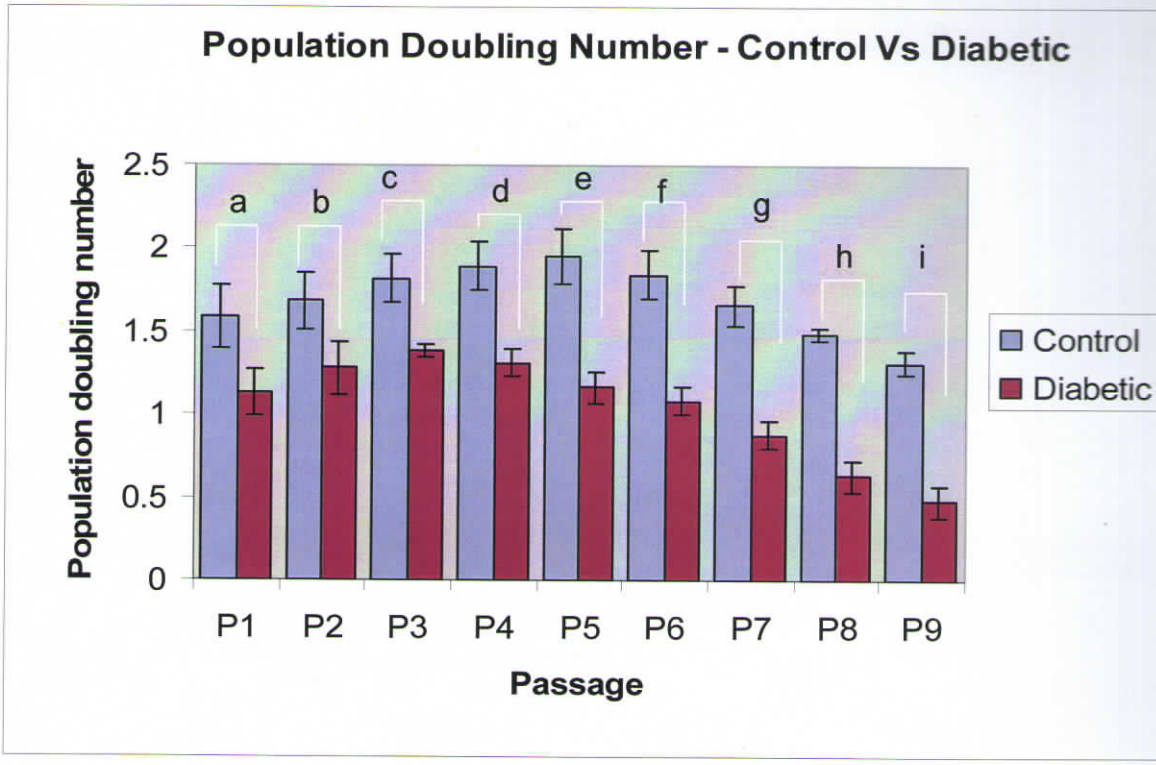


Figure 23- Population Doubling Number of rBMSC cells from control and Diabetic rats (N=6; P Values (a= 0.0008, b= 0.0017, (c,d,e,f,g,h,i) = 0.0001)), The P Value calculated by student t test shows significant difference between control and diabetic rBMSCs.

