Chapter – III

Effects of glucose and their modulation by insulin and estradiol on BMSC differentiation into osteoblastic lineage
Bone marrow stromal cell-lineages

Bone marrow stromal cells (BMSCs) are multipotent cells that differentiate into osteoblasts, chondrocytes and adipocytes (Bennett et al., 1991; Johnstone et al., 1998; Pittenger et al., 1999; Kihara et al., 2004). The multipotent stem cells can be directed towards the osteogenic lineage in vitro when they were cultured in the presence of dexamethasone, β-glycerophosphate (β–GP) and ascorbic acid (Maniatopoulos et al., 1988; Ohgushi et al., 1996; Coelho et al., 2000). Osteogenic differentiation of BMSCs was generally characterized by the appearance of osteoblastic cell morphology and increased synthesis of osteogenic marker proteins such as alkaline phosphatase and osteocalcin. Based on these analyses, in vitro mineralization derived from cultured BMSCs can be characterized as “regenerative cultured bone”.

Development into osteoblastic lineage

During development, pre-osteoblasts undergo a series of stages such as proliferation, differentiation, matrix deposition and matrix mineralization. Osteoblastic differentiation in vitro is marked by three distinct stages of cellular activity: 1) proliferation, 2) extracellular matrix maturation and 3) matrix mineralization (Lian and Stein, 1992). Initially, osteoprogenitor cells are highly mitotic, as demonstrated by their expression of the cell-growth associated genes H4 histone and c-fos (Pockwinse et al., 1992).
During this proliferative stage, genes associated with extracellular matrix formation (type-1 collagen, fibronectin and transforming growth factor-β) are expressed at peak levels (Owen et al., 1990). Alkaline phosphatase values reach a maximum during the matrix maturation stage and are down regulated during the late stages of matrix mineralization. High osteocalcin and osteopontin values reflect the mineralization period. Following the period of matrix maturation, nodule cells begin to mineralize the extracellular matrix. Calcium accumulation of the cultures starts in the matrix development phase and is maximal during mineralization. The expression of osteocalcin and bone sialoprotein increases with mineral deposition (Owen et al., 1990; Pockwinse et al., 1992), while ALP levels decline (Turksen and Aubin, 1991; Malaval et al., 1994).

It is now well documented that the transcription factor, Runx2, is required for commitment to the osteoblast phenotype (Ducy et al., 1997) and that in Runx2 null mice, osteoblast differentiation is arrested in both the endochondral and intramembraneus skeleton (Komori et al., 1997, Otto et al., 1997). Runx2 is known to modulate the transcription of several genes involved in the mineralization process. The bone sialoprotein promoter has a number of functional Runx DNA binding sites and Runx2 mediates repression of this promoter (Javed et al., 2001). In contrast, Runx2 enhances transcription of osteopontin (Sato et al., 1998), type-1 collagen gene (Kern et al., 2001), collagenase-3 (Jimenez et al., 1999), osteoprotegerin (Thirunavukkarasu et al., 2000) and osteocalcin (Gutierrez et al., 2002). Another transcription factor essential for osteoblast differentiation is osterix.
This factor appears to be required at a later stage of differentiation than Runx2 since preosteoblasts of osterix null mice express Runx2 at comparable levels to wild type osteoblasts, while no expression of osterix was apparent in Runx2 null mice (Nakashima et al., 2002). Another transcription factor PPARγ, which has multipotent effects on stromal cells, while activating adipocyte specific genes such as αP2 and phosphos enol pyruvate carboxy kinase (PEPCK), suppresses Runx2 (Lecka-Czernik et al., 1999) and the synthesis of α1(1)-procollagen, osteopontin, alkaline phosphatase and osteocalcin (Lecka-Czernik et al., 2002).

In vitro, as differentiation proceeds, the levels of alkaline phosphatase activity rise and in the presence of organic phosphate will generate free inorganic phosphate (Bellows et al., 1992; Chung et al., 1992). The result of the differentiation process is the formation of hydroxyapatite mineral that is thought to occur through two possible mechanisms, the formation of matrix vesicle, small vesicles that bud from the plasma membrane and accumulate calcium and phosphate (Anderson, 1995) and or the nucleation of collagen, regulated by associated non-collagenous matrix proteins (Glimcher, 1989; Boskey, 1998). Early in the differentiation process (days 1-4) as osteoblasts become confluent, exit the cell cycle and respond to ascorbic acid with deposition of a collagen matrix, the levels of alkaline phosphatase mRNA and activity rise. As the activity of the enzyme increases in the presence of βGP, the amount of inorganic phosphate also rises. Studies investigating the requirement and timing of alkaline phosphatase and βGP in the process of mineralization have revealed that βGP and hence elevated levels of inorganic
phosphate are required for the initiation of mineralization but, once the process
is initiated, mineralization will continue at non-elevated levels in both
osteoblasts (Tenenbaum, 1987; Bellows et al., 1990; Fratzl-Zelman et al.,
1998) and chondrocytes (Zimmermann et al., 1992). There also seems to be a
critical time point during the differentiation process, likely a stage of matrix
maturation, at which the generation of phosphate promotes mineralization
after which no mineralization occurs regardless of amount of phosphate added
(Tenenbaum, 1987; Zimmermann et al., 1992). Hence inorganic phosphate
may be more important for the differentiation process than the actual
hydroxyapatite formation and the ability of phosphate to affect cell function
may be dependent on a particular stage of maturation.

Effect of high glucose on BMSCs and osteoblasts

Osteopenia has been associated with diabetes. Bone mineral density in
both type-1 and type-2 diabetics was reported to be decreased. In those with
type-1 diabetes, the degree of osteopenia correlates with the duration of
diabetes. An increased incidence of osteoporotic fractures is associated with
diabetes. Hyperglycemia is associated with hypercalciurea and impaired vit.D
metabolism. Moroshi and colleagues (1996), demonstrated that high
concentration of glucose induces the production of potent bone resorbing
cytokines, interleukins, tumor necrosis factor-α from human monocytes.

