Effects of pentoxifylline on oxidative stress and levels of EGF and NO in blood of diabetic type-2 patients; a randomized, double-blind placebo-controlled clinical trial

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Abstract

Background. – As oxidative stress contributes to both progression and pathologic complications of diabetes and effective therapeutic strategies to prevent or delay the damage remain limited, the aim of the present study was to assess the efficacy of pentoxifylline in reducing oxidative stress. Since there is a relationship between nitric oxide (NO), epidermal growth factor (EGF) and oxidative stress, we measured the effect of this drug on these parameters in comparison to placebo.

Methods. – Thirty-nine patients with type-2 diabetes mellitus were randomized in a double blind, placebo-controlled clinical trial to receive either pentoxifylline 400 mg four times a day or placebo for 14 days. Blood samples were obtained at baseline and at the end of the study. Samples were analyzed for thiobarbituric reactive substances (TBARS) as a marker of lipid peroxidation, ferric reducing ability (total antioxidant power, TAP), EGF and NO levels.

Results. – Pentoxifylline in comparison to placebo was effective ($P < 0.05$) in reduction of lipid peroxidation in plasma of the patients without significant effects on TAP, levels of EGF and NO in plasma.

Conclusion. – Adding of pentoxifylline to drug regimen of diabetic type-2 patients can be helpful. Exact mechanism of action of pentoxifylline in reduction of blood lipid peroxidation remains to be elucidated.

Keywords: Type-2 diabetes mellitus; Oxidative stress; Nitric oxide; Epidermal growth factor; Pentoxifylline

1. Introduction

Diabetes is recognized as one of the leading causes of morbidity and mortality in the world [1]. Diabetic complications in target organs arise from chronic elevation of glucose. The pathogenic effect of high glucose is mediated to a significant extent via oxidative stress [2]. Oxidative stress occurs where there is an imbalance between free radical production and the body’s antioxidant defense [3].

Recent studies demonstrate that a single hyperglycemia-induced process of over production of superoxide by the mitochondrial electron transport chain seems to be the first and the key event in the activation of all other pathways involved in the pathogenesis of diabetic complications. These include increased polyol pathway flux, increased advanced glycosylation end product formation, activation of protein kinase C, and increased hexosamine pathway flux. Superoxide overproduction is accompanied by increased nitric oxide (NO) generation, due to an endothelial nitric oxide synthase (NOS) and inducible NOS uncoupled state, a phenomenon favoring the formation of the strong oxidant peroxynitrite, which in turn damages DNA. These processes result in acute endothelial dysfunction in diabetic blood vessels that, convincingly, also contributes to the development of diabetic complications [4].

On this matter, it has recently been suggested that antioxidant therapy with vitamin E or other antioxidants is limited to scavenging already formed oxidants and may therefore be considered a more symptomatic rather than a causal treatment for vascular oxidative stress [5].
Mitochondrial radical production associated with hyperglycemia will also disrupt glucose-stimulated insulin secretion by pancreatic beta-cells, because they are particularly susceptible to oxidative damage. Therefore, oxidative stress contributes to both the progression and pathologic complications of diabetes [6].

Although our understanding of how hyperglycemia-induced oxidative stress ultimately leads to tissue damage has advanced considerably in recent years, effective therapeutic strategies to prevent or delay the development of this damage remain limited.

Pentoxifylline is a xanthine derivative which has inhibitory effects on xanthine oxidase [7]. Xanthine oxidase is considered as a candidate for oxygen free radical formation in cells [8]. Pentoxifylline down regulates production of tumor necrosis factor-alpha (TNF-$\alpha$). This cytokine provokes a rise in hydrogen peroxide production from mitochondria [9,10]. This drug is a non-selective phosphodiesterase (PDE) inhibitor [11] and it may have some effects on production of epidermal growth factor (EGF) and NO [12].

Interaction of EGF with its receptor causes transient increase in hydrogen peroxide [10]. EGF-receptor extra cellular signal regulated protein kinase (ERK) pathway as the major signaling pathway that mediates up regulation of aldehyde reductase expression under oxidative stress [13]. Our recent study showed that oxidative stress and changes in EGF and NO present in diabetic type-1 and -2 patients [14,15].

Another factor that increases oxidative stress in diabetic patients is the interaction of advanced glycation end-products (AGEs) with their receptors (RAGE) which can lead to modification in cell signaling and further production of free radicals [16] and it has been reported that this drug has some inhibitory effects on AGE formation [17].

According to the aforementioned preliminary information, this randomized clinical trial was performed to elaborate the value of pentoxifylline as a causal antioxidant, on reduction of oxidative stress and measuring its effect on EGF and NO plasma level in diabetic type-2 patients.

2. Materials and methods

2.1. Materials

Phosphate buffer, 2,4,6-tripyridyl-s triazine (TPTZ), 2-thiobarbituric acid (TBA), 1,1,3,3-tetraethoxypropane (malondialdehyde, MDA), trichloroacetic acid (TCA) and n-butanol were purchased from Merck Chemical Company (Germany). EGF and NO determination kits (Quantikine®) were provided from R&D system GmbH, Germany.

