A study on the osteogenic activity of *Trigonellafoenum-graecum* L. seed extract on osteoblast like cells

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**Abstract** The anti-proliferative and apoptosis inducing effect of hyperglycaemia on osteoblasts cells were well documented. This experiment was based on the idea that antidiabetic constituents from *Trigonellafoenum-graecum* seed extract would lower the effect of high glucose thus restore the osteoblasts like cell proliferation and differentiation. The effect of fenugreek seed extract was studied on MG-63 cells – osteoblasts like cells exposed for 24 hours to high glucose. MTT assay and propidium-iodide staining was performed to study cell proliferation and apoptosis respectively. Alkaline phosphatase assay and alizarin red staining both were performed to study cell differentiation and matrix mineralization. In order to study the possible role of fenugreek seed extract in lowering reactive oxygen species generation under hyperglycaemia, DCFH-DA staining was carried out. The result showed that fenugreek seed extract lowered the high glucose induced inhibition of cell proliferation (*P*<0.001). It also restores cells from oxidative stress generated due to osmotic shock (*P*<0.001). This in turn leads to increase in alkaline phosphatase activity and bone nodule formation (*P*<0.001). These effects are comparable to as observed in insulin treated groups. Thus this study suggests that fenugreek seed extract offer a prospective natural alternative to diabetes associated osteopathy.

**INTRODUCTION**

Today, 382 million people of the world population are having diabetes and this number is expected to rise to 592 million by 2035 (1). Several lines of circumstantial evidence suggest the deterioration in bone quality and increased fracture rates due to diabetes (2-6). Increase in apoptosis (7), reduced osteoblasts activity and increase in adipocyte numbers, increased osmotic shock (8), and oxidative stress (9) are some reasons behind diabetes induced bone loss. High glucose is the key mediator of these effects in diabetic subjects (10). The medications available for treatment of any kind of diabetes include meglitinides, sulfonylureas, dipeptidyl peptidase-4 (DPP-4) inhibitors, biguanides, thiazolidinediones, amylin mimetics and incretin mimetics and all are associated with certain complications ranging from minor side effects like nausea, diarrhoea, weight gain etc. to major complications such as hyperglycaemia, heart attack, osteoporosis etc. chromatography (HPLC). Most existing methods require tedious.

Trigonellafoenum-graecum L. (family: Fabaceae) commonly known as fenugreek is an annual herb native to Indian subcontinents, leaves and seeds of which are consumed as food. The seeds of fenugreek are extensively used as a source of antidiabetic compounds (11,12). The GC/MS analysis of hydroalcoholic extracts of fenugreek seeds revealed the existence of sapogenins, dia genin, trigonelline, 4OH-Ile (4 hydroxy-isoleucine), dietary fibres and minerals and vitamins (13). Besides this fenugreek seeds possess anti-inflammatory (14), anti-hyperlipidimic, anti-oxidative, antiapoptotic (15), estrogenic (16) and antineoplastic (17) properties.

At present, there is no scientific information available on the effect of fenugreek seed extract on bone cells so we carried out our experiment on osteoblasts like MG-63 cells and standardised in vitro model of diabetes to evaluate the effect of fenugreek seed extract under high glucose; a condition comparable to TIDM (type I diabetes mellitus) where bone cells exposed to high blood glucose due to complete absence of insulin which results from selective autoimmune destruction of pancreatic β cells.

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MATERIAL AND METHODS

Chemicals and Reagents

Minimum Essential Medium (MEM), foetal bovine serum (FBS), sodium pyruvate, non-essential amino acids, sodium bicarbonate, L-glutamine, antibiotic solution (streptomycin/penicillin), ascorbic acid, alizarin red powder, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye and dexamethasone were all purchased from HIMEDIA Laboratories Pvt. Ltd. Mumbai, India. Cetylpyridinium chloride (CPC) was purchased from Sisco Research Laboratories Pvt. Ltd. Mumbai, India. Insulin was used 100 IU/mL of Huminsulin (regular) from Eli Lilly and Company, India. 2', 7'-Dichlorofluorescin diacetate (DCFH-DA), Propidium iodide (PI) and β-glycerophosphate were from Sigma-Aldrich Inc. St Louis, USA. DMSO (Dimethyl sulfoxide) was from Merck Specialities Pvt. Ltd. Mumbai, India. All the other reagents were of analytical grade.

Cell Culture

MG-63 cell line was procured from NCCS Pune, India and maintained in MEM medium containing 10% FBS and 1% antibiotic solution at 37°C; 5% CO2 in a CO2 incubator.

Collection and preparation of extract

Seeds of fenugreek were purchased from local market of Lucknow, India and were systematically identified by MuhammadArif, Assistant Professor, Department of Pharmacognosy, Integral University, Lucknow. A specimen (accession no. IU/PHAR/HRB/14/09) was deposited in the same department for reference. Dried seeds were grinded to fine powder. Ethanolic extraction was carried out with 95% ethanol using a Soxhlet apparatus (12) continuously for 48 hours. Extract was pooled evaporated to concentrate at 60°C in a water bath. Extract was kept and stored at 4°C for further usage.