Both osteoblasts and odontoblasts are terminally differentiated cells of
mesenchymal origin and are responsible for secretion and mineralization of
type-I collagen (Linde and Goldberg, 1993). In both bone and dentine,
inorganic calcium phosphate is deposited to organic matrix-forming mineral crystals. Collagen is believed to control mineral crystal orientation and organization and the collagen cross-linking pattern may be important for its mineralization (Linde and Goldberg, 1993). Glucose inhibits collagen fibril formation and subsequent cross-linking in human osteoblast-like cells in vitro, with 50% inhibition occurring at 50 mmol/L glucose (Lien et al., 1984). A very high glucose (up to 49.5 mmol/L) environment has been shown to inhibit basal and IGF-I-induced osteoblastic cell proliferation and 1,25(OH)₂D₃-induced osteocalcin secretion in human MG-63 cells in vitro (Inaba et al., 1995; Terada et al., 1998); this was not replicated in mannitol cultures. High glucose concentration was also found to down-regulate PTH-induced calcium uptake in osteoblasts in vitro (Yoshida et al., 1995).

Even though there are some studies indicating that high glucose does not affect skin fibroblast collagen synthesis (Verhofstad et al., 1998), the skin fibroblast proliferation is suppressed (Hehenberger et al., 1999), morphology is altered and apoptosis is enhanced when the cells are cultured in a high-glucose medium (Solini et al., 2000).

Glucose transporters

In eukaryotic cells, glucose uptake is mediated by transmembrane glucose transporter (GLUT) proteins. Six closely related isoforms have been isolated and cloned and their tissue specificity is extensively characterized (Mueckler, 1994). Normally, GLUT4 is exclusively expressed in insulin-responsive tissues, e.g., heart, skeletal muscle and white brown adipose
tissues (James et al., 1993). In these tissues, insulin stimulates cellular glucose uptake by inducing the translocation of GLUT4 from an intracellular pool to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980; Karnieli et al., 1981). Because they are involved in the first step of the glucose utilization cascade, GLUT4 proteins are highly regulated in physiological as well as pathophysiological states. Indeed, type-2 diabetes mellitus and STZ-diabetic rats are associated with a decrease in cellular number and activity of GLUT4 (Karnieli et al., 1981, 1986; Kahn and Cushman, 1987; Garvey et al., 1988). The reduced gene expression of the GLUT4 isoform is probably regulated at the pre-translational level (Weiland et al., 1991; MacDougald and Lane, 1995).

Insulin and IGF receptors share a high degree of similarity as well as many common components of their signal transduction pathways (Chakravorty et al., 1993). Both hormones stimulate GLUT4 translocation and regulate glucose uptake in insulin-responsive tissues as well as in various cell-lines (Karnieli et al., 1986; Kahn and Cushman, 1987; Garvey et al., 1988; Weiland et al., 1991; MacDougald and Lane, 1995). These hormones also modulate GLUT4 messenger RNA level (Weiland et al., 1991; MacDougald and Lane, 1995; Mora et al., 1995). In diabetic mice, significant growth retardation is expressed as severe malformations in both mandibular condyle and humeral growth plate. Maor and Karnieli (1999) found the presence of GLUT4 mRNA and protein in chondroblastic and hypertrophic cells of mouse bone growth centers. GLUT4 seemed to be regulated through IGF-I receptor signaling cascade rather than with the insulin-receptor
signaling pathway. Further, in the skeletal growth centers of streptozotocin-induced diabetic mice, GLUT4, IGF-I and IGF-IR and insulin receptor levels, but not GLUT1 were markedly reduced. The decrease in GLUT4, IGF-I and insulin receptors were associated with severe histological changes in the mandibular condyles and humeral growth plate. Insulin therapy restored insulin receptor levels to normalcy, whereas IGF-I receptor and GLUT4 levels were only partially restored. Thus, GLUT4 and IGF-IR have a potential role in early bone growth in mice.

High-affinity insulin receptors have been documented in several mature osteoblastic cell lines like UMR-106, a clonal rat osteogenic osteosarcoma cell line (De Luise and Harker, 1988; Hickman and McElduff, 1988; Ituarte et al., 1989; Pun et al., 1989; Thomas et al., 1996) and in ROS-17/2.8, a rat osteogenic osteosarcoma cell-line (Levy et al., 1986).

Osteoblast proliferation, assessed by $[^3]H$thymidine incorporation, is stimulated by physiological concentrations of insulin (0.5 – 1.0 nM) in UMR-106 cells (Hickman and McElduff, 1988). Physiological concentrations of insulin (0.5 – 1.0 nM) stimulated glucose uptake in UMR-106 osteoblastic cells (Ituarte et al., 1989; Thomas et al., 1996). Insulin at $10^{-8}$ M also increased the mRNA level of the glucose transporter GLUT1 by three fold in UMR-106 cells (Thomas et al., 1996). Insulin at 1 nM stimulated the incorporation of collagen synthesis as measured by the uptake of $[^3]H$proline into collagen in UMR-106 cells (Pun et al., 1989), but also in fetal rat calvariae, especially in it osteoblast-rich central bone area (Kream et al., 1985).
Chondrocyte proliferation and $^{35}$SO$_4$ incorporation have been shown to be stimulated by insulin in a number of in vitro systems: organ and tissue cultures of neonatal mouse mandibular condyles (Maor et al., 1993) and chondrocyte cultures from rat chondrosarcoma, rat rib cartilage or fetal limb growth plate cartilage (Foley et al., 1982; Heinze et al., 1989; Hill and De Sousa, 1990). These effects are obtained at physiological levels of insulin, as low as 1 nM (Hill and De Sousa, 1990).

Effect of hormones on BMSCs and osteoblasts

Studies on primary human and rat osteoblast cultures showed that 17β-estradiol dose-dependently stimulated osteoblast proliferation and differentiation as assessed by alkaline phosphatase activity and bone nodule formation (O'Shaughnessy et al., 2000). Estrogen is suggested to stimulate bone formation (Chow et al., 1992) possibly by a direct effect on osteoblast cell proliferation (Ernst et al., 1989). The presence of progesterone receptor mRNA and protein has been demonstrated in primary cultures of human OB cell-lines (HOSTE 85, MG-63 and SaOS-2) (Wei et al., 1993; MacNamara et al., 1995). Slootweg et al. (1992) found that P$_4$ did not affect the proliferation of SaOS-2 cells, but it stimulated the cell growth synergistically with E$_2$.