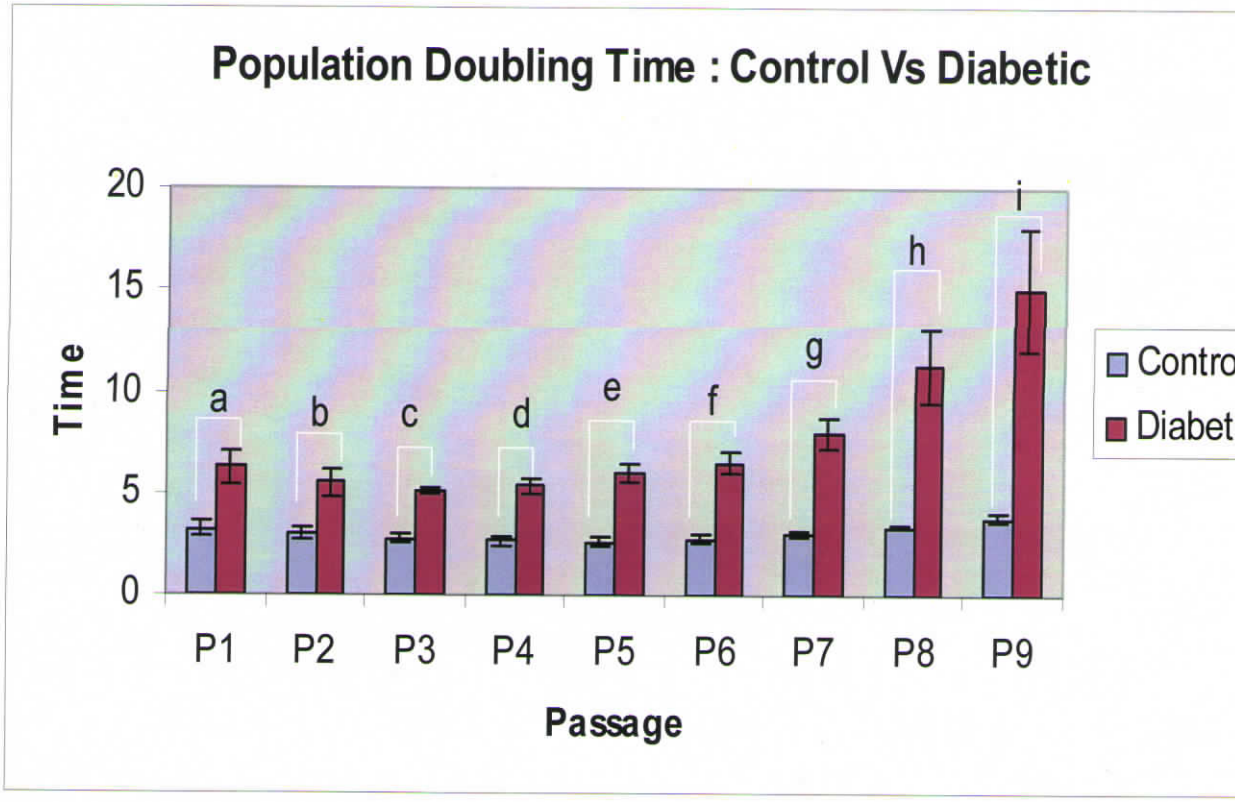


Figure 24- Population Doubling Time of rBMSC cells both control and Diabetic rats (N= 6; P Values (a,b,c,d,e,f,g,h,i) = 0.0001)), The P Value calculated by student t test shows significant difference between control and diabetic rBMSCs.

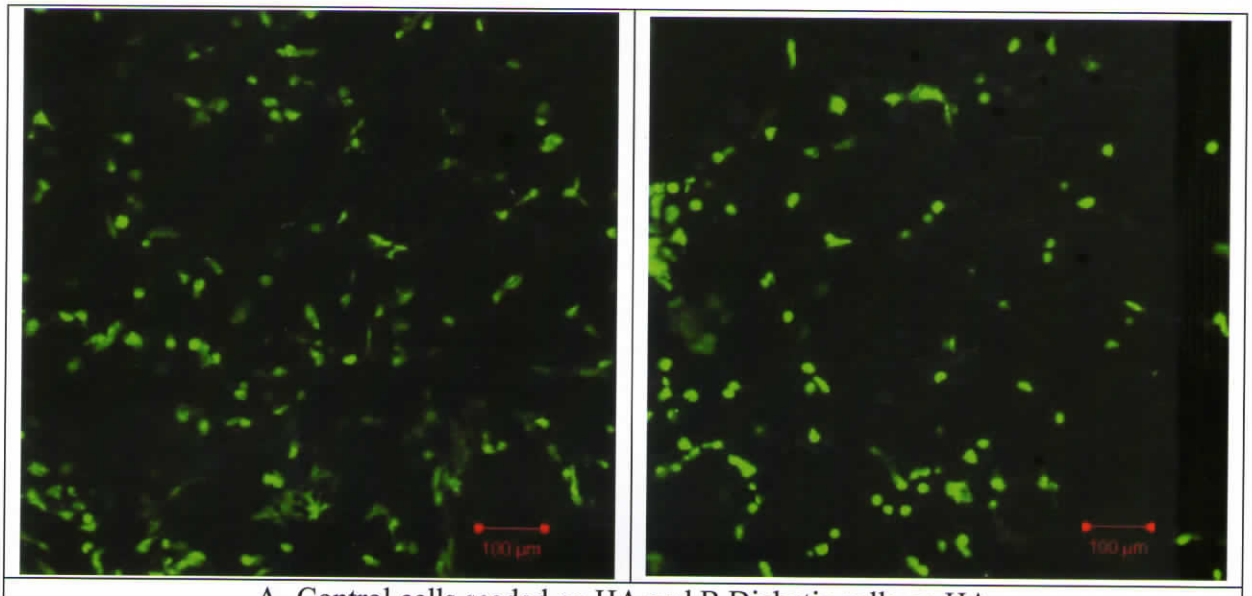
respectively. The P value (0.0001) calculated using student t test shows significant difference in the population doubling time of control and diabetic rBMSCs. This data suggests that the proliferation rate of diabetic rBMSC was low compared to that of the control rBMSC, and the decline is more evident from passage 5. This may be due to the accumulation of AGE, in the bone microenvironment of the diabetic animal. Stolzing *et al.*, have reported elevated level of AGEs in MSC derived from diabetic rats. AGEs is found to inhibit MSC growth and differentiation [S Kume *et al.*, 2005]. Moreover high glucose concentration is shown to impair the proliferation, *differentiation of rBMSC*, by an *in vitro* study [Gopalakrishnan *et al.*, 2006]. In light of these reports, it is reasonable to state that the hyperglycemic condition of the diseased animal is responsible for impaired cellular proliferation and growth. This may also be due to an increase in AGE concentration or apoptosis and (or) cell-cycle arrest as reported by Stolzing *et al.*, [Stolzing *et al.*, 2010] This data suggests the need for a material to support adhesion and proliferation of diabetic rBMSC. Graphs representing Population doubling number and time are depicted in Figures 23 and 24 respectively.