2.2. Participants and study design

In this randomized, double-blind placebo-controlled clinical trial, we recruited 39 patients (22 male and 17 female) with type-2 diabetes mellitus aged (56.26 ± 9.51 years) from the Endocrinology and Metabolism Research Center (EMRC) outpatient clinic in Shariati Hospital, Tehran University of Medical Sciences (TUMS).

Study subjects were chosen from the previously diagnosed diabetic patients according to American Diabetes Association (ADA) criteria [18] which were controlled by diet and oral hypoglycemic therapy. Data on dietary habits, dietary supplements, anthropometric indices including body mass index (BMI), medical history, smoking, drinking and taking medication were obtained from face to face interview.

Exclusion criteria were as follows: documented coronary artery disease, significant renal impairment (serum creatinine > 270 µmol/l), chronic liver disease, diabetic foot ulceration, pulmonary infection, smoking, pregnancy, age less than 35 and supplementation with vitamins or traditional herbs in previous 3 months.

The subjects were fully informed of the purpose, procedures and hazards of the trial and were free to leave the trial at any time. Written informed consent was obtained from all participants. The research protocol was approved by the Ethics Committee on Human Experimentation of TUMS.

The selected subjects were stratified by sex and randomly assigned into two groups. One group ($n = 20$) received 400 mg pentoxifylline four times a day for 14 days and the other group ($n = 19$) received placebo with the same regimen. The subjects were asked not to alter their usual diets and physical activity throughout the study and any changes in their medication were avoided whenever possible.

2.3. Sample collection and handling

After 12–14 h overnight fasting, between 08:00 and 10:30 h and before taking any oral hypoglycemic agent(s), 10 ml blood sample was collected from each subject in tubes containing heparin, before and after administration of pentoxifylline or placebo for 14 days. After centrifugation of blood at 3000 × g for 30 min at 4 °C, the plasma supernatant fluid was separated and stored at –80 °C until analyzed further.

3. Total antioxidant power (TAP) assay

Antioxidant power of plasma was determined by measuring their ability to reduce Fe$^{3+}$ to Fe$^{2+}$ established as named FRAP test and described previously [19]. Briefly, in this test, the medium is exposed to Fe$^{3+}$ and the antioxidants present in medium start to produce Fe$^{2+}$ as an antioxidant activity. The reagent included 300 mmol/l acetate buffer, pH 3.6 and 16 ml C$_2$H$_5$O$_2$ per l of buffer solution, 10 mmol/l TPTZ in 40 mmol/l HCL, 20 mmol/l FeCl$_3$, 6H$_2$O. Working FRAP reagent was prepared as required by mixing 25 ml acetate buffer, 2.5 ml TPTZ solution, and 2.5 ml FeCl$_3$, 6H$_2$O solution. Ten microliters of H$_2$O-diluted sample was then added to 300 µl freshly prepared reagent warmed at 37 °C. The complex between Fe$^{3+}$ and TPTZ gives a blue color with absorbance at 593 nm.
3.1. Lipid peroxidation assay

The method based on the reaction of MDA as the end product of the oxidation of polyunsaturated fatty acids and its concentration in the medium is an established measure of lipid peroxidation extent. In this test the reaction of MDA with TBA creates a complex which is determined spectrophotometrically while lipid peroxidation in samples are assessed in terms of TBA reactive substances (TBARS) produced. Briefly, the samples were diluted by buffered saline (1:5) and 800 µl of TCA (28% w/v) was added to 400 µl of this mixture and centrifuged in 3000 x g for 30 min. Then, the precipitate was dissolved in sulfuric acid and 600 µl of the mixture was added to 150 µl of TBA (1% w/v). The mixture was then incubated for 15 min in a boiling water bath. Following incubation, 4 ml of n-butanol was added, the solution centrifuged, cooled and the absorption of the supernatant was recorded in 532 nm using a UV-160-A Shimadzu double beam spectrophotometer (Japan). The calibration curve of a 1,1,3,3-tetraethoxypropan standard solutions was used to determine the concentrations of TBA-MDA adducts in samples [20].

3.2. EGF determination

Concentrations of EGF in plasma was measured using a Quantikine® EGF Human Immunoassay kit. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for EGF has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any EGF present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for EGF was added to wells. Following a wash to remove any unbound antibody-enzyme reagent, a substance solution was added to the wells and color development in proportion to the amount of EGF bound in the initial step. The color development was stopped and the intensity of the color was measured by ELIZA reader.

3.3. Nitric oxide determination

Concentration of total NO in plasma was measured using a Quantikine® NO Human Immunoassay kit. Total NO was assayed based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is based on the two-step diazotization reaction in which acidified NO\textsubscript{2}\textsuperscript{-} produces a nitrosating agent, which reacts with sulfonic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm and read by ELIZA reader.

3.4. HbA1c determination

It was measured by high performance liquid chromatography (HPLC) as described previously [21].