Various clinical observations and experimental data suggest that estrogen and progesterone can modulate insulin sensitivity (Polderman et al., 1994; Marsden et al., 1996). Molloy et al. (2000) and Lee et al. (1999) showed that estrogen induces the expression of the downstream signaling
molecules, IRS-1 and IRS-2 in insulin signaling pathway. Estrogen induction of IRS-1 expression was associated with increased tyrosine phosphorylation of IRS-1 and correlated with enhanced downstream mitogen-activated protein kinase (MAPK) activation in human breast cancer cells.

**Androgens** increase osteoblast proliferation, collagen secretion and bone formation in bone organ cultures (Kasperk *et al.*, 1997; Davey *et al.*, 2000). Osteoblasts and osteoblast-like cell-lines have high-affinity androgen receptors (Abu *et al.*, 1997; Noble *et al.*, 1998). Androgens also increase gene expression and cellular secretion of TGF-β and decrease secretion of PGE₂ by mixed bone cells *in vitro*. These cytokines have potent effects on osteoclasts and osteoblasts. Androgens can therefore influence bone forming and resorbing cells directly or indirectly via changes in cytokine secretion in bone.

**PTH** has dual (inhibitory and stimulatory) effects on bone collagen synthesis. The inhibitory effect is observed after continuous exposure to the hormone, whereas the stimulatory effect is observed after intermittent exposure of the bone cell to PTH (Canalis *et al.*, 1989; Hock and Fonseca, 1990). PTH at physiological concentrations is a potent suppressor of osteoblast differentiation and that its effect occurs at a late stage in the differentiation of osteoprogenitor cells, probably preventing differentiation of preosteoblasts into osteoblasts (Bellows *et al.*, 1990). *In vitro*, PTH inhibits the expression and synthesis of matrix proteins, including collagen-I, osteocalcin and alkaline phosphatase, regardless of whether exposure is for a few hours or several days in differentiated osteoblasts (Dietrich *et al.*, 1976;
Raisz and Kream 1983a, b; Clohisy et al., 1992; Kream et al., 1993; Tetradi 

Vit.D₃ enhances the synthesis of osteocalcin by the osteoblasts (Lian et al., 1985). It directly inhibits the bone collagen synthesis but increases the binding of IGF-I to its receptor in osteoblastic-lineage and stimulates the synthesis of IGFBPs (Canalis et al., 1992).

Thyroid hormone promotes the proliferation and differentiation of osteoblastic cells (Ohishi et al., 1994; Ishida et al., 1995). Alkaline phosphatase was also increased by thyroid hormone in isolated tibiae (Stracke et al., 1986) and in primary human (Kassem et al., 1993) and rodent (Egrise et al., 1990) osteoblasts.

Glucocorticoids increase the apoptosis of osteoblasts (Weinstein et al., 1998; Gohel et al., 1999; Silverstrini et al., 2000) and osteocytes (Weinstein et al., 1998; Plotkin et al., 1999). In primary fetal rat calvarial cell cultures, the increase in osteoblast apoptosis is associated with a decrease in the Bcl-2/Bax protein ratio (Gohel et al., 1999). Dexamethasone selectively stimulates the proliferation of osteoprogenitor cells and that these progenitor cells have a limited capacity for generating daughter cells capable of expressing the bone phenotype (Bellows et al., 1990).

GH is known to be anabolic for osteoblasts and to stimulate the proliferation of cultured osteoblasts. Some studies, demonstrate that GH regulates the differentiation of cultured osteoblasts (Slootweg et al., 1988;
GH was found to increase the proliferation but not differentiation (ALP activity) of human osteoblasts (Slootweg et al., 1988).

IGFs have been shown to act as a mitogen for normal osteoprogenitor cells and MG-63 cells (Canalis et al., 1988; Micheal et al., 1990), in human osteoblastic cells and human marrow stromal cells (Thomas et al., 1999). IGFs produced by osteoblasts or released from bone matrix have the potential to stimulate proliferation and enhance osteoblastic activity. These effects are mediated through binding of IGF peptides to specific plasma membrane receptors identified on various OB cell models (Centrella et al., 1990). IGFs increase DNA synthesis and replication of cells of the OB lineage and play a major role in stimulating differentiated function of OB. In vitro, human and rodent OB and osteosarcoma cells respond to ligand-activated type-1 IGF-IR stimulation with increase in DNA and protein synthesis (Canalis 1993). Both IGF-I and II increase the type-I collagen expression and collagen degradation in fetal rat OB.
AIM

To study the dose-dependent effects of glucose and their modulation by insulin and estradiol on rat bone marrow stromal cell proliferation and differentiation in vitro.

HYPOTHESIS

High glucose concentration affects BMSC differentiation into osteoblastic lineage osteoblast maturation and mineralization which is reversed by insulin or estradiol.

OBJECTIVES

To expose rat bone marrow stromal cells to different concentrations of glucose and treat them with insulin or estradiol.

To study the marker of bone formation (ALP), histochemical localization of mineralized nodules and matrix components (collagen and GAGs).
MATERIALS AND METHODS

CHEMICALS

Bovine serum albumin (BSA), alizarin red S, Tris-HCl, β-glycerophosphate, vitamin C and dexamethasone were purchased from Sigma chemical Co., USA. Dulbecco’s Modified Eagle’s Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL, sodium bicarbonate solution, penicillin/streptomycin, amphotericin B, trypsin-EDTA and trypan blue solution were purchased from Biochrom, Germany, sodium chloride, sodium hydroxide, sodium carbonate, hydrochloric acid, p-nitrophenol, p-nitrophenyl phosphate, DL-tartrate, sodium thiosulphate and rest of the chemicals were purchased from Sisco Research Laboratories (SRL), India. All the chemicals were of analytical grade. [3H] thymidine was purchased from Amershaw, USA.