7. Viability of cells (rBMSC both control and diabetic) on fabricated cell construct:

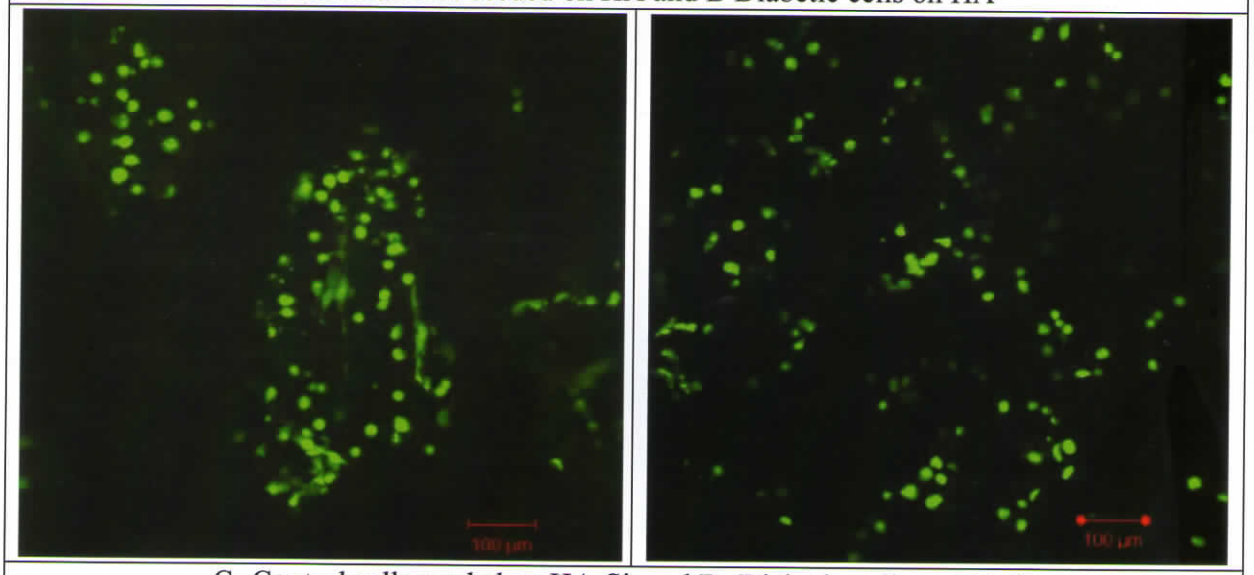
Viability of both control and diabetic rBMSC seeded on the scaffold (HA and HA-Si) was evaluated qualitatively by live-dead assay using acridine orange and ethidium bromide staining and quantitatively by measuring the LDH activity of the intact cells.

7.1. Live Dead Assay:

The live-dead assay is based on the membrane integrity of cells depending on the uptake or exclusion of dye by the cells. Acridine orange is a green fluorescent dye that is permeant to living cell and intercalates between the base pairs in DNA while ethidium bromide can only enter damaged membranes and nucleus, and hence stains



A- Control cells seeded on HA and B Diabetic cells on HA



C -Control cells seeded on HA-Si and D -Diabetic cells on HA Si

Figure 25- Confocal Micrographs of Fabricated Cell Construct stained with Acridine orange and Ethidium Bromide (Live Dead staining).

only cells whose cell membranes are no longer intact. Hence, cells that retain the green stain are live intact cells whereas dead cells take up ethidium bromide and fluoresce red. Confocal micrograph (Figure 25) revealed green viable cells studded around the pores of HA and HASi after 16 days in culture. Cells fluorescing red were not found in both the scaffolds with both the cell types. [Manitha B Nair *et al.,* 2008] The viability of both diabetic and control rBMSCs could be maintained after its expansion on the scaffolds.

7.2. LDH assay:

Viability of normal and diabetic rBMSCs on bioactive ceramics (HA and HA-Si) was quantified by LDH assay at the end of 2nd week and 4th week. Data was represented in figure 26.

Here we observed that the cells were viable on the bioactive ceramic. Though there is an initial increase of viability in case of diabetic cells, subsequently as time advanced the viability was comparable with that of the control cells. The initial increase may be due to the diabetic condition of the animal, wherein the stem cells tries to compensate the loss of bone cells in vivo in the animal model [Stolzing *et al.,* 2010].

The diabetic rBMSC show delayed proliferation in culture plates which is evident by the population doubling number and time data shown above. The time taken by diabetic rBMSC to become confluent is more compared to control rBMSCs. Stolzing *et al.,* has reported that initially after induction of diabetes in rats, there is an increase in MSC number and their proliferative potential, but after 12 weeks this initial increase in number was abolished and replaced by a 50 % loss of MSC committed to become bone which was also paralleled by a massive loss of trabecular bone. They have also reported decrease in colony size of diabetic MSCs in the 4 week diabetic animal as well as increased expression of senescence markers p21 and p53.

This data suggests the positive role of bioactive ceramic in supporting growth and differentiation of MSCs. In particular, the impaired proliferation rate of diabetic cells

