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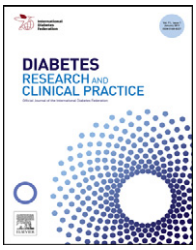

Effect of metformin on oxidative stress, nitrosative stress and inflammatory biomarkers in type 2 diabetes patients

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ABSTRACT

Aim: Advanced research has radically changed both diagnosis and treatment of diabetes during last three decades; a number of classes of oral antidiabetic agents are currently available for better glycemic control. Present study aims to evaluate the effect of metformin on different stress and inflammatory parameters in diabetic subjects.

Methods: 208 type 2 diabetes patients were randomly assigned for metformin and placebo.

Results: Reactive oxygen species generation, advanced oxidation protein products (179.65 ± 13.6, 120.65 ± 10.5 μmol/l) and pentosidine (107 ± 10.4, 78 ± 7.6 pmol/ml) were found to be reduced by metformin treatment compared to placebo. On the other hand metformin administration enhanced total thiol and nitric oxide level ($p < 0.05$). But nutrient level (Mg^{+2}, Ca^{+2}) in plasma was not altered by the treatment. Significant restoration of C reactive protein ($p < 0.05$) was noticed after metformin therapy. Metformin administration also improved Na^+K^+ATPase activity (0.28 ± 0.08, 0.41 ± 0.07 μmol Pi/mg/h) in erythrocyte membrane.

Conclusions: This study explores that metformin treatment restores the antioxidant status, enzymatic activity and inflammatory parameters in type 2 diabetic patients. Metformin therapy improves the status of oxidative and nitrosative stress altered in type 2 diabetes. This study unfolds the cardio protective role of metformin as an oral hypoglycemic agent.

1. Introduction

Type 2 diabetes is a multifactorial metabolic disorder characterized by abnormal insulin secretion caused by impaired β cell function and insulin resistance in target tissues [1,2]. India leads global top 10 in terms of the highest number of people with diabetes mellitus, though very few clinical studies have been performed on Indian diabetic population. Despite significant recent advances in hyperglycemia, real cure of type 2 diabetes is still beyond the horizon. Different oral hypoglycemic drugs are being used nowadays among which metformin (Biguanide family) has greatly improved the prognosis of diabetic patients by improving insulin sensitivity and protecting against vascular complication, especially for the patients accompanied with obesity and insulin resistance.

Oxidative stress and inflammation resulting tissue damage are hallmarks of chronic diseases like diabetes. Increased production and/or ineffective scavenging of reactive oxygen species (ROS), advanced oxidation protein product (AOPP) and accumulation of advanced glycation end products (AGE) play crucial role in diabetes pathogenesis. Nitric oxide (NO), an inorganic molecule formed by vascular endothelial cells is now thought to be a messenger molecule that also plays a significant role in various biological processes. Mg^{+2} and Ca^{+2} are important

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Abbreviations: AOPP, advanced oxidation protein products; CRP, C reactive protein; NO, nitric oxide; NOS, nitric oxide synthase; OHAs, oral hypoglycemic agents.

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nutrients as well as cofactors in several enzymes critical for carbohydrate metabolism and glucose homeostasis [3]. Diabetes has great biological impact on the activity of enzyme like Na^+K^+ATPase present in erythrocyte membrane and on the level of C-reactive protein (CRP). CRP is a phylogenetically highly conserved acute phase plasma protein that participates in the systemic response to enhance inflammation during deregulation of glycemic states. In recent past, important research works in the area of diabetes mellitus have established that inflammation related parameters have also been emerged to be crucial factors in disease pathogenesis.

Our study explores the effect of metformin on the status of some important oxidative stress markers, namely ROS, AOPP and pentosidine. We have also evaluated nitrosative stress, antioxidant level, concentration of nutrients, enzymatic activity of Na^+K^+ATPase and inflammatory parameter like C reactive protein in subjects suffering with type 2 diabetes mellitus treated with metformin.