Reagents

1. DMEM (pH-7.4)

10 g of DMEM was dissolved in 800 ml of sterile distilled water. To this solution, 49.3 ml of sodium bicarbonate solution was added followed by addition of 10 ml penicillin/streptomycin solution (10,000 IU/10,000 µg/ml) and 1 ml of amphotericin B solution (250 µg/ml). The pH was adjusted to 7.4 using 1 N HCL and 1 N NaOH. The final volume was made upto 1 litre with distilled water. Then the medium was sterile filtered (0.22 µ) inside the hood. The medium was then dispensed into sterilized container and stored at 4°C.
Testing viability

The viability of cells was assessed by trypan blue exclusion test (Aldred and Cooke, 1983).

Reagents

Trypan blue solution

0.5% trypan blue (w/v) in physiological saline was used.

Procedure

100 μl of trypan blue solution was mixed with 100 μl of cells contained in the medium and incubated for 5 min at 37°C. The cells were then washed thrice with saline and 10 μl of this suspension was placed in a haemocytometer and viewed under microscope. The unstained cells represented the viable cells whereas the damaged cells were stained. The number of stained and unstained cells was counted and the percentage of viable cells was calculated using the formula:

\[
\text{% of viability} = \frac{\text{No. of unstained cells}}{\text{Total cell count}} \times 100
\]

The viability of the cells was found to be between 90-95%.
Treatment protocol

The experimental groups consist of:

1. Glucose 5.5 mM
2. Glucose 16.5 mM
3. Glucose 49.5 mM
4. Glucose 5.5 mM + Insulin (0.6 µg/ml)
5. Glucose 16.5 mM + Insulin (0.6 µg/ml)
6. Glucose 49.5 mM + Insulin (0.6 µg/ml)
7. Glucose 5.5 mM + E2 (10 nM)
8. Glucose 16.5 mM + E2 (10 nM)
9. Glucose 49.5 mM + E2 (10 nM)

Measurement of $[^{3}H]$ Thymidine incorporation

$[^{3}H]$ Thymidine incorporation assay was performed as described by Terada et al. (1998).

Reagents

1. Phosphate buffered saline (PBS; pH 7.4)

0.63 g of sodium phosphate monobasic (Na$_2$HPO$_4$), 0.17 g of sodium phosphate dibasic (Na$_2$HPO$_4$) and 4.5 g of sodium chloride (NaCl) were dissolved in 500 ml of sterile distilled water. The pH was then adjusted to 7.4 using either 1 N HCl or 1 N NaOH, sterile filtered (0.22 µM) and then stored in a sterile container.
Scintillation fluid

4.0 g of 2,5-diphenyl oxazole (PPO) and 400 mg of 1,4-bis 5-phenyl-2-oxzolyl)-benzene-2,2'-p-phenylene bis 5-phenyl oxazole (POPOP) were dissolved in 100 ml toluene, mixed well and kept in dark.

Cells were plated in 96 well plates at 25,000 cells/well with DMEM with 10% FBS for 12 h for attachment. Then DMEM with different doses of glucose with or without insulin or estradiol were added and maintained for 3 days of experimental period.

After 18 h of culture with different concentrations of glucose alone or treated with insulin or estradiol, cells were incubated for 12 hr with 1 μCi of $^3$H thymidine. The cells were then washed twice with cold phosphate-buffered saline, scraped off with a cell scraper in 5% trichloric acetic acid (TCA) and centrifuged at 400 xg for 20 min at 4°C. After a second wash with 5% TCA, the cell pellet was heated at 80°C for 20 min in 1 ml of 5% TCA. After centrifugation, 100 μl of the supernatant was added to a vial containing 3.0 ml of scintillation liquid and the radioactivity was counted in a liquid scintillation counter.

Estimation of Alkaline phosphatase (Orthophosphoric monoester phosphohydrolase (E.C.3.1.3.1))

Alkaline phosphatase (ALP) activity was determined by the method of Andersch and Szecypinski (1947) using p-nitrophenyl phosphate as the substrate as described in the chapter-II.
Cell lysate preparation

After the experimental period, the media were removed from the wells, the cells were washed twice with PBS. Then 0.5 ml of lysis buffer (10 mM Tris HCl, 2 mM MgCl2, 0.1% Triton-X) was added to each well and allowed to lyse the cells. The cells were freeze–thawed twice and a homogenous cell lysate was prepared by going back and forth with the pipette. The lysate was collected from each well using individual micropipette tips and used for the estimation of ALP activity.

Cell culture protocol for histochemical localization of collagen, GAGs and mineralized nodules

Cells were plated at 2 x 10^5 cells in 24 well culture plates with DMEM containing 10% FBS for 24 hrs for attachment. Then the cells were treated with different doses of glucose with or without insulin or estradiol for 28 days for histochemical staining of mineralized nodules, collagen and glycosaminoglycans. The medium was replaced every 2 days.

Collagen

Collagen histochemical localization of collagen in the BMSC culture was made using van Giessonn staining technique (Conn et al., 1965). The stained cells were then photographed at 4 and 50 x magnification under a Nikon microscope.
Glycosaminoglycans

Histochemical localization of GAGs were made using method of Luna (1960). The cells were first fixed in formaldehyde (10%, v/v) in PBS for 30 min. After two washes with PBS, water and acetic acid (3%, v/v), cells were incubated with alcian blue (0.25%, w/v) in acetic acid (3% v/v) for 30 min. Cells were then washed twice with acetic acid (3%) and twice with water. The stained cells were then photographed at 4 and 50 x magnification under a Nikon microscope.

Mineralization

Mineralization was assessed after 28 days by staining with 40 mM Alizarin red (pH 4.1) by the method of DiGirolamo et al. (1999). The bone nodules stain highly for Alizarin red S after mineralization. The stained cells were counted macroscopically on a 10 mm grid and then photographed at 4 and 50 x magnification under a Nikon microscope.

Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) followed by Student-Newman-Keul (SNK) test for multiple comparisons using a computer-based software, Statistical package for Social Sciences (SPSS).