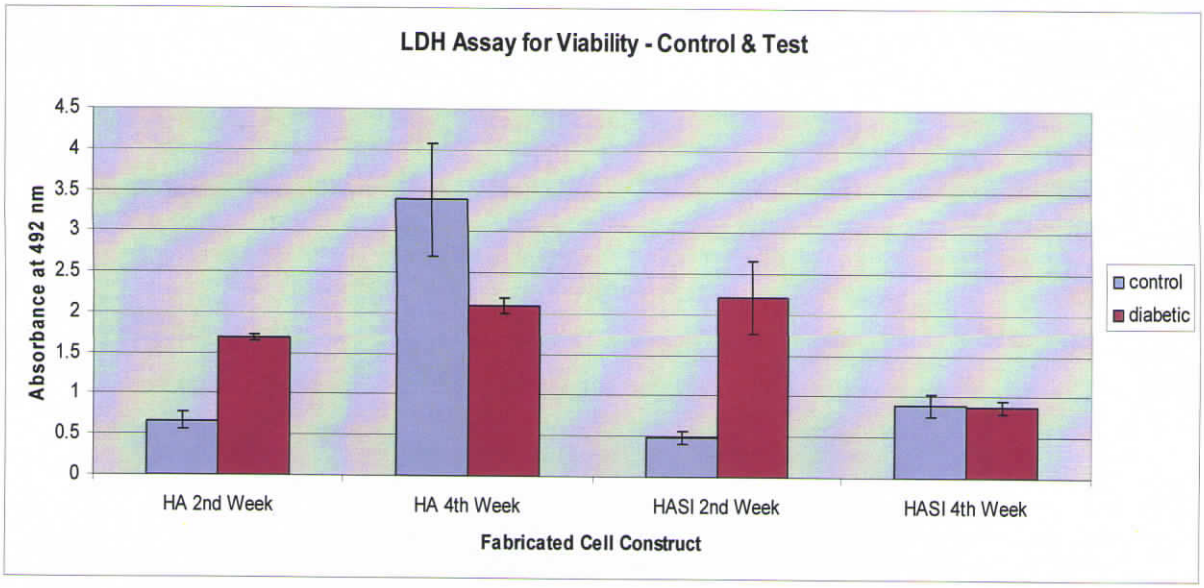


Figure 26 Graph representing Cell Viability on fabricated cell construct - Lactate Dehydrogenase assay.

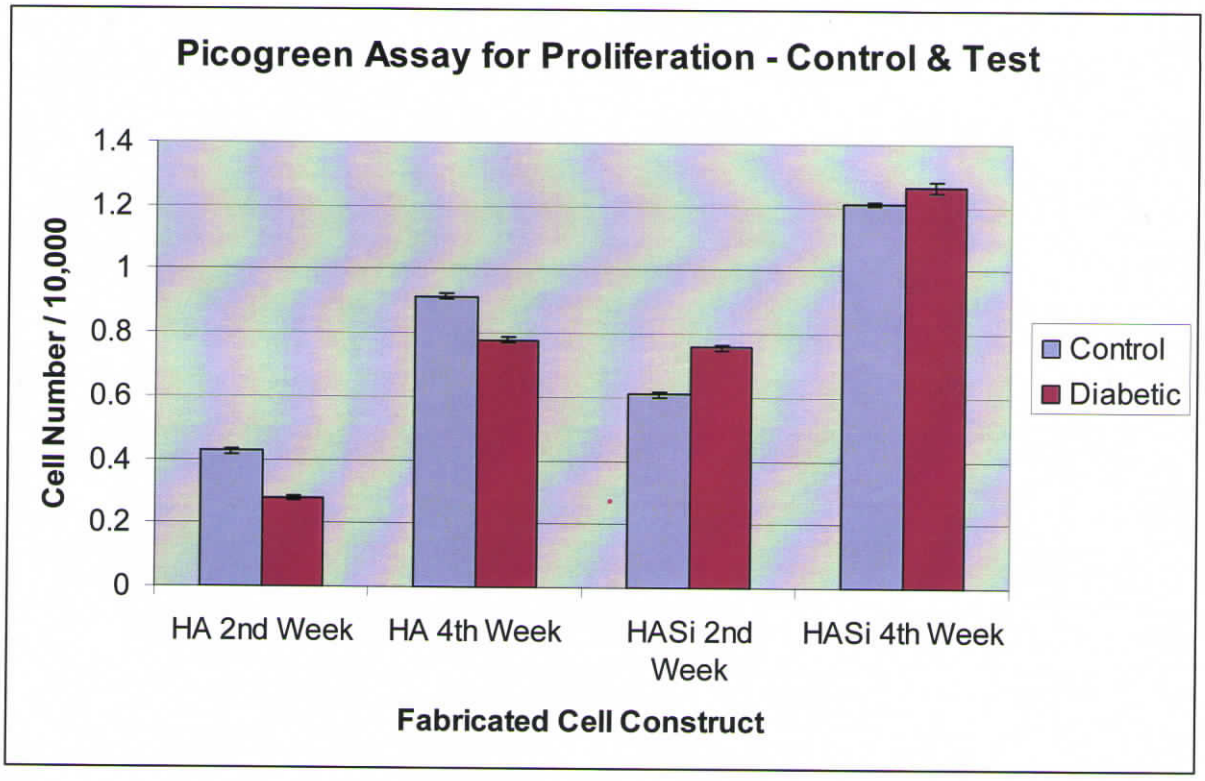


Figure 27 Graph representing Cell proliferation on fabricated cell construct - Picogreen assay

grown on TCPS was considerably enhanced when allowed to expand and proliferate on favourable bioactive scaffolds. The scaffolds might have provided the stimulus for the enhanced growth and proliferation of the diabetic cells, as reported in our previous studies [Manitha B Nair *et al.*, 2008].

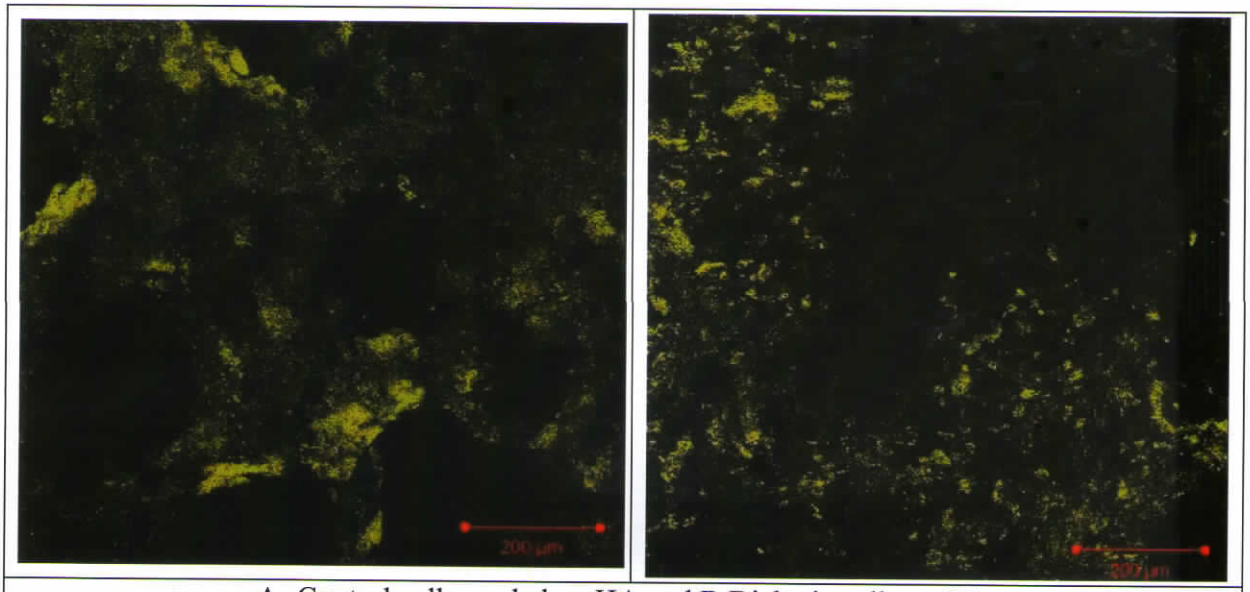
8. Proliferation Assay- Pico green Assay:

Proliferation of cells on HA and HASi was quantified by picogreen assay. Picogreen is a very sensitive fluorescent dye that intercalates to DNA and is used effectively to evaluate cell proliferation (Kee WN *et al.*, 2005). The proliferation of diabetic cells was comparable with that of control cells (Figure 27). The impaired proliferation, of diabetic rBMSC, which is substantiated by population doubling time study, has been over come by the material used, as the material favors adhesion and spreading of cells, mimicking the bone microenvironment, which is not affected by microangiopathy, hence the proliferation of diabetic rBMSCs are as comparable to that of control rBMSCs.

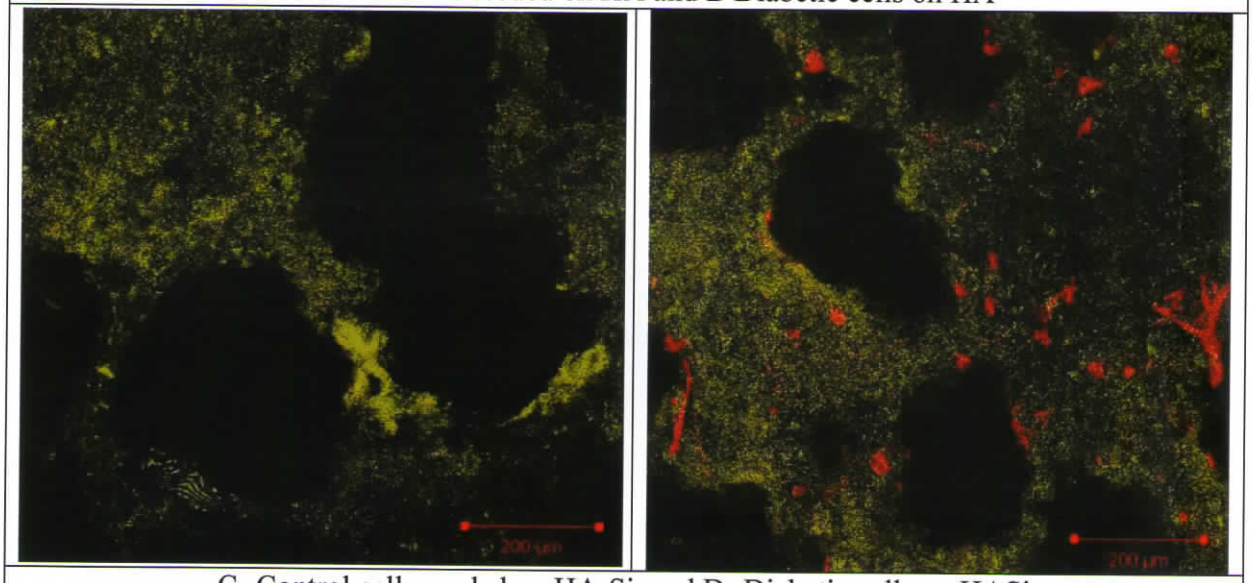
9. Alkaline phosphatase activity on cell seeded scaffolds:

ALP, an **essential enzyme, responsible for the deposition of minerals,** is present in pre-osteoblast cells at an early stage of osteoblast differentiation. The activity of the enzyme is at its peak around the second week of *in vitro* culture, and is considered as the classical marker for increased osteoblast activity [zur Nieden NI *et al.*, 2003; Stein GS *et al.*, 1990].

The cell seeded scaffolds were examined for endogenous alkaline phosphatase activity by staining with ELF-97 and visualized through cLSM. Scattered yellowish green granules were distinctly visible suggesting ALP activity of the cells expanded over the scaffolds (Figure 28). The staining indicates that both the cells (rBMSC & rADMSC) have been differentiated to osteoblast-like lineage. Interestingly cells from the diabetic origin also favoured the differentiation when exposed to a favourable



A- Control cells seeded on HA and B Diabetic cells on HA



C -Control cells seeded on HA-Si and D -Diabetic cells on HASi

Figure 28- Confocal Micrographs of ALP Staining of Fabricated Cell construct.

culture condition and thereby enhanced the osteogenic property of the in house developed scaffold [Manitha B Nair *et al.*, 2008]. Sun *et al.*, (2010) has also reported that the differentiation capacity of bone marrow-derived MSCs in diabetic patients was intact as the BMSC from diabetic patients could differentiate into insulin-producing cells in vitro.

