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A Study on the Effect of High Glucose on Osteoblasts like Cell Proliferation and Apoptosis

Madhu Gupta, Vineeta Rawat and Md Arshad*
Molecular Endocrinology Laboratory, Department of Zoology,
University of Lucknow, Lucknow, (UP) - India

Abstract

Incidences of diabetes (both type I and type II) are increasing worldwide. The increased blood glucose affects the functioning of all tissues. Various complications of diabetes include neuropathy, nephropathy, retinopathy, micro-vascular complications and osteopathy. It is now becoming clear that high blood glucose have an osmotic effect on bone cells. To know what would be likely effect of glucose on bone cells, MTT assay for cell proliferation, haematoxylin and eosin double staining and Hoechst-propidium iodide double staining for apoptosis was carried out. It is reported that high glucose induces in a concentration dependent manner apoptosis and inhibits proliferation in MG-63 cells, a human osteoblasts like cell line. The harmful effects of glucose were found reduced when these cells were exposed to insulin. Thus our study suggests that glucose may induce osteopenia in diabetes by increasing apoptosis and reducing cell proliferation.

Key-Words: Hyperglycaemia, Cell proliferation, Apoptosis, Osteopenia

Introduction

Bone is a multifunctional, dynamic tissue that is being continuously remodelled throughout the life span of an individual. Bone remodelling is the result of an intricate balance between two processes viz., bone formation by osteoblasts and bone resorption by osteoclasts. Shifting balance in either direction will lead to bone diseases like osteoporosis or osteosarcoma. Various pathological conditions pose threat to bone health and hyperglycaemia as a consequence of diabetes is one such threat.

Diabetes is an epidemic disorder with a range of complications. A recently noticed problem of diabetic patients is osteopenia [1,2]. The chances of diabetes occurring in both men and women are common though the females at the postmenopausal stage are at increased risk of developing diabetic osteopathy [3,4]. In the Iowa women's Health Study, women with type I diabetes were 12.25 times more likely to report having had a fracture than women without diabetes [5]. Being an anabolic agent for bone [6] insulin exercises control over extracellular blood glucose and plays a key role in regulating fracture healing [7,8] and peri-implant bone repair [9] in diabetes mellitus. Studies have shown that insulin can influence bone growth by directly binding to its receptors present on osteoblasts [10,11].

*** Corresponding Author**
E-mail: molendolab@gmail.com
Mob.: +91-9453303114

What causes osteopenia in diabetic patients and how it develops? Although a concrete mechanism is lacking but the answer to the first part of this question may largely rely on the osmotic effect of glucose [12]. This study was carried out to answer though in part, how presence of high glucose leads to osteopenia.

Material and Methods

Materials

Minimum Essential Medium (MEM), Foetal Bovine Serum (FBS), sodium pyruvate, non-essential amino acids, sodium bicarbonate, L-glutamine, antibiotic solution (streptomycin/ penicillin), ascorbic acid, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye, haematoxylin and eosin were all purchased from HIMEDIA Laboratories Pvt. Ltd., Mumbai, India. Insulin used was 100 IU/mL of Huminsulin (regular) from Eli Lilly and Company, India. Double Stain Apoptosis Detection Kit (Hoechst 33342/PI) (Cat. No. L00309) were from GenScript, USA. All the other reagents were of analytical grade.

Cell culture

MG-63 cell line was procured from NCCS, Pune. The cell lines was maintained in MEM media and grown at 37ºC in a humidified air containing 5% CO_2 till confluence. Cells were detached using Trypsin-EDTA solution and used for sub-culturing or treatment.

MTT assay

MTT or cell proliferation/cytotoxicity assay was carried out to study the effect of glucose in a

concentration dependent manner [13]. Briefly, cells were seeded at a density of 5 × 10^3/well in 96 well microplate for 24 h. Cells were exposed to varying concentrations of glucose for next 44 h. Ten µL of MTT (5 mg/mL) was added in each well. After 4 h supernatant was discarded carefully and formazan crystals were dissolved in100 µL of DMSO and absorbance was read at 490 nm by microplate reader (BIORAD 680). The percentage cell viability was calculated by following formula:

$$\frac{\text{Absorbance of Treated} - \text{Absorbance of blank}}{\text{Absorbance of Ph. Ctrl} - \text{Absorbance of blank}} * 100$$

The group INS (insulin - 0.5 IU per 200µl + 5.5 mmol/L glucose) served the positive control in all experiments.

Haematoxylin-eosin double staining

Following 48 hour exposure of varying concentration of glucose, media was aspirated and cells were washed two to three times with phosphate buffered saline (PBS) and one time with distilled water. The sequential steps of staining are as follows:

$$\text{Haematoxylin} \xrightarrow{1\,min} \text{acid water} \xrightarrow{30\,sec} 50\% \xrightarrow{2\,min} 70\% \xrightarrow{2\,min} 90\% \xrightarrow{2\,min} 2\%\ \text{eosin} \xrightarrow{2\,min} 90\% \xrightarrow{2\,min} 100\%$$

Cells were visualized by phase contrast microscope (Nikon, ECLIPSE T*i*-Series). All the experiments were performed in quadruplets and in 96 well culture plates with lid.

