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Research Article

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Antidiabetic action and significant cell viability of bezafibrate-in HEPG2 cell line analysis compared with a biguanide-A potential in the treatment of metabolic syndrome/syndrome X

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ABSTRACT

Metabolic Syndrome (MetS) has been highlighted as a major socioeconomic problem throughout the world as the burden of MetS along with its individual risk factors-central obesity, insulin resistance, dyslipidaemia, and hypertension is evident throughout all ethnicities studies. This study was conducted to evaluate the anti-diabetic property and the cell viability of Bezafibrate, an activator of Lipoprotein Lipase(LPL), when compared to a standard drug used in the treatment of type II Diabetes Mellitus, Metformin (Biguanide – Oral Hypoglycaemic Agent). In-Vitro analysis with HepG2 tissue culture was done. Glucose consumption of the test drug Bezafibrate was analysed using Glucose oxidase method and the cell viability was determined by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay. The test drug Bezafibrate showed increased glucose consumption level in HepG2 cell lines and had a higher cell viability percentage than the control drug, Metformin.

Keywords: Metabolic syndrome, Bezafibrate, PPAR-α agonist, Anti-Diabetic action, cell viability, In-Vitro analysis, Glucose Oxidase method, MTT assay

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INTRODUCTION

The term Metabolic syndrome (MetS), first coined by Haller and Hanefeld in 1975, is characterised as a combination of underlying risk factors that when –occurring together- culminate in adverse outcomes, including Type II Diabetes Mellitus (T2DM), cardiovascular disease (CVD) and thus an approximately 1.6-fold increase in mortality. Insulin resistance is thought to be underlying feature of the metabolic syndrome. Genetic abnormalities, foetal malnutrition, and visceral adiposity may play roles in the pathophysiology of insulin resistance and the metabolic syndrome. The concurrence of disturbed glucose and insulin metabolism, overweight and abdominal fat distribution, mild dyslipidaemia, and hypertension and its association with subsequent development of type 2 Diabetes Mellitus and cardiovascular disease has given rise to the concept of the metabolic syndrome also known as the Insulin Resistance syndrome. The National Cholesterol Education Program's Adult Treatment Panel III report (ATP) identified the metabolic syndrome as a multiplex risk factor for cardiovascular disease (CVD) that is deserving of more clinical attention. In 1988, Reaven noted that several risk factors (dyslipidaemia, hypertension, and hyperglycaemia) commonly cluster together. This clustering is called Syndrome X, Reaven and others postulated that insulin resistance underlies Syndrome X (Hence the commonly used term Insulin resistance syndrome). [1-3, 11]

According to the Third National Health and Nutrition Examination Survey(NHANES III) criteria, about 47 million people have metabolic syndrome, including 44 percent of those in the >/= 50 year age group. Metabolic syndrome is present in 10percent of women and 15percent of men with normal glucose tolerance; 42 percent and 64percent of those with impaired fasting glucose; 78percent and 84percent of those with type II Diabetes. [12]

Components of metabolic syndrome: ATP III identified 6 components of metabolic syndrome that relate to CVD: abdominal obesity, atherogenic dyslipidaemia, raised blood pressure, insulin resistance +/- glucose intolerance, proinflammatory state, prothrombic state. The major risk factors are cigarette smoking, hypertension, elevated LDL cholesterol, low HDL cholesterol, Family history of premature coronary heart disease (CHD), and aging. [4]

The ability of bezafibrate to reduce Triglycerides, Cholesterol, and blood glucose levels in patients with diabetes was first reported over 30years ago, and the drug has been used for treating dyslipidaemia, particularly to improve TG and HDL-C levels. Bezafibrate functions as an agonist of PPAR nuclear transcription factors, which play an important role in glucose and lipid metabolism. This In-Vitro study in Hepatic cell tissue was henceforth conducted to observe the glucose lowering capacity and bezafibrates' improved cell viability. Both of which are a potential in the treatment of metabolic syndrome. [5]

EXPERIMENTAL SECTION

GLUCOSE CONSUMPTION ASSAY

Glucose levels are maintained within tight limits to ascertain sufficient energy supply to the brain and other peripheral organs and to prevent accumulation to toxic concentrations. Homeostasis is achieved by the hormones insulin and glucagon, affecting glucose uptake, release and metabolism in glucose-responsive tissues. The response of mammalian cells to fluctuations in glucose levels is of major relevance, since alterations are important for pathological conditions, i.e. obesity or type-2 diabetes (Hitomi Takanaga, 2007).

