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PHARMACOLOGICAL EVALUATION OF GLIMEPIRIDE FOR DIABETES MELLITUS AND ASSOCIATED COMPLICATION IN RATS

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ABSTRACT

Present study is designed to evaluate the possible involvement of glimepiride in complication of diabetes mellitus in wistar rats.3 groups of rats with 6 rats per group were taken in the study. Diabetes was induced with high fat diet for 63 days. Glimepiride was given in a dose of 17.5 mg/kg for 90 day.5 parameter viz Post Prandial blood sugar, Uric acid, C-peptide, Micro RNA 21 and total Protein were investigated.Complication of diabetes in a form of diabetic nephropathy was seen in the animal and it was correlated to the parameter taken in the study. Finally it was concluded that the glimepiride indirectly involved in diabetic nephropathy apart from its existing pharmacological role. Micro RNA expression performed in the present study also favors the occurrence of complication of diabetes in glimepiride treated groups of rats.

KEYWORDS: Glimepiride, Diabetes mellitus, Micro RNA, Protein.

INTRODUCTION

Diabetes is not a disease of blood sugar, rather a disorder of insulin and leptin signaling**.** Mainstream medicine largely fails in treating diabetes, even worsens it because it is not based on underlying cause. Insulin sensitivity is the key regarding this finding (Meek TH, Morton GJ, 2012). Your pancreas secretes insulin into your blood stream, lowering your glucose. Insulin is meant to control the lifespan in some organisms, but what is its true purpose in humans? (Kenyon C, 2011).

Diabetes occurs in people of all ages, but it’s more common in older adults (Diabetes care, 2012).

Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased pre-prandial and post prandial blood sugar levels (Diabetes care, 2016).

Insulin, a hormone released from the pancreas, controls the amount of glucose in the blood. Glucose in the bloodstream stimulates the pancreas to produce insulin. Insulin allows glucose to move from the blood into the cells. Inside the cells, glucose is converted to energy, which is used immediately, or the glucose is stored as fat or glycogen until it is needed (Cooke DW, Plotnick L, 2008).

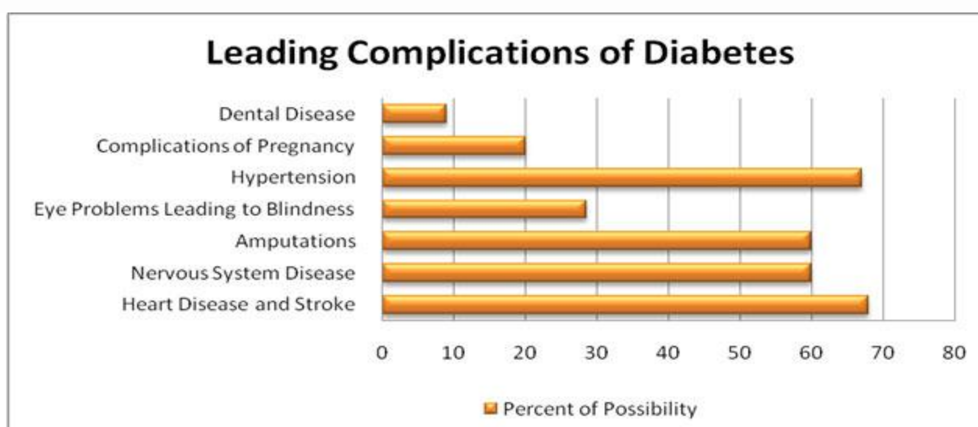
The global prevalence of diabetes is estimated to increase, from 4% in 1995 to 5.4% by the year 2025. WHO has conjectured that the major burden will occur in developing countries? Studies conducted in India in the last decade have highlighted that the prevalence of diabetes is not only high in rural area but increasing rapidly in the urban population (Ramachandran et al, 2002). It is estimated that there are approximately 33 million adults with diabetes in India. This number is likely to increase to 57.2 million by the year 2025.

DM associated morbidity and mortality

- In 2009, diabetes mellitus was the seventh leading cause of death in the United States(ADA,2016) .In addition, diabetes is a contributing cause of death in many cases, and it is underreported as a cause of death (Kochanek KD, Xu J, Murphy SL, et al., 2011).
- Overall, the death rate among people with diabetes is about twice that of people of similar age, without diabetes (National diabetes fact sheet, 2011).
- The American Diabetes Association estimated that in 2007, direct medical costs due to diabetes in the United States were $116 billion, with another $58 billion in indirect costs (eg, disability, work loss, premature mortality). Approximately 1 in 5 health care dollars in the United States was spent caring for someone with diagnosed diabetes, while 1 in 10 health care dollars was attributed to diabetes (Economic costs of diabetes in the U.S., 2007).