3.5. Statistical analysis

The statistical analysis was performed with the use of SPSS ver 11.5. Continuous data are expressed as mean ± S.E. General characteristics such as age, sex, duration of diabetes, BMI, and blood glucose concentrations were compared between groups with the use of unpaired 2-sample Student’s t-test for continuous data and with the use of Chi-square test for categorical data if applicable. Paired t-test was applied to compare before and after intervention levels of favorable parameters in each group. A P value < 0.05 was considered to be statistically significant.

4. Results

As shown in Table 1, at the beginning of the study, the groups were similar with respect to the sex, age, duration of diabetes, BMI, blood pressure and HbA1c. Two patients were diet control only and the others were treated with sulfonylurea and/or metformin. During the study period, there were no significant changes in physical activity, dietary intake or medication.

Table 2 shows the oxidative stress parameters, EGF, NO, and HbA1c levels before and after the drug or placebo administration. Pentoxifylline in comparison to placebo was effective (P < 0.05) in reduction of plasma TBARS but it didn’t show any effects on TAP, and levels of EGF and NO in plasma. There was no significant change in HbA1c levels between groups during the study.

5. Discussion

Increased oxidative stress in type-2 diabetes could result from increased generation of reactive oxygen species or diminished antioxidant defense or both. Lipid peroxidation is a central feature of oxidant injury [22] and leads to formation of MDA. The end-products of lipid peroxidation including unsaturated aldehydes and other metabolites have also cytotoxic and mutagenic properties [23].

Some new findings implicates that classic antioxidants, such as vitamin E, which work by scavenging already formed toxic oxidation products, have failed to show beneficial effects.
on diabetic complications and may suggest new and attractive causal antioxidant therapy [15]. Our data also showed that pentoxifylline therapy was more effective than placebo in reduction plasma lipid peroxidation. Pentoxifylline is a PDE inhibitor capable of increasing cAMP and cGMP levels [24]. There are good evidences that cyclic nucleotides are able to prevent from oxidative stress by reduction of lipid peroxidation [25,26]. Therefore, it is concluded that pentoxifylline reduces the cellular injury resulted from free radicals. This process demonstrates itself as reduced levels of lipid peroxidation end-products. Pentoxifylline do this by inhibiting PDE the enzyme responsible in degradation of cellular cyclic nucleotides. The present study also showed that pentoxifylline administration did not change blood TAP. This means that pentoxifylline shows this protective effect by inhibiting free oxygen radicals to a greater extent than developing the antioxidant capacity. This is in concordance with report of Noyan et al. [27] and the theory that this drug acts as a causal antioxidant rather than a scavenger of already formed oxidation products.

Since many investigators have demonstrated different effects of this drug on NO synthesis, we measured the plasma levels of nitrite/nitrate (stable metabolites of NO) to assess the effect of pentoxifylline on NO level in this group of patients. Although usage of this drug in patients with HIV infection decreased NO production [28], our results did not show any difference between pentoxifylline and placebo on NO level in this group of patients. Nevertheless, simultaneous measurement of circulating cGMP, a second messenger produced by soluble guanylate cyclase following stimulation by NO is required to allow differentiation between NO synthesis and oxidative inactivation [29]. Therefore, regarding the ability of pentoxifylline in reduction of lipid peroxidation level, it is suggested that although pentoxifylline did not show to have any effects on NO level, but may protect it from inactivation by free radicals by reducing free radical formation.

The present results also showed that pentoxifylline does not affect blood EGF level. Since there is evidence that EGF may have a role in pathogenesis of diabetes-related kidney disease [30] and oxidative stress, it can be considered as a good point.

Although it has been reported that this drug has some inhibitory effect on AGE formation [17], in this study, pentoxifylline did not change HbA1c levels significantly and our finding is in concordance with report of Harmankaya et al. [31].

There are some studies in which pentoxifylline (1200–1600 mg/day) has been used safely in diabetic patients without significant adverse effects [32,33] and this is in accordance with our findings that pentoxifylline was well tolerated in all patients except one case of headache.

Taking collectively, we conclude that adding of pentoxifylline to diabetic type-2 patients drug regimen can be helpful. Exact mechanism of action of pentoxifylline in reduction of blood lipid peroxidation remains to be clarified by further experimental studies.

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Table 2

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<th>Pentoxifylline</th>
<th>Placebo</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>TBARS (µmol/l)</td>
<td>152 ± 8.52</td>
<td>110 ± 7.61</td>
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<tr>
<td>FRAP (mmol/l)</td>
<td>454.20 ± 13.91</td>
<td>418.78 ± 13.55</td>
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<tr>
<td>EGF (µg/ml)</td>
<td>428.83 ± 18.85</td>
<td>490.07 ± 18.37</td>
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<tr>
<td>NO (µmol/l)</td>
<td>829.48 ± 7.89</td>
<td>805.38 ± 6.43</td>
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<td>HbA1c (%)</td>
<td>7.26 ± 1.44</td>
<td>7.15 ± 1.31</td>
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</table>

*a Represents that the difference between before and after intervention is significant at P < 0.01.

References