REAGENTS

1. 10%, 2% Minimum essential medium
2. 2% trypsin in 5% EDTA
3. D- Glucose
4. Insulin
5. Metformin

CELL CULTIVATION

HepG2 cells were cultured in open vented 75 cm^3 culture vessels (Corning, NY) in a standard horizontal laminar flow hood and incubated in a NUAIR cell incubator at 37°C in an atmosphere of 5% CO2 and 95% Air. HepG2 cells were provided with a growth media of 90% Minimum Essential Medium (MEM), 10% Foetal Bovine Serum as well as 5% Streptomycin-Penicillin (Sigman-Aldrich, MO). Medium was refreshed at least two times a week (15 mL) and aseptic techniques were employed rigorously to avoid cell culture Contamination.

HepG2 cell were sub-cultured when 80% confluence was reached. Sub-culturing consisted of dispensing old media with a vacuum and addition of 5 ml of 0.25% Trypsin-0.03% EDTA solution to remaining cell layer followed by incubation for 6 min at 37°C. The activity of trypsin was stopped by washing the cells with a 10 mL aliquot of MEM media. HepG2 cells were then transferred to a 50 mL falcon centrifuge tube and centrifuged for 5 min at 1000 rpm. MEM and trypsin mixture was decanted and cells were aspirated with fresh media, transferred to new 75 cm^3 culture vessels and incubated with 5% CO2 at 37°C. [6]

PROCEDURE

The glucose consumption assay was carried out using the method described by scientist Yin et al. (2002) with slight modifications.

HepG2 cells were grown in 10% MEM with 11.1mmol/L glucose concentration medium. Two days before the experiments, the cells were plated into 96 well, tissue culture plates with some wells left bank. After the cells reached 80% to 90% confluence, the medium was replaced by MEM supplemented with 2% FBS. Two hours later, the medium was removed and the same culture medium containing different concentration of extracts and metformin with and without glucose insulin was added to all wells, including the blank wells. After 24 hours of treatment, the medium was removed and its glucose concentration was determined by the glucose oxidase method (Lott and Turner, 1975). The amount of glucose consumption was calculated by the glucose concentrations of blank wells subtracting the remaining glucose in the cell plated wells. [7]

MTT ASSAY:

MATERIALS AND REAGENTS

1. 96 well Tissue culture plates
2. 0.45 microns filter
3. Tissue culture flasks 25 cm2

4. 5 mL, 10 mL, pipettes
5. Trypsin Phosphate buffered saline Versene Glucose (TPVG)
6. 10 mL Syringe
7. DMSO

PRINCIPLE

MTT is a colorimetric assay that measures the reduction of yellow
3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent (e.g. isopropanol) and the released, solubilised formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells. [8]

MAINTENANCE OF CELL LINES

Tissue culture bottles that showed confluent monolayer were selected by observing them under an inverted microscope. Growth medium was removed from the bottle, washed with PBS 5 mL of TPVG (for 25 cm2) was added dispersing evenly on the monolayer and left in contact with the cells for 2-3 min. TPVG was removed and the bottle was incubated at 37ºC, until all the cells detach from the surface. The cells were re-suspended in 5 mL of growth media. (For RD cell line 10% growth medium and for Hep2 cell line 5% growth medium was used). The suspension was aspirated few times to break cell clumps. The cell concentration was determined by counting the cells in haemocytometer.

PROCEDURE

HepG2 cell culture bottles were cultured before a week to the test to make the confluent growth of the cells. To the selected cell culture bottle 5 mL of TPVG solution was added (for 25 cm2 flask) dispersed evenly on to the monolayer. The bottle was kept flat with the cell surface side down on the table for 2-3 min. Then, TPVG was removed using pipette and the flask was placed in incubator, until the cells detach from the surface, re- suspended the cells in 5 mL of growth medium. The suspension was aspirated few times with pipette to break cell clumps. Sterility was put up in nutrient agar tubes and the sterility tubes were labelled. To the 50 µL of cell suspension an equal volume of trypan blue was added and mixed well. From the mix 50 µL of solution was charged using a pipette to a haemocytometer. The cell suspension will pass under the cover slip by capillary action. The cell concentration per mL was determined by counting the cells in haemocytometer. The live cells as clear form were counted and dead cell as blue cells were left.