The results are expressed as means ± SEM of 6 observations of quadruplicate cultures. Significance of differences was determined using one-way analysis of variance (ANOVA).
RESULTS

\(^3\text{H}\text{thymidine incorporation}

Effect of different concentration of glucose on rat bone marrow stromal cell growth: role of insulin or estradiol

To determine whether high glucose might impair the growth of BMSCs, different concentrations (5.5, 16.5 and 49.5 mmol/l) of glucose were added to the culture medium. Higher concentration (16.5 and 49.5 mmol/l) of glucose significantly inhibited the proliferation of BMSCs in a dose-dependent manner as reflected by the thymidine incorporation by the cells. Addition of insulin to the cultures significantly improved the growth of the cells in higher concentrations but decreased thymidine uptake by cells cultured in medium containing 5.5 mmol glucose/l. Estradiol treatment to the cultures did not affect thymidine uptake in any of the treatment groups (Fig-3.1).

Effect of different concentrations of glucose on rat bone marrow stromal cell alkaline phosphatase activity: role of insulin or estradiol

In all the treatment groups studied, the ALP activity registered a small but significant decrease was observed compared to 5.5 mmol glucose alone treated. Further, within the low glucose group, addition of insulin or estradiol significantly reduced the ALP activity. While the high glucose (16.5 mmol and 49.5 mmol) glucose-containing medium was used for culture,
the addition of insulin slightly increased the ALP activity. Nevertheless, addition of estradiol to culture medium containing normal (5.5 mmol) and high (49.5 mmol) glucose concentrations reduced the ALP activity remarkably where the decrease was more pronounced in the high glucose-containing medium (Fig-3.2).

Effect of different concentrations of glucose on the number of nodules formed in rat BMSC cultures: role of insulin or estradiol

At the end of the experimental period (day 28), culture dishes were stained with Alizarin red S calcium stain. Bone marrow stem cells incubated with the basal glucose concentration (5.5 mmol/l), exhibited nodules with large calcifying foci surrounded by small foci in different stages of development. The prominent nodules were counted under microscope (4x). Regular culture media contain the basal glucose level of 5.5 mmol under basal glucose supplementation, 232 nodules were counted. As the supplementation of glucose is increased to 16.5 mmol and 49.5 moles, the number of nodules formed was reduced to 185 and 107, respectively. To determine whether addition of insulin (0.6 μg/ml) or estradiol (10 mM) (the doses were used on the basis of information from the literature) modifies the effect of glucose these hormones were added individually to the culture medium along with different concentrations of glucose. Addition of insulin and estradiol to the cultures supplemented with 5.5 mmol glucose increased the number of nodules to 258 and 268, respectively.

SCAP
Addition of insulin and estradiol to the cultures supplement with 16.5 mmol glucose increased the number of nodules to 216 and 211 from 185 respectively, which were more or less close to the basal glucose concentration i.e., 232.

Addition of insulin or estradiol to the cultures supplemented with 49.5 mmol/l also increased the number to 154 and 159 from 107, respectively (Fig-3.3).

**Effect of high glucose on matrix deposition**

In the process of mineralization, differentiated osteoblasts first secrete the matrix and mineralize them into nodules.

The bone marrow stromal cells were grown in culture media containing DMEM with 10% FBS, 5.5 mmol/l glucose and osteogenic substances. The test wells had high glucose concentrations (16.5 and 49.5 mmoles) either with or without insulin (0.6 μg/ml) or estradiol (10 nM). The cultures were maintained for 28 days.

Plate-3.1 depicts the photomicrographs (4x) of culture wells stained with van Giesson staining for collagen. The varying glucose concentrations are depicted in rows (A, B and C) and the hormone treatments are shown in columns (I, II and III). A look at the staining pattern reveals that the area stained with van Giesson was reduced in BI (16.5 mmol/l) and CI (49.5 mmol/l) wells, which received high glucose than AI (5.5 mmol). Culture wells received insulin or estradiol show an increase in the stained area.
which are more or less close to the normal glucose containing wells. A higher magnification (50 x) of the same is represented in plate-3.2. There is a drastic reduction in collagen matrix deposited by cells exposed to 49.5 mmol/l glucose. However, the staining for collagen is increased in cells exposed to insulin or estradiol.

GAG is again a component of the matrix, which stains with alcian blue. The results are depicted in plates-3.3 and 3.4.

Under low magnification (4 x) the stained area is reduced in wells that contained high glucose (BI and CI) compared to AI. Treatment with insulin or estradiol improved the same.

The higher magnification (50 x) reveals that high glucose (49.5 mmol) is not as detrimental as collagen when compared to the staining for GAGs. However, the size of the deposited area is comparatively smaller than normal glucose containing wells (AI). Again the presence of insulin or estradiol shows larger area of depositions.

Effect of high glucose on matrix mineralization

Osteoblasts secreted matrix is mineralized into nodules. Alizarin red S is a very powerful stain that stains red the calcium deposits (mineralized nodules). The histochemical staining of mineralized nodules with Alizarin red (1%) at the end of 28 days of culture is presented in plates-3.5 and 3.6.
The area stained with Alizarin red is comparatively less in BI and CI wells. However, the stained area was improved in wells that received insulin or estradiol.

A higher magnification (50 x) of the same reveals some interesting features. Mineralization is very much affected in wells that contained 16.5 mmol (BI) and 49.5 mmol (CI) glucose. While 5.5 mmol/l glucose (AI) containing well shows a well mineralized larger nodules, the nodules are smaller and poorly mineralized in wells with high glucose. Interestingly, wells that received insulin or estradiol depicts a better sized mineralized nodules when compared to high glucose containing wells (BI and CI). The quality and size of mineralized nodules are comparatively less than the nodules shown in AI. In short, insulin or estradiol treatment was able to attenuate the deleterious effects of high glucose on mineralization.
Fig. 3.1. $[^3H]$Thymidine incorporation in rat bone marrow stromal cells cultured with different doses of glucose: role of insulin or estradiol

Each value represents Mean ± SEM of 6 observations. Significance of the data at p<0.05.

- a - 5.5 mmole +V Vs rest;
- b - 16.5 mmole +V Vs rest;
- c - 49.5 mmole +V Vs rest

V-Vehicle  I-Insulin  E-Estradiol
Fig. 3.2. Alkaline phosphatase activity in the bone marrow stromal cells cultured with different doses of glucose for 3 days: role of insulin and estradiol

Each bar represents Mean ± SEM of 6 observations. Significance of the data at p<0.05.
- a - 5.5 mmole + V Vs rest;
- b - 16.5 mmole +V Vs rest;
- c - 49.5 mmole +V Vs rest
Fig. 3.3. Number of nodules formed in the rat bone marrow stromal cell cultures treated with different doses of glucose for 28 days: role of insulin and estradiol

Each bar represents Mean ± SEM of 6 observations. Significance of the data at p<0.05.