Hoeschst-PI double staining

Hoeschst-PI double staining made it easier to detect normal, apoptotic and dead cells in the same culture well. Hoeschst is a kind of vital dye that stains chromatin of apoptotic cells more brightly than normal cells. Propidium iodide on the other hand stains the chromatin of dead cells only. Staining procedure was based on the manufacturers (GenScript) protocol. After exposing cells to varying concentration of glucose for 48 hours, media was aspirated and cells were washed with cold PBS twice. 1µl Hoechst 33342/100 µl PBS was loaded in each well and incubated at 37°C for 10 minutes and then aspirated. 100 µl of 1X buffer A mixed with 0.5 µl PI was then loaded. Plate was incubated at room temperature in dark conditions for 5 minutes. Immediately after staining cells were visualized under inverted fluorescence microscope (Nikon, ECLIPSE T*i*-Series).

Statistical analysis

Statistical analysis was done using Graphpad Prism 5.0 software and data was represented as mean ± SD. One way analysis of variance following Tukey's post hoc test was performed. $P<.05$ was considered significant.

Results and Discussion

Photographs from haematoxylin and eosin (HE) double staining reveal that the numbers of cells were decreasing with the increase in glucose concentration (Figure 2). It was reduced to below 50% in groups G, H and I (glucose concentration 45mmol/L, 55mmol/L and 65mmol/L respectively). The data was quantified by calculating percentage cell viability in MTT assay which also verified results from haematoxylin and eosin staining. This shows that cell proliferation was reduced under high glucose. Percentage cell viability in INS (insulin - .5 IU per 200µl + 5.5 mmol/L glucose) and 5.5 mmol/L glucose group was almost similar (Figure 1).

Hoechst-PI double staining showed increased rate of apoptosis with increasing glucose concentration (Figure 3). At higher glucose concentrations (35 mmol/L and above) the cells with bright blue and white fluorescence were undergoing apoptosis and the cells showing pink fluorescence were dead cells. Cells were in good numbers and seen healthy in groups A, B and C (control, insulin and group with physiological glucose concentration i.e., 5.5 mmol/L).

Diabetes is a serious metabolic disorder and is most common in the world. It can be due to complete lack of insulin (type I diabetes mellitus) [14] or due to insulin resistance (type II diabetes mellitus) [15] and result in hyperglycaemia. It has been found that hyperglycaemia affects various tissues functioning viz., eyes, kidneys, heart and nerves leading to retinopathy, nephropathy, atherosclerosis and neuropathy respectively [16]. Recently it has been observed that bone formation was impaired during diabetes due to hyperglycaemia [12,17,18]. In our study, we evaluated the effect of hyperglycaemia on human osteoblasts like MG-63 cells. Cell proliferation rate was found deteriorated below 50% versus control ($P< 0.001$) as the concentration of glucose was increased above 25 mmol/L. At 5.5 mmol/L glucose concentration, percentage cell viability was raised to 153%. This indicated that glucose at physiological level promotes cell growth and was related to low percentage of cells undergoing apoptosis. As the concentration of glucose increased, the resulting increase in osmolarity caused osmotic shock to cells leading to cell death. This is related to the reduction in bone mineralization [18]. When cells treated with insulin were studied the proliferation and apoptosis rates were restored to physiological control group (Fig.1). This shows the anabolic action of insulin on bone cells [11,19]. This is due to the presence of a functional insulin receptor on bone cells [10].

Conclusion

Thus we may say that it is the reduced rate of proliferation of osteoblasts like cells and increased rate of apoptosis responsible for impaired bone remodelling under high glucose.

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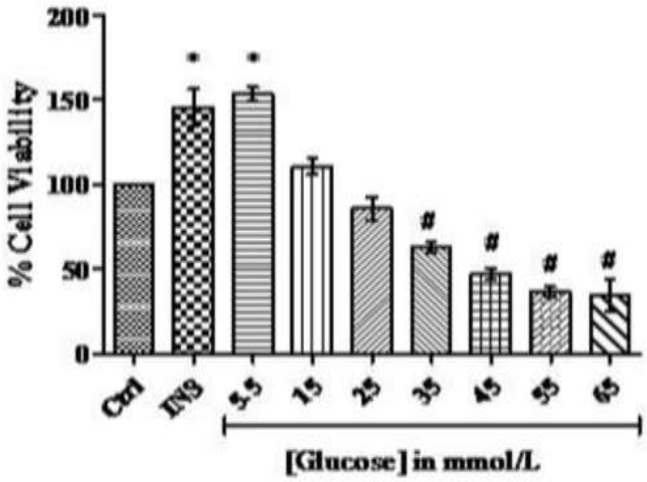

Fig. 1: Effect of increasing glucose concentration on cell proliferation of MG-63 cells. INS is insulin and 5.5 mmol/L represents physiological glucose concentration. *P* < 0.0001 (* percentage cell viability was significantly increased in INS and 5.5 mmol/L glucose group than Ctrl; # cell proliferation rates went on decreasing with the increasing concentration of glucose.)

