Materials and methods
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1. Place of study: Burdwan medical College & Hospital
   
   a) Medicine outpatient department
   
   b) Department of Biochemistry
   
   c) Diabetic clinic
   
2. Study population: The Type-2 Diabetes Mellitus (t2dm) patients coming to MOPD and able to give informed written consent. And also some impaired glucose tolerant pts.
   
3. Study period: 3years
   
4. Sample size were 200 patients but 164 patients had completed the stipulated followed up period.
   
5. Selection of study population was done following pre fixed inclusion and exclusion criteria.

Case definition of study population:

Type 2 diabetes mellitus: if fasting plasma glucose ≥126mg/dl, 2hrs post prandial plasma glucose ≥200mg/dl on consecutive two days. Fasting plasma glucose is defined as no calorie intake for at least 8 hrs.
Impaired glucose tolerance: if 2hrs plasma glucose between 140-199mg/dl

Inclusion criteria

a) Age—more than 18yrs

b) able to give written informed consent

c) sex-- no bar

d) t2dm patients:

   newly diagnosed

   getting oral antidiabetic agents

   getting insulin.

e) impaired glucose tolerance pts

Exclusion criteria

a) Unable to give informed written consent

b) Renal impairment

c) H/ hepatic disease

d) H/O heart failure

e) H/O lactic acidosis
f) Alcoholics

g) Pregnancy

h) Female subjects planning for pregnancy during study period

i) H/O chronic hypoxic lung disease (like bronchial asthma, COPD)

j) H/O taking very low calorie diet regularly and long standing repeated fasting

examinations done

A) Biochemical parameters:

a) For diagnosis: 1) Fasting blood glucose (fbg) 2) Post prandial blood glucose (ppbg) 3) HbA1c 4) s. insulin &c-peptide, urine albumin, Apo-A1 lipoprotein & Apo-B lipoprotein.

b) To measure the outcome 1) fasting blood glucose (fbg) 2) postprandial blood glucose (ppbg) 3) HbA1c 4) serum lipid profile

c) For monitoring safety profile 1) SGPT 2) s. lactic acid 3) s. pyruvic acid 4) haemoglobin%

d) To determine exclusion criteria 1) s. urea 2) s. creatinine 3) pregnancy test as it was required 4) s bilirubin 5) SGOT 6) ECG

B) Physical parameters:
1) Body weight 2) Height 3) Body mass index 4) Waist circumference
5) Hip 6) waist: hip ratio 6) thigh circumference

C) Other parameters:

1) ECG 2) Physical Examination 3) Adverse reaction query

7. Study design: Open level randomized self controlled sequential prospective interventional study

8. Informed written consent had been taken from the study subjects at the time of allocation

9) Every patients had been followed up for more than two months in each step

Of drug treatment. Hence each patients had been followed for at least six months

10) Intervention:

For impaired glucose tolerance: study subjects were selected from medical out patients department and diabetic clinic of Burdwan medical college following the inclusion criteria of fasting plasma glucose ≥126mg/dl and post prandial plasma glucose ≥200mg%. Infomed written consent had been taken at that time. They were initially 50 in number. Preliminary examination had been done along with base line investigation to find out any exclusion criteria. Then a two weeks period had been
spent to educate the patients (pts) regarding medical nutrition therapy (diet), exercise, physical activity, importance of adherence to the drug therapy (compliance). Then metformin was introduced at a dose 500mg three times daily. Few patients complained of nausea, abdominal distension, reduced appetite. At this time 15 patients lost follow up. Remaining pts continued metformin after proper reassurance. Then dose escalation was done gradually over a period of two weeks up to 850mg thrice daily. Then they were followed for next two weeks. Blood examination was done in the department of biochemistry for all the criteria like fbg, ppbg, sgpt, total cholesterol, LDL-c, HDL-c, S.lactic acid, s. pyruvic acid, TGL. After a proper wash out period of two weeks metformin (met) withdrawn, pioglitazone (pio) was started at a dose 15mg initially, asked the to come after 2 weeks to examine for any adverse effect, then they were asked to come again after two weeks; dose escalation was done at that time to 30mg daily at morning. Followed for next 8 weeks, pts were then again asked to come and all the physical parameter were measured during examination and all biochemical examinations were done in the department of biochemistry.

For t2dm pts: pts were selected 100 in number. Most of them were in the treatment of some antidiabetic drug and not under proper glycemic control and some are new. They were allocated in the similar manner as previously. The antidiabetic drugs they were taking, was gradually withdrawn and glibenclamide (gbcm) administered gradually with a initial
dose of 2.5mg daily gradually escalated to 10mg (maximum) daily dose.

All the physical parameter was measured during examination and biochemical parameter measured the biochemistry department as baseline. Pts were educated regarding the importance of MNT, drug compliance, physical exercise as before at that time. All these were done over a period of 4 weeks. Then physical and biochemical examination was done to measure the effect of glibenclamide. Later MET was started along with GBCM initially 500mg thrice daily-folowed by 850mg thrice daily keeping the GBCM dose fixed. At this 11 pts did not come for follow up. The remaining 89 pts were examined for all the physical and biochemical parameters after a period 8 weeks. Then MET was withdrawn, and after 1 week washout period, PIO started gradually in a similar fashion 15mg/d initially followed by 30mg/d keeping GBCM dose fixed in period of 8 weeks. Then after 8 weeks all the examination (physical and biochemical) was done.