- a - 5.5 mmole +V Vs rest;
- b - 16.5 mmole +V Vs rest;
- c - 49.5 mmole +V Vs rest

[Graph showing the number of nodules for different glucose doses with annotations for significance]
Plate 3.1: Histochemical staining of mineralized nodules with van Giessen staining at the end of day 28 of culture. Row A, B & C - in the presence of 5.5, 16.5 and 49.5 mmole glucose, respectively. Columns I, II & III - without insulin or estradiol, with insulin (0.6 µg/ml) and with estradiol (10 nM) respectively (magnification 4x)
Plate 3.2  Histochemical staining of mineralized nodules with van Giessen staining at the end of day 28 of culture. Rows A, B & C – in the presence of 5.5, 16.5 and 49.5 mmoles glucose, respectively. Columns I, II & III – without insulin (0.6 μg/ml) or estradiol (10 nM), with insulin and with estradiol respectively (magnification 50x)
Plate 3.3: Histochemical staining of mineralized nodules with Alcian blue staining at the end of day 28 of culture. Row A, B & C - in the presence of 5.5, 16.5 and 49.5 mmole glucose, respectively. Columns I, II & III - without insulin or estradiol, with insulin (0.6 μg/ml) and with estradiol (10 nM) respectively (magnification 4x)
Plate 3.4  Histochemical staining of mineralized nodules with Alcian blue at the end of day 28 of culture. Rows A, B & C – in the presence of 5.5, 16.5 and 49.5 mmoles glucose, respectively. Columns I, II & III – without insulin (0.6 μg/ml) or estradiol (10 nM), with insulin and with estradiol respectively (magnification 50x)
Plate 3.5: Histochemical staining of mineralized nodules with Alizarin red S (1%) at the end of day 28 of culture. Row A, B & C - in the presence of 5.5, 16.5 and 49.5 mmole glucose, respectively. Columns I, II & III - without insulin or estradiol, with insulin (0.6 μg/ml) and with estradiol (10 nM) respectively (magnification 4x)
Plate 3.6  Histochemical staining of mineralized nodules with Alizarin red S (1%) at the end of day 28 of culture. Rows A, B & C - in the presence of 5.5, 16.5 and 49.5 mmoles glucose, respectively. Columns I, II & III - without insulin (0.6 µg/ml) or estradiol (10 nM), with insulin and with estradiol respectively (magnification 50x).
DISCUSSION

It is well known that diabetes affects bone in human and animal models leading to osteopenia and osteoporosis. Bone mineral density and other biochemical markers of bone turnover are very much affected in these subjects. High glucose (hyperglycemia) in the diabetics leads to this complication. In this regard, only few in vitro studies using bone cell-lines have been carried out to address this problem.

To the best of the investigator's knowledge, this is the first in vitro study employing primary cultures of BMSC derived from rat femurs to simulate diabetes-associated derangements in bone metabolism. Bone marrow stromal cells adhere to culture dishes and grow rapidly under defined culture conditions. The progenitor cells grown in the presence of βGP, dexamethasone and ascorbic acid differentiate into osteoblastic lineage.

Glucose concentrations used in the present study correspond to healthy individuals (control, 5.5 mmol/l glucose) and two other levels frequently recorded in patients with poorly controlled diabetes (16.5 mmol, 3 fold more than normal) and severe hyperglycemic situation (49.5 mmol, 9 fold greater than normal).

The cell proliferation assay measured by [³H] thymidine incorporation reveals that glucose supplementation to the poorly controlled hyperglycemic status and or severe hyperglycemic status leads to a decreased [³H] thymidine uptake by 20% (16.5 mmol) and by 40% (49.5 mmol) indicating the
inhibitory effects of high glucose concentration on cell growth. The present findings are in agreement with an earlier observation in MG-63 cells, wherein 49.5 mmol/l glucose even impaired the responsiveness of cells to IGF-I, a well-known mitogen and cell survival factor (Terada et al., 1998). Further, they also demonstrated that the inhibitory effect was not mediated by hyperosmolarity as high mannitol concentration did not inhibit \[^{3}\text{H} \text{H}\] thymidine incorporation. The authors have also ruled out the possibility of a cytotoxic effect of high glucose, as these cells resumed proliferation upon their return to normal glucose concentration.

In lieu of these reports it is reasonable to state that high glucose concentration is responsible for the diminished cellular DNA synthesis and growth. This may further be due to an increase in apoptosis and or cell cycle arrest. In this regard, studies from other systems have shown that incubation of cells with high glucose have promoted: (i) intracellular free radicals and the associated activation of mitochondrial apoptotic program in mesangial cells (Kang et al., 2003), in proximal tubular epithelial cells (Allen et al., 2003) and human aortic endothelial cells (Sekiguchi et al., 2004); (ii) the intrinsic proapoptotic signaling pathway in mesangial cells (Mishra et al., 2005). Mesangial cells exposed to high ambient glucose concentration also exhibited cell cycle arrest at G1 phase (Wolf et al., 2001). Thus in the current study, the bone marrow stromal cells exposed to high glucose concentration could have been subjected to apoptotic cell death and or cell cycle arrest.

In another study, glucose (15.5 mmol/l) significantly increased \[^{3}\text{H} \text{H}\] thymidine uptake and thus cell proliferation in MC3T3 cells (Baliant et al.,
2001). The present findings do not support this earlier observation. The discrepancy may be due to differences in the experimental protocols. While \[^{3}\text{H}]\text{thymidine incorporation was studied at the end of 3 day culture in the current study, they have studied at the end of day 30 cultures.}

In the presence of insulin, the suppressive effect of high glucose (16.5 and 49.5 mmol/l) not only prevented the fall in \[^{3}\text{H}]\text{thymidine incorporation but also raised the same by } \sim 35\%. It appears that insulin treatment increases the utilization of glucose and thus prevents the apoptotic cell death due to hyperglycemic condition. In this regard it is appropriate to point out that IGF-I has protected human mesangial cells from hyperglycemia induced apoptotic cell death (Kang et al., 2003). As in the case of IGF-I, insulin might have protected bone marrow stromal cells from death.