Chapter 4 - Summary and conclusion

Diabetes causes structural abnormalities on bone, which predisposes to fractures, which may occur spontaneously or with minimal trauma in patients. Alterations in bone mass and mineral metabolism have been shown to occur in both human and experimental diabetic animals. [Verhaeghe J et.al. 1997; Schwartz AV et.al. 2001] Though there is controversy in BMD of type 1 and type 2 diabetes mellitus, in both the cases bone metabolism is affected, which is evident by cohort studies of different populations, which shows increased fracture risk in diabetic populations compared to that of normal population [Janghorbani M *et.al.* 2007]. From the literature survey, it is well documented that diabetes mellitus causes loss of bone mass, which may occur as a result of a decrease in the deposition of calcium, as a direct consequence of hyperglycemia and impaired osteogenesis of bone marrow mesenchymal stem cells.

In Vitro studies on Diabetic rBMSCs have been found to possess impaired proliferation and differentiation capability. Several hypothesis have been proposed to substantiate this observation such as hyperglycemic condition of diabetes which causes impaired proliferation and differentiation of MSCs, is alleviated when treated with insulin and estradiol.[Gopalakrishnan et.al. 2006], Accumulation of AGEs in bone microenvironment, as over expression of AGEs by MSCs found under diabetic conditions, inhibits its growth and differentiation. Over expression of apoptosis markers and cell cycle arrest have also been reported, which parallels with loss in trabecular bone, which shows high correlation between impaired MSC and osteogenesis in diabetic animal.[Stolzing et.al. 2010] The osteoblast to adipocyte shift of MSCs have also been proposed to cause reduction in the number of differentiated osteoblast available for bone formation. [Wongdee K and Charoenphandhu N, 2011]

Though literature survey shows increased morbidity in orthopedic patients with diabetes which has been well documented by cohort studies, therapeutic regimens for this condition (Diabetes associated osteopenia and fractures) are limited. In spite of

the positive effect of calcium-phosphate ceramics and its composites in fracture healing, studies on the influence of this composite on fracture healing in diabetics are limited. Previous studies on cellular responses in these constructs with HASi (Silica coated Hydroxy apatite) and HA showed good cytocompatibility, cell adhesion, osteoconduction, osteoinduction and osteointegration, including a positive response both *in vivo* and *in vitro* studies, suggesting that this material is appropriate for bone tissue engineering.[Manitha B. Nair *et.al.* 2008] The aim of the present study is to evaluate the adhesion, proliferation, viability, morphology and osteogenic activity of diabetic and control rADMSC and rBMSC in the construction of *in vitro* tissue constructs (Stem cells and scaffold) in perspective of bone healing and repair under diseased conditions.

In this study we used pharmacologically induced diabetic rat model was developed. The induction of experimental diabetes in rats using Streptozotocin (STZ), which selectively destroy pancreatic beta cells, is very convenient and simple to use. This type of animal model is used to induce insulin-dependent type 1 diabetes. The dosage of STZ used in this study (single intraperitoneal administration of 45 mg/kg body mass) maintained diabetic status throughout the study period. The diabetic experimental animals suffer a significant weight loss and elevated blood glucose compared to buffer injected control animals, at all time points throughout the experiment. Serum calcium and alkaline phosphatase level was also increased compared to that of control animals, which clearly manifests the disease condition of the animal.

MSCs from two different sources - bone marrow and adipose were used in this study to substantiate the delayed proliferation of rBMSC as reported in some *in vitro* studies [Stolzing et.al. 2010]. Population doubling time of rBMSC was evaluated and found to be impaired compared to that of control cells. This clearly states the need for a substrate to support adhesion, viability and enhance the proliferation rate of diabetic rBMSC. Yield of adipose tissue from diabetes induced rats was scarce.

Here in we seeded the diabetic and control (rBMSC and rADMSC) cells on scaffolds and differentiated the cells into the osteogenic lineage and maintained the cell culture for 28 days and the constructs were evaluated in terms of cell morphology and adhesion (ESEM); cell viability (acridine orange / ethidium bromide staining by cLSM and LDH assay) and cell proliferation (picogreen assay). The results of both diabetic and control cells were comparable, which suggests that the bioactive ceramic scaffolds influenced the expansion and proliferation of both diabetic and control MSCs. Further more the impaired proliferation observed in PDT evaluation was surmount by seeding on to the scaffolds, which is clearly depicted by the *in vitro* studies using ceramic (HA and HASi) wherein the viability and proliferation of the diabetic MSCs where similar to that of the control MSCs.

The cellular activities (both diabetic and control cells) were comparatively enhanced on HASi and HA emphasizing the importance of surface chemistry on cell behaviour. It is reported that the surface chemistry of silica-based materials are amenable to ECM proteins that improve cell adhesion and other cellular activities. The highly interconnected porous nature of HASi (50-500 μm pore size) is in par with the appropriate chemical composition which improved the migration limitation and distribution of cells towards the internal voids of the material, which is a major drawback in many static culture systems.

The ability of HASi to maintain cell proliferation and osteogenic differentiation in a parallel relation indicated that the ultimate fortune of cells is determined by the respective matrix / substratum. The response of diabetic and control rat stem cells in terms of adhesion, viability, morphology and proliferation was approximately same on HA and HASi, implying the comparability between these two species for *in vivo* studies. However the efficacy of the material on implantation can be determined only by *in vivo* studies. As HA has already been proven to effectively increase the biomechanical properties of bones in diabetic rats [Parizi et al 2010], this cell seeded HA construct may favor fracture healing under diabetic conditions as it contains both stem cells and bioactive ceramic.