Status of DM in USA (National Diabetes Fact Sheet regarding diabetes mellitus, USA – 2011)

- 8.5% of the US population has diabetes including 25.8 million children.
  Researchers from the Jefferson School of Population Health published a study which estimates that by 2025 there could be 53.1 million people with the disease.
- 18.8 million people have been diagnosed with diabetes.
- About 7 million people with diabetes have not been diagnosed.Even though type 2 diabetes rates in the USA have raised sharply, Timothy Lyons, MD, who is presently Director of Research of the Harold Hamm Diabetes Center in Oklahoma City says that the disease is still not being detected promptly. He added that the lag in diagnosis involves both patients and doctors. About 79 million people have pre-diabetes.
- 1.9 million people aged 20 years or more were newly diagnosed with diabetes in 2010.
- 215,000 (0.26%) people younger than 20 years have diabetes.
- Approximately 1 in every 400 kids and teenagers has diabetes.
- 11.3% of people aged 20+ years have diabetes; a total of 25.6 million individuals.
- 26.9% of people aged 65+ years have diabetes; a total of 10.9 million people.
- 11.8% of men have diabetes; a total of 13 million people.
- 10.8% of women have diabetes; a total of 12.6 million people.



Glimepiride and other sulfonylureas are associated with weight gain, hence should not be used in obese patient.

Hypoglycemic episodes are less reported with glimepiride (2% to 4%) as compared to glibenclamide (20-30%). Glimepiride does not interfere with the normal homeostatic suppression of insulin secretion in reaction to hypoglycemia, whereas glibenclamide interferes. Glimepiride increases glucagon secretion in reaction to hypoglycemia, while glibenclamide diminishes it (Davis, Stephen N, 2005).

The complications of diabetes mellitus are broadly classified as microvascular, including neuropathy, nephropathy, and retinopathy, or macrovascular, including cardiovascular and peripheral vascular disease. The risk for developing complications is influenced by many factors including duration of diabetes and genetic factors.

MATERIALS AND METHODS

Animals

18 male wistar rats with body weight 200-250 gm were taken from animal house facility, Department of Pharmacology, SunRise University and were divided into 3 groups with 6 rats per group. Animals were maintained on pellet feed diet and water ad libitum until the time of experiment. All required condition was maintained as per guidelines of CPCSEA. Animals were kept overnight fasting before the day of experiment.

Chemicals

Only analytical grades chemicals and reagents were used. High fat diets were purchased from Grover (Spectrochem Pvt. Ltd. Mumbai, India) other reagents and kits were procured from Chopra chemicals (Delhi, India) and CDH.

Drugs

Glimepiride was procured from CDH (Manufacturer ---Sanofi-Aventis Ltd).

Induction of diabetes

High Fat Diet with different composition like beef tallow, casein protein, corn starch, vitamin mixture, mineral mixture have been mixed in specified quantity and spherical balls were formed. These spherical balls were placed on the cage of rats for free access on daily basis 24X7(Vogel HG, 2002).

Group of rats with treatment

Group	Rats	Drugs, route of administration, dose
1	Normal control	No drug was administered, only 0.9% normal saline was given.
2	Disease control	High fat diet was given for 63 days
3	Glimepiride control	Glimepiride (17.5mg/kg/day, P.O.) was administered after inducing diabetes for 90 days

Experimental Protocol for separation of Serum

Blood sample was collected whenever required after overnight fasting from tail vein under ketamine- xylazine anesthetic agent without any anticoagulant. After that allowed to stand for

30 minutes at room temperature then centrifuged at 2500 rpm for 10 minutes to separate the serum. The serum obtained was kept at 2ºC - 4ºC. (Takashashi M et al, 2014).

Estimation of uric acid (Thomas L, 1998)

In vitro quantitative determination of uric acid in serum was done using enzymatic kit (Nicholas India Pvt. Ltd.).

Mixed 4 parts of reagent 1 with 1 part of reagent 2(Monoreagent). Left the monoreagent for at least 10 min and protect it from light.

	Blank	Standard	Test
Distilled water	20 µl	-	-
Uric acid standard	-	20 µl	-
Test	-	-	20 µl
Monoreagent	1000 µl	1000 µl	1000 µl

Mixed, incubate for 30 min at room temp. Read absorbance against reagent blank within 60 min at 520 nm.