Since growth process of cells requires high glucose utilization, the importance of glucose transport by specific transporters and their regulation assumes significance. The glucose transporter (GLUT) proteins found in cells are of different isoforms (Mueckler, 1994). Of these isoforms, GLUT1 is the major isoform found in most cells, while other GLUTs show tissue-specific distribution (Czech et al., 1992). GLUT-4 is exclusively expressed in insulin responsive tissues (James et al., 1993). The insulin-sensitive GLUT-4 is reported to be involved in early bone growth in normal as well as diabetic mice (Maor and Karnieli, 1999). Indeed, diabetic conditions in rats are associated with a decrease in GLUT-4 activity (Karnieli et al., 1981, 1986; Kahn and Cushman, 1987; Garvey et al., 1988; Maor and Karnieli, 1999).
High affinity insulin-receptors have been documented in several osteoblastic cell-lines like UMR-106 (Hickman and McElruff, 1988; Thomas et al., 1996) and Ros-17/28 (Levy et al., 1986). The distribution pattern of insulin receptor is found to be regulated by the stage of differentiation in bone marrow stromal cells. It is shown that the mature osteoblast cell-line UMR-106-01 possess higher levels of insulin receptor, insulin binding and insulin stimulated glucose uptake and insulin-upregulated GLUT-1 mRNA. In contrast, in the clonal pre-osteoblast-like cell-line, UMR-201-10B, negligible insulin binding and lower expression of insulin-sensitive glucose uptake system were reported (Thomas et al., 1996). These observations suggest that the stage of differentiation of osteoblasts influence the distribution pattern of insulin-receptor coupled effector system.

It is interesting to note that insulin while could counter the pro-apoptotic effects of high glucose, decreased [³H] thymidine incorporation in cells cultured under normal glucose (5.5 mmol/l) condition. This may be due to alterations in the sensitivity of signaling molecules to insulin action under normo-glycemic condition. Because the status of glucose is known to regulate the synthesis and secretion of insulin, they may also regulate the sensitivity of molecules in insulin signaling pathway.

Insulin and IGF-I initiate cellular responses by binding to distinct cell surface receptor tyrosine kinases that regulate a variety of signaling pathways controlling metabolism, growth and survival. Insulin receptor substrates (IRS-1 and IRS-2) are essential for intracellular signaling of insulin and IGF-I. IRS-1 expression is limited to osteoblast, while IRS-2 is expressed in
haemotopoietic cell, osteoblasts and osteoclasts as well (Sun et al., 1992; Ogata et al., 2000). IRS-2 deficiency in osteoblasts causes osteopenia through impaired anabolic function and enhanced osteoclastogenesis. Thus, IRS-2 is needed to maintain the predominance of bone formation (Akune et al., 2002). Although insulin causes potent anabolic actions on bone, it may be transiently catabolic to BMSC under normoglycemic conditions. This transient catabolic action of diminished cell survival or increased apoptosis may be due to a transient dysjunction in the signaling of insulin to its down-stream molecules like IRS-2. However, it needs further study to prove this contention. Thus, under normal glucose concentration, the stimulation of cell proliferation by insulin might have been attenuated.

In the present study, BMSCs might have been in the different phases of differentiation in response to the available glucose concentration. This in turn could have influenced the distribution pattern of insulin-receptor coupled glucose utilization system. Thus, these arrangements may differ between BMSCs grown in 5.5 mmol/l and 16.5/49.5 mmol/l glucose. If the presumption is correct, insulin might have increased the utilization of glucose by activating GLUT 4 and induced proliferation of cells grown in high glucose media (16.5 and 49.5 mmol/l) and suppressed the same in cells grown in normal glucose containing media. There is also a report divergence with this view, wherein GLUT 4 is reported to be much under the control of IGF-I rather than insulin in a diabetic mice model study (Maor and Karnieli, 1999). Clarification of these assumptions and divergent reports need to be ascertained through further investigations.
Unlike insulin, addition of estradiol to BMSC cultures did not modulate the suppressive effect of high glucose on cell proliferation. There are reports for and against the role of estradiol on bone cell proliferation. Estradiol has been reported to stimulate cell proliferation in primary and immortalized fetal rat calvarial cells (Ernst et al., 1989), normal adult human osteoblasts (Schevan et al., 1992), mouse MC3T3E1 cells (Majeska et al., 1994) and human osteosarcoma cells (Ikegami et al., 1994). In contrast to these proliferative effects of estradiol on bone cells, estradiol was found to have no significant effect on cell proliferation in rat BMSC derived osteoblasts (Rickard et al., 1995). The later observation is in agreement with the present study, wherein estradiol had no significant effect on BMSC proliferation. It appears that increased glucose utilization is the principal mechanism that mitigate high glucose-induced fall in \[^{3}\text{H} \] thymidine incorporation and thus DNA synthesis.

Estradiol plays an important role in the regulation of insulin and glucose homeostasis in a dose and duration dependant manner. While high doses of estradiol impair insulin sensitivity, low doses improve it. It could increase the levels of insulin receptor and its gene expression in the cell membrane and the phosphorylation rate of insulin receptors at low doses. The reverse is true in high doses in liver, muscle and adipose tissues of ovariectomized rats (Gonzalez et al., 2002). Estradiol has also been reported to regulate the expression of GLUT1 in endothelial cells and immature rat uterus (Welch and Gorski, 1999).
Alkaline phosphatase is expressed early in the developing osteoblast during the phase of matrix deposition and down regulated in calcifying osteoblasts. After 3 days in culture, rat BMSCs cultured in the presence of high glucose (16.5 and 49.5 mmol/l) registered a small but significant decrease, compared to 5.5 mmol/l glucose. These cells also exhibited reduced $[^3]H$ thymidine incorporation. Thus, exposure to high glucose concentration appears to depress cell proliferation as well as its differentiation. The high glucose might have affected the normal phases of differentiation in these BMSCs. Nevertheless, as in the case of $[^3]H$ thymidine incorporation, insulin treatment to these cultures increased ALP activity. Insulin stimulates cell proliferation as well as its differentiation.