In conclusion, this study showed enhanced viability, proliferation and osteogenic activity of diabetic rBMSC on HASi and HA scaffolds, which is comparable to that of the control cells. Hydroxyapatite has many advantages, as it mimics the natural bone and also it possesses biocompatibility. Its easy availability, non-toxicity and non-immunogenicity render it to be a suitable candidate for bone implants. Silica coated hydroxyapatite are being preferred since it is proven to enhance apatite formation for osteogenesis. Cell-seeded ceramic scaffolds could be a possible option for autologous cell therapy in diabetic patients. The cells exhibited enhanced growth and expansion in conjunction with the appropriate scaffold paving way for its use in clinical situations of delayed fracture healing in diabetes.

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ANNEXURE

Dulbecco's Modified Essential Medium-Low Glucose (DMEM-LG) – 15% FBS

DMEM- LG	1litre pack
Antibiotics	1ml
$NaHCO_3$	3.7g
FBS	150ml

Dissolved in 1000ml deionised water and then filter sterilized.

Dulbecco's Modified Essential Medium-High Glucose (DMEM-LG)

DMEM- LG	1litre pack
Antibiotics	1ml
$NaHCO_3$	3.7g
FBS	100ml

Dissolved in 1000ml deionised water and then filter sterilized.

Phosphate Buffer saline (PBS) - 1000 ml

NaCl	8.0 g
KCl	2.0 g
Na_2HPO_4	1.15g
KH_2PO_4	2.0 g

Dissolved in 1000ml deionised water and then autoclaved at 121^{o}C for 20 minutes.

Osteogenic Medium (100ml)

ß Glycerophosphate	1ml
Dexamethasone	2.456µl
Ascorbic acid 0.05 mg/ml	1ml
DMEM-HG containing 15% FBS	

Sorensen's Phosphate Buffer (100ml):-

NaH2PO4 19ml
Na2HPO4 81ml

Paraformaldehyde (100ml):-
Paraformaldehyde 3.7g
Sorensen's buffer 50ml
Distilled water 50ml

3% Gluteraldehyde (50ml):-

Gluteraldehyde (25%) 6ml
Sorensen's buffer 44ml

1% Glutaraldehyde (50ml):-

Gluteraldehyde (25%) 2ml
Sorensen's buffer 48ml

International Conference on Futuristic Science and Technology in Frontier areas &
2nd Annual Conference of Indian JSPS Alumni Association
Thiruvananthapuram, Kerala, India
August 5-6, 2011

Certificate

This is to certify that.....

Mr./ ~~Ms./Dr./Prof~~.....Dinesh Kumar K.....has participated as a

~~delegate/Chairperson/Guest speaker~~/presented a paper at the International Conference

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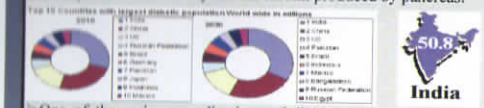
e-mail: dinesh_watson@yahoo.com

INTRODUCTION

➢Diabetes mellitus (DM) is a pandemic metabolic disease, which occurs either due to lack of insulin production by the pancreas, or when the body cannot effectively use the insulin produced by pancreas.



➢One of the major complications of diabetes is osteopenia coupled with decreased bone mineral density (BMD) in Type 1 and increased BMD in type 2 diabetes. It is evident from clinical & animal studies that in spite of the lowered or higher BMD, diabetic bone appears to be fragile & thus it is more prone to undergo fracture [1,2,3].

➢Literature reports states that diabetic Bone marrow mesenchymal stem cells (BMSC) have impaired proliferation & differentiation which alters bone metabolism in diabetes [4] Hormone (Insulin & estradiol) administration enhances MSC proliferation & differentiation [5].

➢Herein, we developed a diabetic animal model (rat) & stem cells (rBMSC and rADMSC) were isolated from the animals to fabricate an engineered construct (*in vitro*) towards bone tissue engineering in diabetic osteopenia & associated fracture.

METHODS

1. Development Of Diabetic Animal Model

Wistar rats were divided into two groups –

a. Test (induced diabetes by intraperitonial administration of Streptozotocin (45 mg/kg bwt) dissolved in 0.1 M cold citrate buffer (pH-4.5)

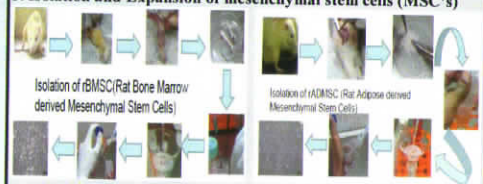
b. Control (injected with citrate buffer alone)

- Blood glucose and body weight were monitored
- Serum calcium and ALP estimation before and after induction.
- Histology of pancreas of both test and control animals

2. Material characterization

- Bioactive ceramic Hydroxyapatite (HA) & Silica Coated Hydroxyapatite (HA-Si) were used for the fabrication of tissue engineered construct.
- Materials were characterized by XRD, FTIR and SEM

3. Isolation and Expansion of mesenchymal stem cells (MSC's)



4. Differentiation potential of MSCs

- MSCs ($1*10^5$) were cultured on cover slips, induced to osteogenic lineages (DMEM-HG with osteo inducers)
- Alizarin red and Von kossa staining