Calculation:

Uric acid (mg/dl) =A_{sample} / A_{std} X conc. of Std

C-Peptide

All single volume dispensing was done with eppendorf fixed volume pipettes. A new tip is used for each new specimen (Beyer J, Krause U, Cordes, 1979). All multiple volume dispensing is done with an eppendorf 5.0 mL. Repipet with combitip graduated in 100-µL increments (e.g. a setting of "1" dispense a volume of 100 µL; a setting of "3" dispense a volume of 300 µL).

After the assay is counted on the gamma counter, the following information is recorded: total counts, % B0/TC, smoothing factor, detection limit, goodness of fit, and control results. The mean and range of each control are used to determine assay acceptance or rejection (Heding, LG, 1975).

- Pipette 100 µL of the zero calibrator A into the NSB and A tubes, and 100 µL of each of the calibrators B through G into corresponding control into the appropriate tubes.
- Add 100 µL of I-125-C-Peptide (color coded yellow) to all tubes. Shake rack. Add 100 µL of C-Peptide Antiserum (color coded red) to all tubes.

- Cover the rack with foil, label with using a Cornwall repeating syringe, a green to all tubes except TC. Vortex.
- Centrifuge for 30 minutes at 2000 × g at 4°C.Place all tubes except TC into a decanting rack, and invert on to a radioactive waste receptacle.

Allow the rack to remain inverted on a stack of paper towels for 10 minutes, and then blot gently. Check the background activity of the gamma counter counting racks by counting the empty rack for two minutes prior to placing any RIA tubes in the racks. Background activity should be less than 100 CPM. If the background exceeds 100 CPM, do not aside until its radioactivity has deteriorated below the limit. Count the radioactivity in each tube for 2 minutes.

After the protocol information is completed, the gamma counter screen displays a graphic of the tube sequence. Place the first 12 RIA tubes in the plastic counting rack. Match the tube sequence with the screen display.

Press the red button to initiate the counting. The counting time is indicated on the gamma counter.

At the end of two minutes, a beep sound to alert you to place the next twelve RIA tubes in the rack.

Glucose estimation (Braham and Trinder, 1972)

20µl of blood sample was taken with freshly made trichloro-acetic acid (TCA 4%) solution and was centrifuged at 3000 rpm for 10 minutes. The supernatant of this was used for the estimation of blood glucose. Preparation of the Blank, Standard and Tests was carried out as follows:

Reagents	Blank(ml)	Standard(ml)	Tests(ml)
Working Reagent	1.5	1.5	1.5
Distilled Water	1.5	1.5	1.5
TCA (4 %)	0.2	0.18	_
Std. Glucose sol.	_	0.02	_
Supernatant	_	_	0.2

The solutions was mixed and incubated for 15 minutes at 37 °C. The optical density (OD) was measured at 505 nm.

Calculation

- Glucose (mg / dl) = $\frac{\text{OD of test}}{\text{OD of standard}} \times 250^*$
- (* Dilution factor)

Micro RNA (Ma J,Wang J et al,2017)

The northern blot or RNA blot is a technique used in molecular biology research to study gene expression by detection of RNA or isolated mRNA in a sample (Albert B et al, 2008).

Micro RNA expression before and after treatment was done. The serum (400 micro litre) isolated from each sample was centrifuged at 6,000 RPM at 4 °C for 15 min prior to RNA extraction. Mi RNA was isolated from DNA and T2DM serum samples using QIAzol Lysis Reagent (Qiagen, Hilden, Germany) as part of the mi RNA essay Serum/Plasma Kit (Qiagen). Then, 3.5 ml of synthetic miRNA 21 family was added to 4 extracted mi RNA as a spike-in control (1.6 x 108 copies/ml working solution) before the samples were reverse transcribed to complementary DNA. RNA concentration and purity were determined using an Agilent 2100 Bioanalyzer and RNA 6000 Nano/Pico Lab Chip (Agilent Technologies, Boeblingen, Germany).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Each reverse transcription (RT) reaction contained 1 ml of mi Script Reverse Transcriptase Mix, 4 ml of 5x mi Script RT Buffer, 13 ml of RNase-free water and 2 ml of RNA template. The 20 ml RT reaction was incubated at 37 °C for 1 hour followed by 5 min at 95 °C using an I Cycler system (Bio-Rad, Hercules, CA). The cDNA was diluted 10-fold before being added to each quantitative polymerase chain reaction (qPCR), with spiked-in cel miR 21 family serving as the external control for normalization. To improve quantification accuracy, each sample was analyzed in triplicate, and both the melting curve and amplification plot analyses were used to confirm the specificity of the reactions. Each 12.5 ml quantitative real time PCR reaction contained 6.2 ml of SYBR Green PCR Master Mix, 1.2 ml of mi Script universal primer, 1.2 ml of specific primer, 2 ml of cDNA and 1.9 ml of RNase-free water. The amplification protocol consisted of an initial activation step at 95 °C for 15 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s,70 °C for 30 s, and was carried out on the Mx3005P qPCR system (Stratagene, USA). The levels of circulating miR-21 were analyzed quantitatively using the 2-DDCt (cycle threshold) method after normalization to the cel-micro RNA control.