There is a reciprocal correlation between osteoblast proliferation and differentiation. There are tissue-specific transcription repressors and activators of proliferation and differentiation (Siddhanti et al., 1994; Lian and Stein, 1995), which act reciprocally to counter these different functions. These observations deviate from the held view of reciprocal correlation between osteoblast proliferation and differentiation. Insulin appears to reverse the changes brought about by high glucose concentration rather than maintaining the reciprocal correlation between cell proliferation and differentiation. BMSCs exposed to normoglycemic condition (5.5 mmol/l) and treated with insulin also responded with diminished ALP activity like $[^3]H$ thymidine incorporation. Thus, insulin exerts similar effects on BMSCs proliferation and differentiation.
Addition of estradiol to high glucose (49.5 mmol/l) and normoglycemic (5.5 mmol) cultures decreased cellular ALP activity. This effect differs from estradiol action on cell proliferation wherein it did not have any significant effect. The decrease in ALP activity is well pronounced in high glucose exposed BMSCs. While estradiol is reported to stimulate differentiation and mineralization of matrix in mouse bone marrow cultures (Qu et al., 1998), such a stimulation on differentiation is not evident in the presence of high glucose. Estradiol has been shown to restore normal glucose uptake that was decreased by ovariectomy in the skeletal muscles of rats (Campbell and Febbraio, 2002). In the present study under *in vitro* condition estradiol does not appear to counter the effects of high glucose on cell proliferation as well as on cellular ALP activity. The hyperglycemic condition may modify either the action of estradiol or the response of cells to estradiol. Nevertheless, its stimulatory effect on the number of nodules formed reiterates the positive regulatory effects of estradiol on matrix mineralization.

*In vitro* mineralization of matrix is achieved in cells of osteoblastic lineage, which have undergone well-regulated proliferation, and early phases of matrix formation. During this period (up to 18-21 days) no significant amount of calcium is deposited in to the extracellular matrix. Nevertheless, the phase of intensive matrix calcification starts at day 21. Exposure of BMSC to glucose, insulin and estradiol during this period could influence the changes at molecular level and phenotypic characteristics of the mineralized nodules such as shape and pattern of calcification.
In the present study, high glucose might have activated collagenase and contributed for the decrease in collagen. BMSC cultures exposed to high glucose (16.5 and 49.5 mmol/l) exhibited reduced number of nodules formed, compared to cultures exposed to normal glucose. This is in correlation with reduced cell proliferation and differentiation under these conditions. So also treatment with insulin increased the number of nodules formed in BMSC cultures exposed to high glucose concentrations. The severity of high glucose on bone nodule formation varied according to the level of glucose contained in the culture media.

Osteoblasts cultured on glycated collagen registered reduced proliferation and differentiation (Katayama et al., 1996). The high glucose may also have interfered with collagen synthesis or degradation. Collagenase activity was reported to be increased in the skin and periodontium of diabetic rats (Bain et al., 1997).

Surprisingly, estradiol which did not show much effect on the possible growth arrest or apoptotic cell death and diminished cellular ALP activity induced by high glucose concentrations, registered a remarkable increase in the nodule formation. It appears that estradiol is found to have positive effects on the later phases of in vitro mineralization wherein it might have mitigated the deleterious effects of high glucose on the maturation of osteoblasts and calcification of matrix deposited by them. It is not clear how estradiol have managed to overcome the high glucose-induced impairment on nodule formation. Estrogen has been reported to induce the expression of insulin down-stream signaling molecules, IRS-1 and IRS-2 (Lee et al., 1999; Molloy
et al., 2000). Estrogen induction of IRS-1 expression was associated with increased tyrosine phosphorylation of IRS-1 and correlated with enhanced down-stream mitogen activated protein kinase (MAPK) activation in human breast cancer cells. Probably, the molecule(s) and the mechanism(s) involved in estradiol regulated glucose utilization might have responded to the action of estradiol during the late phases of matrix mineralization. However, this speculation needs to be confirmed with further investigation.

Conclusion and future directions

The present study clearly demonstrates that hyperglycemic condition interferes with the successful differentiation of BMSCs into osteoblast lineage and their mineralization into nodules. Insulin or estradiol ameliorates the damage caused by high glucose. The effects of estradiol are comparable to that of insulin. High glucose appears to affect not only the matrix formation but also mineralization.

The findings are inconclusive, until the specific bio-molecules such as non-collagenous proteins and proteoglycans involved in mineralization are identified. The molecules may exhibit different pattern of expression under high glucose condition. It is also essential to know the role of estradiol on glucose uptake, GLUT and its cross-talk with insulin signaling pathway. It is also pertinent to know the effect of high glucose on the regulation of local growth factors, especially the IGFs and their binding protein production by osteoblastic lineage cells.
SUMMARY AND CONCLUSION

Impaired bone formation resulting from a decline of osteoblast activity has been implicated in the pathogenesis of diabetic osteopenia. In the in vitro model, supplementation of the culture media with higher concentration of glucose mimics the in vivo hyperglycemic situation. In the present study, different doses of glucose (5.5, 16.5 and 49.5 mmol/l) were added to the culture media containing rat BMSCs. Insulin or estradiol was added to the culture media. BMSC proliferation, ALP activity and the number of nodules formed were estimated. Histochemical staining for collagen, GAGs and mineralization were done.

- High glucose significantly decreased the cell growth of BMSCs in a dose-dependent manner. Insulin treatment alone significantly increased the [3H]-thymidine uptake under high glucose condition.

- ALP activity was significantly decreased in the BMSCs cultured at high glucose concentrations. Insulin treatment alone significantly increased the ALP activity in the BMSCs cultured with high glucose concentration.

- The number and size of nodules were decreased in BMSCs cultured with high concentrations of glucose. Treatment with insulin or estradiol prevented the same.
- Under high glucose condition, histochemical staining for collagen and GAGs were very much reduced. Insulin or estradiol treatment reversed the same and was comparable to the control level.

In conclusion, high glucose appears to affect both matrix formation and mineralization. Insulin or estradiol ameliorates the damage caused by high glucose. The effects of estradiol are comparable to that of insulin.