Percentage of cell viability was calculated using formula:

% viability = (live cell count/total cell count)*100

The MTT method described by Mosmann (1983) was used to quantitatively detect living but not dead cells. In brief, approximately 2x104 cells/well were seeded onto 96 wells plate.100 µL of MEM medium was added and incubated at 37ºC for 24 h. After 24 hour, medium was discarded and fresh medium was added with different concentrations of drug. The setup was incubated for 1-3 days at 37ºC in CO2 incubator. After respective incubation period, medium was discarded and 100 µL fresh medium was added with 10 µL of MTT (5 mg/mL). After 4 hour, of incubation, the medium was discarded and 100 µL of DMSO was added to dissolve the formazon crystals. Then the absorbance was read in a spectrophotometer at 570 nm. [8]

$$\text{Viable cell (\%)} = \frac{\text{Test OD} \times 100}{\text{Control OD}}$$

RESULTS

GLUCOSE CONSUMPTION ASSAY

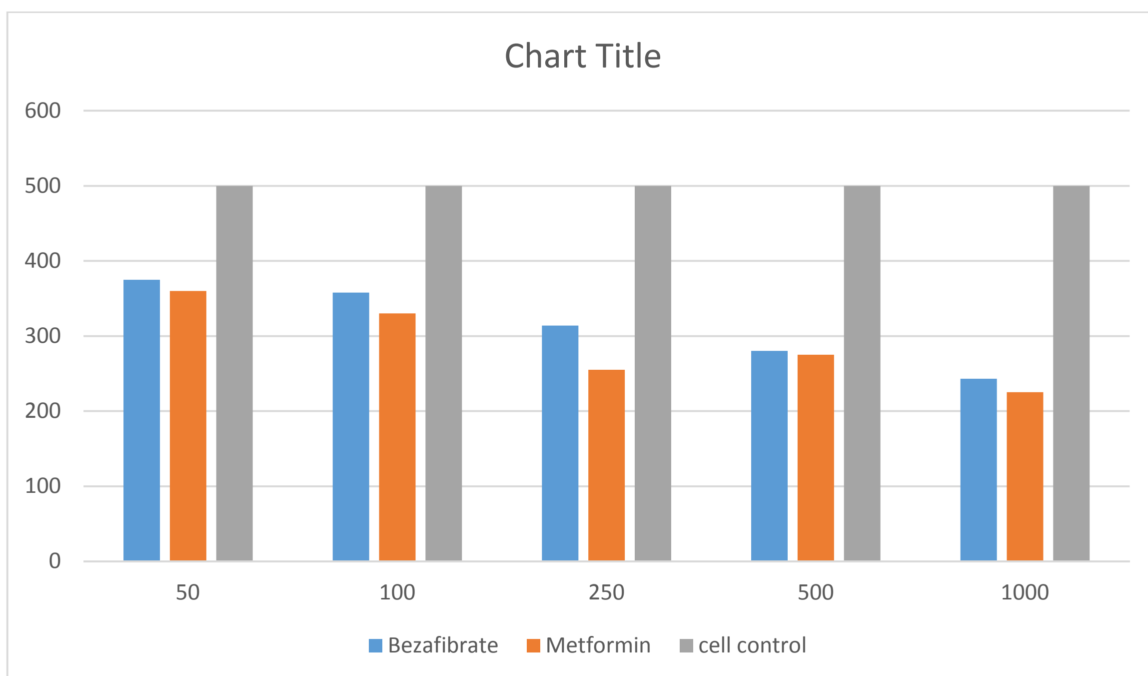
Glucose consumption assay for the drugs were studied by in vitro method using HepG2 cell culture plates. Drug bezafibrate showed increased glucose consumption level in HepG2 cells. The plates were read and corresponding glucose values for the different concentration of bezafibrate were tabulated. Bezafibrate showed the significantly

reduced the glucose value of 500 mg to 375mg at 50 µg concentration. Further it reduced to 275 µg, at 1000 µg showed significant decrease in glucose level when compared to control and known type 2 antidiabetic drug metformin.

Table 1. Results of In-Vitro Glucose consumption assay

S. No	Extract conc. µg/mL	Glucose consumption (mg/dL)		Cell Control (Untreated)
		Bezafibrate	Metformin	
1	50	375	360	**500 mg**
2	100	358	330	
3	250	314	255	
4	500	280	275	
5	1000	243	225	