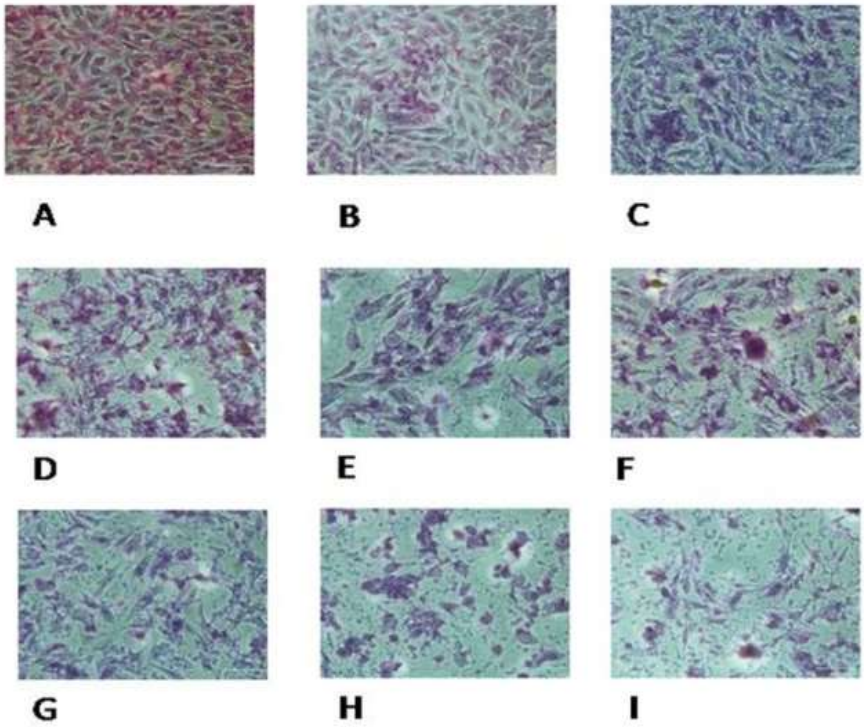

Fig. 2: Haematoxylin-eosin double staining. (A= control, B= insulin, C= 5.5 mmol/L, D=15 mmol/L, E=25 mmol/L, F= 35 mmol/L,G= 45 mmol/L, H= 55 mmol/L, I= 65 mmol/L)

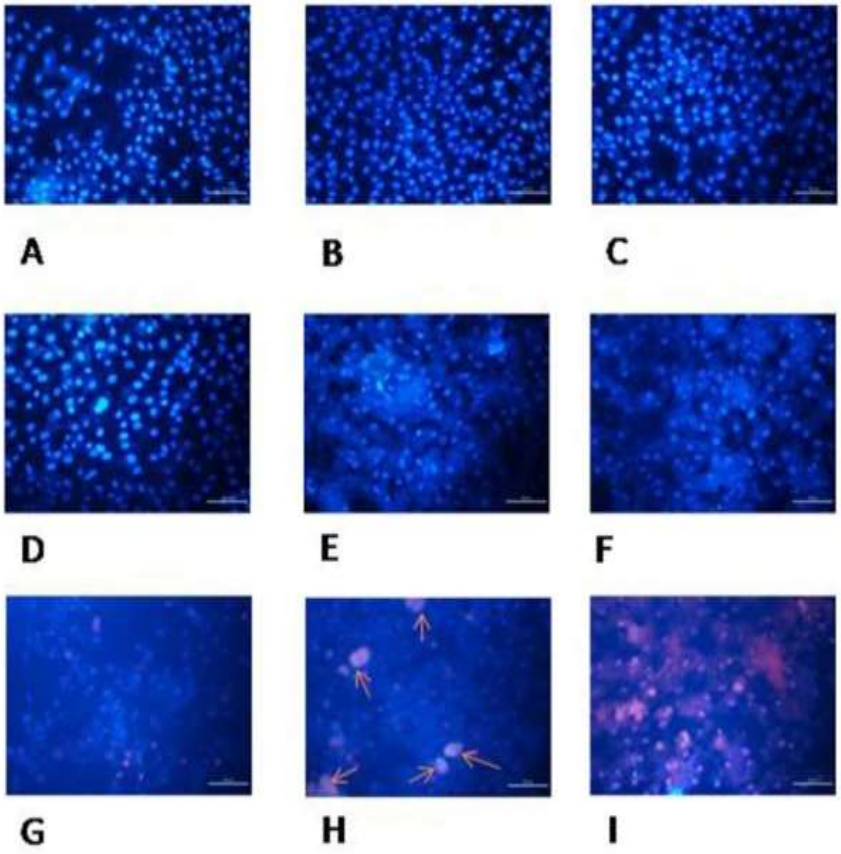

Fig. 3: Hoechst-propidium iodide double staining. (A= control, B= insulin, C= 5.5 mmol/L, D=15 mmol/L, E=25 mmol/L, F= 35 mmol/L, G= 45 mmol/L, H= 55 mmol/L, I= 65 mmol/L). Red coloured arrows indicate apoptotic cell mass.

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