Protein estimation by Folin's reagent (Lowry et al., 1951)

- 0.1 ml of 10% homogenate was diluted with 0.9 ml of water. Then, the resultant mixture was mixed with 5 ml of alkaline solution and allowed to stand at room temperature for 10 min. This was followed by addition of 0.5 ml of Fc reagent into each test tube. The test tubes were shaken immediately for a thorough mixing. After 30 min, the absorbance was read at 750 nm against a reagent blank.
- **Standard Curve:** 5 ml of BSA solution (0.5 mg/ml) was prepared and different volumes were taken in 6 tubes. To all tubes distilled water was added to make up the volume in tubes to 1 ml. The protein in the above 6 tubes was estimated in the same way as for the sample. A graph was plotted between concentration of protein and Optical Density (OD).

RESULT AND DISCUSSION

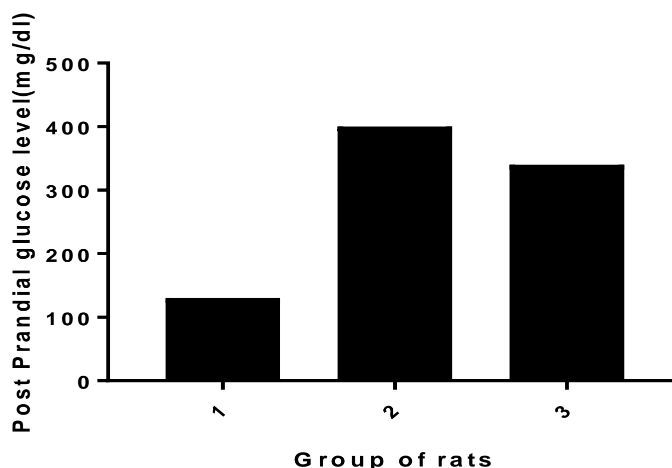
Group of rats	Post Prandial Glucose(mg/dl)	Uric Acid(mg/dl)	C-Peptide (nmol/L)	miRNA 21	Total Protein (mg)
1	130±2.25	6.90±1.50	0.80±1.20	3±0.43	55.50±0.48
2	400.10±2.12$^{###}$	8.80±0.80$^{###}$	1.00±0.45$^{##}$	5±3.05$^{###}$	60.44±0.52$^{##}$
3	340.10±0.98**	7.95±1.10$^{##}$	0.87±0.42**	8.5±0.60**	63.44±0.53$^{###}$

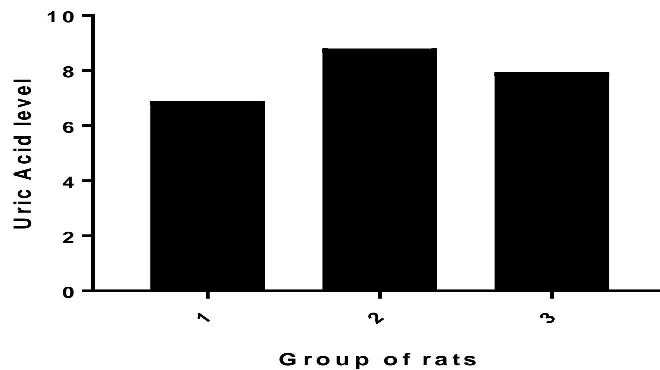
Values are given as Mean ±S.E.M. for six rats in each group (n=6). $^{##}$ (P<0.01) compared with the corresponding value for normal control (group I); ** (P<0.01) compared with the corresponding value for diabetic control (group II).

***Highly significant(P<0.001),**Moderately Significant(P<0.01) and *Less significant(P<0.05) as compared to diabetic control (group II).