Bar Chart 1. Results of In-Vitro Glucose consumption assay



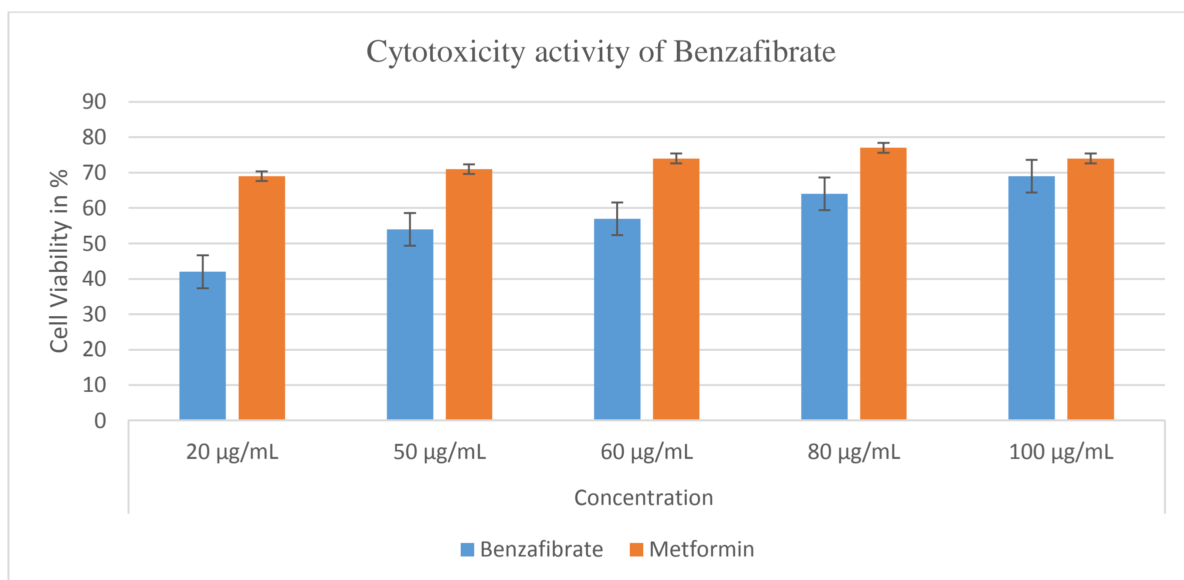
RESULTS OF IN-VITRO CELL VIABILITY ASSAY

Drug bezafibrate were tested for cytotoxicity on HepG2 cells. Drug bezafibrate does not show any toxic effect to the cells up to 80 µg/ml concentration. But there is slight decrease in cell viability percentage. These results were compared with metformin, indicated that bezafibrate had higher cell viability percentage than metformin used as drug for type 2 diabetes.

Table 2 Results of In-Vitro cell viability assay

S.No	Sample	Cell viability (%)				
		20 µg/mL	**50 µg/mL**	**60 µg/mL**	**80 µg/mL**	**100 µg/mL**
1.	Bezafibrate	42	54	57	64	69
2.	Metformin	69	71	74	77	74

Bar Chart 2 Results of In-Vitro cell viability assay



DISCUSSION

The aetiology of metabolic syndrome is complex and characterises defects in several homeostatic regulatory systems that, when coexisting, increase the risk of cardiovascular events and diabetes. The clustering of some risk factors and their shared responsiveness to lifestyle modification suggests that they are not independent of one another and that they share underlying causes, mechanisms and features. Identifying metabolic syndrome is important for several reasons; patients who are at high risk of developing atherosclerotic CVD and type 2 Diabetes (T2D); considering the relationship between the components of metabolic syndrome and understand the pathophysiology, thirdly facilitates the pharmacological, lifestyle and preventive treatment approaches. Pharmacological intervention for metabolic syndrome would require multiple agents, which increases the costs and risk of adverse effects [4, 9]

Bezafibrate, is a PPAR-α agonist, activator of lipoprotein lipase, a key regulatory enzyme responsible for the hydrolysis of triglycerides(TG) rich lipoproteins, and its treatment resulted in significant decrease in the serum concentrations of triglycerides, total cholesterol and LDL- cholesterol and also VLDL, where HDL-cholesterol serum levels increased, uniquely affecting insulin resistance. [10]

Klein et al. identified an approximately 35-fold increase in T2DM risk for individuals with four or more components compared with those, with none of the MetS components at baseline. It is possible, therefore, that a decrease in one or two of the components of MetS could potentially reduce the risk of CVD and T2DM. Not only does it save lives but reduces the burden throughout the world. [11]

CONCLUSION

Studies have shown that all fibrates except bezafibrate are far more selective for PPAR-α, assuming bezafibrate Pan-PPAR agonist status with unique antidiabetic property, which should be promoted for its use in India widely, for its potential effect to delay and prevent the complications of metabolic syndrome. Recent studies have also reported so far, MetS or its components may play a role in the aetiology, progression or prognosis of certain cancers. Hence accurate and timely diagnosis and treatment of MetS- or better still, prevention – is therefore crucial to the health of the world’s population but also to the global economy. [11]

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