In inslin naïve subjects: 50 pts were selected in similar fashion. All the parameters were examined and all the subjects made euglycemic by appropriate insulin dose. Then MET started reducing the dose of insulin keeping the subject euglycemic with every checkup. Then MET was withdrawn and after a proper wash out period, PIO started gradually. Maximum dose of MET and PIO were 850mg thrice daily and 30mg once daily respectively. Insulin dose was noted at the beginning, along with
MET and along with PIO. Dose reduction was observed along with other parameters in every steps.

11) Statistics: Analysis was done by employing SPSS -17version software. Here paired sample t- test is used, as because MET and PIO administered in the same group of pts, to observe the efficacy of MET and PIO individually and along with GBCM & insulin. Pearson correlation had also been observed to find out the difference of effect among different physical and biochemical results. Statistical significance was considered when p≤ 0.05.

12) Ethical consideration: The proposal had been approved by the institutional ethics committee.

13) This study was conducted in accordance with the ICMR guidelines for Biomedical Research on Human Subjects, 2006 and the declaration of Helsinki.

14) Waist, hip and thigh circumference was measured by using measuring tape in inch by following usual measuring procedure. Body weight is measured by using electronic weighing machine.

15) Biochemical measurement was done by using autoanalizer and colorimeter available in the Biochemistry department. Appropriate test kit was used for different measurement. They were as following

A) Hba1c :
Principle – A hemoglobinised preparation of whole blood is mixed continuously for 5 minutes with a weak binding cation exchange resin. During this time, HbA0 binds to the resin. After the mixing period, a filter is used to separate the supernatant containing the glycohaemoglobin from the resin.

Hemoglobin whole blood preparation + cation exchange resin-------mixed for 5 minutes-----fast fractions (HbA1a, HbA1b, and HbA1c)

The glycohaemoglobin present is determined by measuring the absorbance at 415 nm of the glycohaemoglobin fraction. The ratio of the two absorbance gives the percentage of glycohaemoglobin.

b) Lactic acid;

Principle—lactate is oxidized by lactate oxidase to pyruvate and hydrogen peroxide, which, in presence of peroxidase (POD), react with TOOS* forming a compound, which colour intensely is proportional to the concentration of lactate in the examined sample.

*=N-ethyl-n-(2-hydroxy-3 sulfopropyl)-3-methylaniline.

c) Plasma glucose: Estimation by glucose oxidase and peroxidase method;

Principle: glucose is oxidized by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of enzyme peroxidase (POD) oxidizes phenol which combines
with 4-amino antipyrine dye to produce red colored quinoneimine. The intensity of the color developed is proportional to glucose concentration in the sample.

**GOD**

\[
\text{D-glucose +H}_2\text{O+O}_2 \rightarrow \text{D-gluconic acid+H}_2\text{O}_2
\]

\[
\text{H}_2\text{O}_2 +\text{4-Aminopyrine+phenol} \rightarrow \text{red quinoneimine dye +H}_2\text{O}
\]

Reagent used 1) enzyme reagent 2) buffer solution 3) glucose standard 100mg/dl

The reagents were stored between 2-8 degree celcius.

d) Insulin:

Principle -by immunoenzymometric assay. The essential reagents required for an immunoenzymometric assay include high affinity and specificity antibodies (enzyme conjugated and immobilized) with different and distinct epitope recognition, in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-insulin antibody.
Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing the native antigen, reaction results between the native antigen and the antibodies without competition or steric hindrance, to form a sandwich complex. The interaction is occurred through a complex equation.

Streptavidin immobilized on well and this complex is the sandwich complex bound to the solid surface.

After equilibrium is attained, the antibody bound fraction is separated from the bound antigen by decantation of aspiration. The enzyme activity in antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a response curve can be generated from which the antigen concentration of an unknown can be ascertained.

e) Lipid profile and SGPT: These are measured by using appropriate test kit and auto-analyzer present in the department of Biochemistry of Burdwan medical college.
nC-peptide measurement principle:

Immunoenzymometric assay: the essential reagents required for immunoenzymometric assay include high affinity and specificity antibodies (ab) (enzyme conjugated and immobilized), with different and distinct epitope recognition. In excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated on the well and exogenously added biotinylated monoclonal anti-insulin antibody.

Upon mixing monoclonal biotinylated antibody, the enzyme-labeled antibody and a serum containing native antigen, the reaction results between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. Simultaneously, the complex is deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody.

After equilibrium is attained the antibody bound fraction is separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody bound fraction is directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen values, a dose response curve can be generated from which the antigen concentration of unknown can be ascertained.

g) Apolipoprotein A1 & Apolipoprotein B:


Principles: Antigen antibody reaction occurs between apolipoprotein A1 or B in the serum and antihuman apolipoprotein A1 or B antibody, which causes turbidity. Apolipoprotein A1 or B can be obtained by measurement of turbidity. This method is a 2-point assay removing a blank of test sample.

Apolipoprotein A1 or B + Antihuman apolipoprotein A1 or B antibody ----→ antigen-antibody precipitate ----→ Measurement of absorbance.

h) Urinary albumin test: this was done by MICRAL-TEST (An ACCU-CHEK product).
Test principle: immunological detection of human albumin by means of soluble antibody-gold-conjugate. Excess conjugate is retained in a separation zone containing immobilized human albumin. Cross reaction with other human protein, such as hemoglobin, transferin, bence-jones protein, α1 antitrypsin, acidic α1-glycoprotein, α1 amylase, Tamm-Harsall protein and retinol binding protein, as well as with IgG, IgA, human leucocyte and erythrocyte have been found to be < 0.05%.

Specimen Material:

i) For blood—specimen collected as clotted blood from which serum has been separated and stored at -37°C in deep freeze for testing collectively.

ii) For urine—the first morning urine voided after rising was used for testing of urinary albumin.