Experimental protocol

MG-63 cells, after seeding were divided into 5 groups; each with at least three wells. The groups are - 1) Ph. Ctrl: Physiological control (cells were given 5.5 mmol/L of glucose - the concentration of glucose in the blood of a healthy human being); 2) HG: high glucose (45 mmol/L); this is the concentration at which ~50% of the cells were surviving. This was chosen so as to develop a diabetic model. In this and rest of the three groups cells were exposed to only HG for 24 h which is similar to bone cells exposed to high glucose present in blood of a diabetic patient. We standardized hyperglycaemic in vitro model with slight modification in protocol developed by Botolin and McCabe (2006). HG group also represents negative control in our experiment; 3) INS: insulin (high glucose exposed cells treated with 0.5IU insulin); 4) 1 µg: cells treated with 1 µg fenugreek; 5) 10 µg: cells treated with 10 µg fenugreek.

MTT assay for cell viability

This assay was performed according to Faucheux et al. (18). Briefly, cells were seeded in 96 well plates at a density 5 x 10³/well for 24 h. Cells were then exposed to high glucose (45 mmol/L) for next 24 h. Treatment was given in the high glucose medium as mentioned in the experimental protocol section. 10µl of MTT reagent was added in each well after 44 hours and left for next 4 hours for reaction to occur. Supernatant was discarded and formazan crystals were dissolved in 100 µl of DMSO. Absorbance was read at 490nm using microplate reader (BIORAD-680). Following formula was used to calculate the percentage cell viability.

Percentage cell viability = \( \frac{A_T - A_R}{A_C - A_R} \times 100 \)

Where \( A_T \) is absorbance of treated, \( A_R \) is absorbance of blank and \( A_C \) is absorbance of physiological control.

Propidium Iodide staining for apoptosis

Propidium iodide (PI) is fluorescent molecule used to stain cells in either late apoptosis or undergoing necrosis. This staining was carried out according to Kaviaras et al., (15) After following above mentioned protocol, cells were washed with PBS and fixed in 50µl of 4% paraformaldehyde for 10 min. After fixing 1 µl of PI dye in 100 µl of PBS was loaded in each well and left for 10 min at 37 °C for 10 min. Images were snapped using fluorescence microscope (Nikon, ECLIPSE Ti-Series). Images were analysed using microscope’s NIS Elements Documentation software and percentage of apoptotic cells were calculated in each group.

Alkaline phosphatase (ALP) Assay

ALP assay was performed according to Faucheux et al. (18). Briefly, after treatment, the plate was washed with PBS and kept at -70°C for 20 min followed by exposure to 37°C for 10 min. 75µL of chilled pNPP substrate (9.4 mg pNPP/5 mL of milliQ water) was loaded in each well. Cells were incubated for another half an hour at 37°C for colour development. Finally, absorbance was taken at 405 nm in micro plate reader (BIORAD 680).

Alizarin staining for matrix mineralization

It was according to Jeong et al. (19). Cells were seeded at a density of 10⁶ cells per well in 6 well plate and were cultured in differentiation medium containing 10 mmol/L β-glycerophosphate, 50 µg/mL of ascorbic acid and 10⁻³ mol/L dexamethasone. Media was changed on each alternate day for a fortnight. On day 15, cells were fixed in 10% formalin followed by staining with alizarin red stain (40 mmol/L) 10 min washed 5 times with distilled water. Images were taken under inverted microscope (Nikon, ECLIPSE Ti-Series). Cells were appeared bright orange due to formation of alizarin red S-calcium complex which is then solubilised using 10% cetylpyridinium chloride in 10 mmol/L sodium phosphate for 30 min. The absorbance was read at 610 nm in a multi-well ELISA plate reader to determine the concentration of released alizarin red stain.

DCFH-DA staining for reactive oxygen species (ROS)

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To assess oxidative stress due to ROS generation DCFH-DA dye was employed (15). 12h after treatment, freshly prepared DCFH-DA (10 mmol/L) was loaded in 24 well plates for 30 minutes at 37°C. Cells were photographed under fluorescence microscope (Nikon, ECLIPSE Ti-Series). Relative percentage of ROS generating cells was calculated using NIS Elements Documentation software provided with microscope.

**Statistical analysis**

All the data were presented as the means ± S.D. One way ANOVA method followed by the Tukey’s test of significance was employed using GraphPad Prism 5.0 software. Probability values of $P < 0.05$ were considered statistically significant.

**RESULTS**

**Fenugreek seed extract restores cell proliferation and reduces rate of apoptosis**

MTT assay was performed for evaluating cell proliferation under high glucose. The basis was the reduction of yellow tetrazolium salt MTT to purple coloured insoluble formazan crystals in part by the action of dehydrogenases present in metabolically active cells. Thus, there was a linear relationship between formazan crystals formed and number of viable cells which was expressed here as percentage cell viability. In high glucose there was reduction in percentage cell viability (55.4%±4.1; $P<0.01$ versus Ph.Ctrl) (Fig. 1). When treated with 0.5IU insulin the cell proliferation rate was restored approximately to the Ph.Ctrl (94.5%±5.6). Percentage cell viability was found increased in both TFG treated groups i.e., 82.6%±11.9 in 1 µg ($P<0.05$ vs. HG) and 121.3%±16.7 in 10µg treated group ($P<0.001$ vs. HG) (Fig. 1).