2. Materials and methods

2.1. Study design

A double blind randomized study was conducted for 24 weeks among type 2 diabetes patients in IPGMER/SSKM hospital, Kolkata, India. Eligible patients were 30–55 year old, had a BMI of 23–29 kg/m^2, HbA1C $>$ 7% and suffering from type 2 diabetes for at least 1 year but no longer than 5 years. Patients were capable to carry out self-monitoring of blood glucose concentration. Patients, who had been treated with hypoglycemic agents, had been free from therapeutic drugs for at least 2 weeks before screening. After primary screening the baseline data was recorded and patients were randomly assigned for metformin (Group I) and placebo (Group II) treatment. The study was approved by the Calcutta University Biosafety and Ethics Committee and informed consent form was signed by the patients. Patients randomized to metformin took (850–2000 mg)/day. After randomization study medications were administered once daily (before breakfast) during the 1st week and twice daily (before breakfast and evening meal) during the 2nd week. Amendment of medications was performed if the mean daily glucose level was greater than 130 mg/dl and the HbA1C was greater than 7.5%. The maximum allowable total daily doses were 2000 mg of metformin. Patients with the subsequent conditions were eliminated from the study: noticeable abnormal renal function (considered as serum creatinine $>$ 125 μmol/l), addicted to smoking, previous history of severe cardiomiopathy and taking vitamin capsule and calcium tablet. Lifestyle was not changed during the entire study in order to eliminate other factors influencing the estimated parameters. Physical check-up, glucose monitoring, counseling on diet, exercise and laboratory data analysis were performed during clinical visits

2.2. Sample size

Initially 285 patients were enrolled for the study, after primary screening 250 patients were randomized, 127 to metformin (Group I) and 123 to placebo (Group II). Among all the patients 110 metformin administered and 98 placebo were finally able to continue the study and rest of the patients were excluded due to loss of follow up, lack of efficiency and personal conflict.

2.3. Isolation of plasma, serum and ghost membrane

Blood samples were collected from the subjects after 12 h of fasting in heparin vial as well as in a separate vial in the absence of any anticoagulant. Blood plasma was separated by centrifugation of the blood sample and was stored in −80 °C for analysis. The buffy coat was removed by gentle aspiration and the red cells were washed three times with 310 imOsM Tris buffer pH – 7.6 at 4 °C. Washed red blood cells were then hemolysed by forcefully blowing 20 imOsM Tris buffer pH – 7.6 into the cell suspension followed by centrifugation at 4 °C. The supernatant was removed; the pellet containing the membrane was given three more identical wash cycles until the membrane was colorless [4]. Non-sealed erythrocyte ghost was obtained as almost white pellet. The serum was isolated from clotted blood by centrifugation and was stored in −80 °C for analysis.

2.4. Estimation of total protein content in serum and ghost membrane

The amount of total protein content was estimated according to the method of Lowry et al. [5] using standard protein bovine serum albumin (BSA) (Sigma chemicals).

2.5. Investigation of AOPP (advanced oxidation protein product) level

Determination of AOPP was carried out using plasma from different groups of patients, according to the method of Witko-Sarsat et al. [6]. Plasma was diluted 5 times with PBS (pH 7.4), chloramin-T (1-100 μmol) (LOBA-CHEMIE) was used as a calibrator. The reaction was initiated by the addition of 50 μl of 1.16 mol/l potassium iodide (KI), after 2 min 200 μl of glacial acetic acid was added into it. The absorbance of the reaction mixture was monitored immediately at 340 nm. The level of AOPP was expressed in μmol of chloramin-T equivalent per litre of plasma (μmol/l).

2.6. Fluorescence microscopic analysis of intracellular ROS (reactive oxygen species) generation

White blood cells were isolated from whole blood using lysis buffer (pH 7.4) [7]. After centrifugation of 10 min at 350 *g* and 4 °C, leukocyte pellet was suspended in Hanks' balanced salt solution (HBSS; pH 7.3). Leukocytes were then incubated at 37 °C for 15 min with 50 μmol/l 2′,7′-dichlorofluorescein diacetate (DCFH-DA). ROS production was then monitored by fluorescence microscope by measuring the fluorescence emission at 525 nm.

2.7. Evaluation of AGE (advanced glycation end product)

Plasma pentosidine (the index for estimating AGE) content was determined [8] using reverse phase HPLC (Water). Plasma was lyophilized and hydrolyzed by equal amount of 6 N HCl at 110 °C under nitrogen atmosphere for 16 h. It was subsequent-