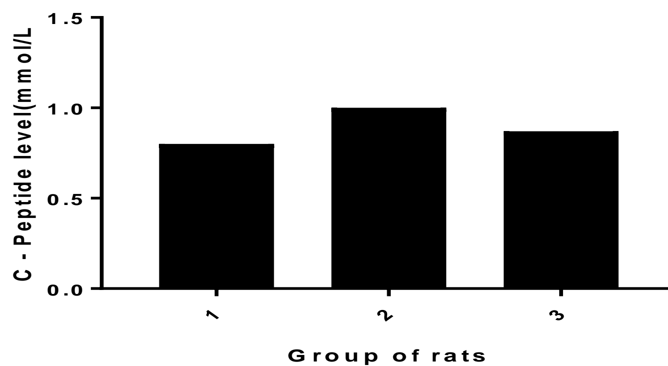
$^{###}$ Highly Significant(P<0.001),$^{##}$Moderately significant(P<0.01) and $^{#}$Less significant(P<0.05) as compared to normal control (group I).

Graph for different parameter

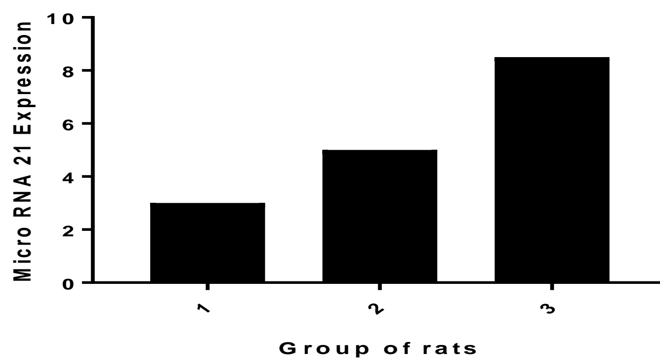




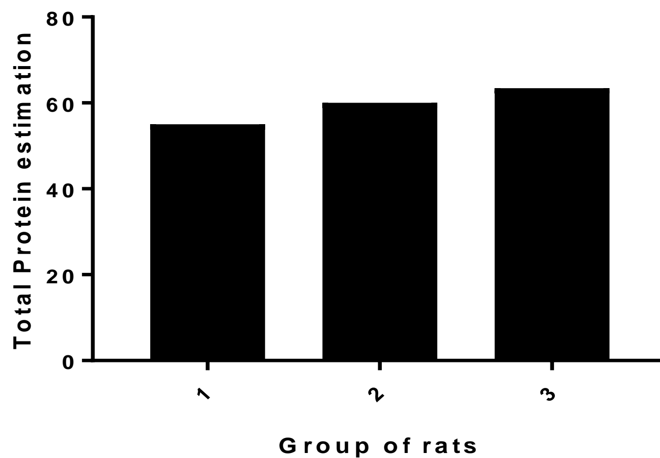
Uric Acid level
10
8
6
4
2
0
1
2
3
Group of rats



C - Peptide level(mmol/L
1.5
1.0
0.5
0.0
1
2
3
Group of rats



Micro RNA 21 Expression
10
8
6
4
2
0
1
2
3
Group of rats



Total Protein estimation
80
60
40
20
0
1
2
3
Group of rats

DISCUSSION

Graph for postprandial glucose level clearly indicates that the level of glucose was lower in group 3 in comparison to group 2.Glimepiride treated group controlled blood glucose more significantly but the level was still higher than normal control group. This increased blood glucose is converted to sorbitol which has higher osmotic pressure than glucose; accumulates water around it and causes diabetic diabetic nephropathy.

Graph for uric acid clearly indicates that the level of uric acid was lower in group 3 in comparison to group 2.Although the level of uric acid was more in glimepiride control than normal control. As glucose is soluble in synovial fluid so it accumulates in joints of upper and lower limbs & interstitial cells of kidney leading to diabetic nephropathy.
Graph for C-peptide clearly indicates that the level of C-peptide was higher in group 2 in comparison to group 1 & 3.Glimepiride treated group has more c-peptide than normal control.

Actually pro-insulin is converted into C-peptide and insulin. Insulin along with C-peptide are responsible for glucose homeostasis.

Graph for Micro RNA expression clearly indicates that the glimepiride control group has expressed micro RNA 21 more than group 1 & 2.This micro expression is indirectly linked to diabetic nephropathy, a chronic microvascular complication of diabetes mellitus.

Graph for total protein level clearly indicates that the level of protein was highest in glimepiride treated group. It means microalbuminaria a common complication in diabetic animals has occurred in diabetic control and glimepiride control group.

CONCLUSION

From the data in results the present study concludes that the glimepiride reduce the post-prandial plasma glucose level significantly. Besides this it was involved indirectly in complication of diabetes like diabetic nephropathy.

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