For cells undergoing apoptosis, plasma membranes of these cells become more permeable to PI stain. From the images it can be observed that shapes of the cells were changed. It was more rounded than polygonal, an initial mark of apoptotic cells, as can be seen in images snapped for high glucose group (Fig. 2A). An increase in percentage apoptotic cells (42.8%±1.2; $P<0.001$ vs. Ph. Ctrl) was observed in HG group (Fig. 2B). All the treatment groups (INS, 1µg and 10µg) were showed reduction in the rate of apoptosis ($P<0.001$ vs. HG) under hyperglycaemic condition.

**Fenugreek seed extract increases bone mineralization and differentiation**

*In vitro* increase in ALP activity and matrix mineralization were the sign of enhanced rate of osteogenesis in Ph. Ctrl. Cells expressing ALP were able to dephosphorylate pNPP (pNitro Phenyl Phosphate) to yellow soluble p-nitrophenol. Bone nodule formation was directly correlated with the formation of alizarin red - calcium complex. The inhibitory effect of HG on MG-63 cells was explicit as shown by the reduction in alkaline phosphatase activity ($P<0.001$ vs. Ph. Ctrl) (Fig. 3) and rate of mineralization ($P<0.001$ vs. Ph. Ctrl) (Fig. 4). Both ALP activity and bone nodule formation were improved in all the treatment groups ($P<0.001$ vs. HG). Fenugreek seed extract at 10 µg dose was most effective in recovering cells from high glucose induced stress and enhancing ALP activity and mineralization process.

**Fenugreek seed extract recovers cells from high glucose induced oxidative stress**

When DCFH-DA dye was applied to cells, cells in HG group were able to convert it into DCF, a fluorescent compound, due to excess reactive oxygen species (ROS) generation (Fig. 5). In Ph. Ctrl group Percentage of cells showing DCF fluorescence were 3.8±0.9 and in HG was 71.7%±3.5. This was reduced in both insulin and fenugreek treated groups ($P<0.001$ vs HG).

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We also observed that in all the experimental setups, the effect of treatment of insulin and 10µg fenugreek seed extract was consistent and comparable to Ph.Ctrl.

**DISCUSSION**

Osteoporosis has now become established as a secondary complication of diabetes. The aim of present study was to investigate prospective anti-osteoporotic effects of fenugreek seed extract using an in vitro model of diabetes. The effect of high glucose was evident from the above experiment. It was reported that hyperglycaemia induced inhibition of osteoblast like cell proliferation was the result of increased production of reactive oxygen species, reduced rate of bone mineralization and alkaline phosphate activity thus increased rate of cell death i.e. apoptosis (8, 20).

Fenugreek’s is well known for possessing antidiabetic and antihyperlipidimic bioconstituents but its effect on bone cells under diabetic conditions is yet not evaluated. Thus we decided to carry out our experiment on MG-63 cells; the osteoblast like cells to study the effect of TFG extract on the osteoblastogenesis, mineralization, reactive oxygen species generation and apoptosis. Our results indicated that TFG extract not only recovers cells from high glucose induced oxidative stress and apoptosis but also augmented the process of bio-mineralization thus improving cell’s health under hyperglycaemia. We also reported that the effect of TFG extract given in 10µg doses was almost equivalent to insulin treatment. This means that fenugreek seed extract can be considered as better alternative to insulin therapy for diabetic patients as a natural remedy.

Now coming to the question, what in TFG causes MG-63 cells to recover from inhibitory effects of high glucose? Much of the antidiabetic and hypoglycaemic effect (12) of TFG has been largely attributed to its fibre contents and saponins (diasgenin). Though the research works on effect of fenugreek on bone like cells was unavailable to the best of our knowledge, but its effect as anti-hyperglycaemic agents was well established. Also fenugreek seed extract is rich source of saponins, flavonoids and alkaloids (13). Some flavonoids such as quercetin and kaempferol were reported to stimulate osteoblast proliferation (21). In our study also the seed extract stimulated MG-63 cells proliferation. In an in vivo study on alloxan induced diabetic rats, fenugreek seed powder has also been demonstrated to modulate the expression of glycolytic and gluconeogenic enzymes. This could be one mechanism among others that results in lowering of blood glucose levels. Another mechanism that may reduce the severity of high glucose is the cellular uptake of glucose via GLUT-4, an insulin sensitive glucose transporter also present in osteoblasts.
CONCLUSION

Finally, it may be said that fenugreek seed extract was proved to be an alternative remedy to insulin for curing secondary osteoporosis associated with diabetes. Further experiments were required at clinical level to evaluate its effect on bone loss in type I diabetic subjects.

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