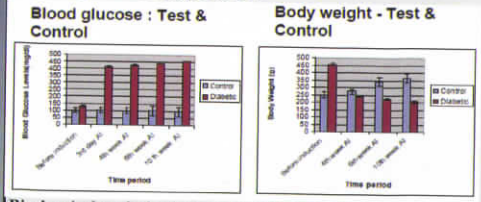
5. Evaluation of cell seeded tissue construct

rBMSC (P2) were seeded on to the conditioned scaffold (1×10^5

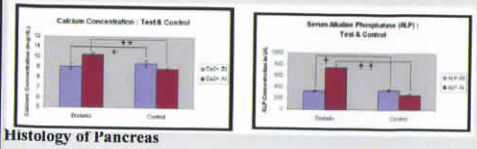
RESULTS

Development of diabetic animal model

Body weight and blood glucose level

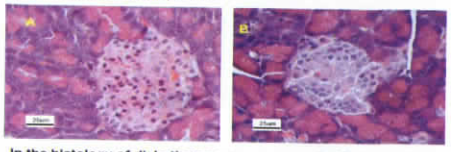


Biochemical analysis of serum (experimental animals)



Histology of Pancreas

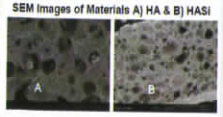
Light microscopic images of H& E stained sections of A) normal and B) diabetic pancreas



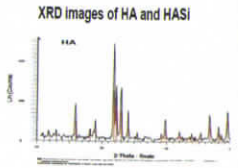
In the histology of diabetic pancreas, represented in Figure B, the acinar cells were seen to be normal, but the islets were seen shrinked (insulinitis), compared to the normal pancreas (Figure A)

Material characterization

SEM Images of Materials A) HA & B) HASi



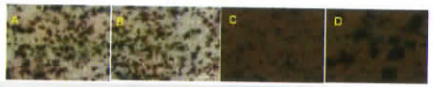
XRD images of HA and HASi



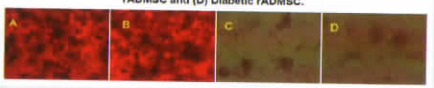
FTIR images of HA and HASi

Differentiation potential of diabetic and control cells

Differentiation studies- (Osteogenic Induction) Light Micrographs of cells stained with von Kossa (A) Control rBMSC (B) Diabetic rBMSC (C) Control rADMSC and (D) Diabetic rADMSC.



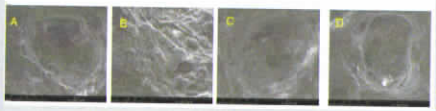
Differentiation studies- (Osteogenic Induction) Light Micrographs of cells stained with Alizarin red (A) Control rBMSC (B) Diabetic rBMSC (C) Control rADMSC and (D) Diabetic rADMSC.



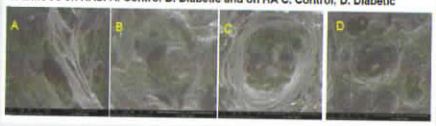
Cell adhesion on bioactive ceramics

Scanning electron micrographs of cell seeded scaffolds revealed that both diabetic and control rBMSCs as well as rADMSCs adhered and expanded over HA and HASi scaffolds. Cells had spherical morphology initially and further flattened to spread over the entire surface of the scaffolds.

Scanning Electron Micrographs of fabricated cell construct-28 days in Culture rBMSCs on HASi A. Control B. Diabetic and on HA C. Control, D. Diabetic

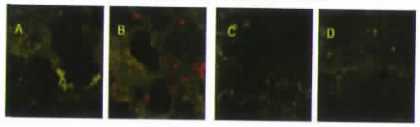


Scanning Electron Micrographs of fabricated cell construct-28 days in Culture rADMSCs on HASi A. Control B. Diabetic and on HA C. Control, D. Diabetic

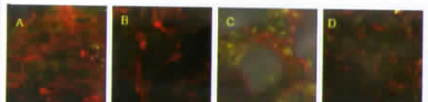


Osteogenic differentiation of MSCs on bioactive ceramics stained for ALP

Confocal Micrographs of fabricated cell construct-20 days in Culture rBMSCs on HASi A. Control B. Diabetic and on HA C. Control, D. Diabetic



Confocal Micrographs of fabricated cell construct-20 days in Culture rADMSCs on HASi A. Control B. Diabetic and on HA C. Control, D. Diabetic



SUMMARY

❖ The diabetic experimental animals had significant weight loss and elevated blood glucose level compared to buffer injected control animals, at all time points.

❖ Serum calcium and alkaline phosphatase level was also increased compared to that of control animals, which clearly manifests the disease condition of the animal.

❖ Deposition of abundant mineralized matrix comprising of calcium &phosphate was visualized by Alizarin red & Von kossa staining. Calcium deposition was stained orange-red in Alizarin staining whereas the Von kossa depicted black phosphate deposits in the matrix of both diabetic and control cells.

❖ The impaired proliferation of diabetic MSCs[] were surmount by seeding on the scaffolds (HA and HASi), wherein the adhesion and expansion of the diabetic MSCs were enhanced depicting the cell-friendly nature of the ceramics.

CONCLUSION

❖Though literature survey shows increased morbidity in orthopedic patients with diabetes, therapeutic regimens for this condition (Diabetes associated osteopenia and fractures) are limited. In spite of the positive effect of calcium-phosphate ceramics and its composites in fracture healing, studies on the influence of this composite on delayed fracture healing in diabetes are limited.

❖The cellular activities (both diabetic and control cells) were enhanced on HASi and HA emphasizing the importance of surface chemistry of the scaffolds on cell behaviour.

❖The ability of HA and HASi to maintain cell proliferation and osteogenic differentiation in a parallel relation indicated that the ultimate fortune of cells is determined by the respective matrix / substratum.

❖The response of diabetic and control rat stem cells in terms of adhesion, viability, morphology and proliferation was approximately same on HA and HASi, implying the comparability between these two species for *in vivo* studies.

❖The cell seeded construct may accelerate the fracture healing under diabetic conditions and hence can be utilized as a treatment option fordiabetic osteopenia & associated fracture.

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