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Potential Therapeutic Effects of *Morus alba* Leaf Extract on Modulation Oxidative Damages Induced by Hyperglycemia in Cultured Fetus Fibroblast Cells

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Abstract: Reactive oxygen species (ROS) generation plays a central role in pathogenesis of diabetes mellitus. Therefore, antioxidant therapy may inhibit progress diseases and thereby prevent the clinical complications in these people. In this study, the protective effects of alcoholic extraction *Morus alba* leaves on fetus fibroblast cells under high glucose condition were assessed. The fetus fibroblast cells were incubated with high dose of glucose and treated with plant extraction. Total antioxidant, glutathione content and lipid peroxidation were measured. The results obtained in the oxidative stress markers support this hypothesis that *Morus alba* may protect the cells toxicity induced by high glucose via antioxidant system.

Key words: *Morus alba* · Diabetes · Oxidative Stress

INTRODUCTION

Diabetes is associated with hyperglycemia and as a major non-epidemic disease caused a considerable cost of the total health budget [1]. The number of people with diabetes is increasing due to population growth, urbanization and increasing prevalence of obesity and physical inactivity [2]. The worldwide figure of diabetic patients is set to rise from 150 million in the year 2000 to 300 million in 2025[3].

Diabetic patients have significant defects of antioxidant defense elements and it is generally accepted that reactive oxygen species (ROS) generation is one of the major determinants of diabetic complications [3-5]. Lipid peroxidation and alternation of antioxidants was induced in diabetic patients and oxidative stress has been implicated in the pathology of diseases [6]. ROS have been implicated in causing cell damage and cell death. Treatment by ROS scavengers has potential to delay diabetic complications. Diabetes patients have greater antioxidant requirements.

Plants antioxidants are known to inhibit oxidative damages. Their antioxidants can scaverage free radicals. *Morus alba* leaves are useful for prevention of diabetes. The inhibitory effect on human intestinal disaccharidase activity had been approved. This plant has a suppressive effect on both the blood glucose response and on the secretion of insulin [7]. It suppresses blood glucose and insulin by inhibiting the intestinal sucrose activity [8].

In our laboratory, the antioxidant level of Red Blood Cell that incubated with this plant extraction was markedly increased. Further, in our study, among of selected medicinal plants, a potent antioxidant activity of *Morus alba* leaves extract was observed [9]. Thus, a fundamental question addressed that whether *Morus alba* leaves are effective on damage result of ROS in addition to decrease blood glucose level. Therefore, this study was conducted to assess the protective effects of *Morus alba* leaves on fetus fibroblast cells from a view point of oxidative stress under hyperglycemia condition.

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MATERIALS AND METHODS

Plant Material: Morus alba leaves were separated, air dried in the shade and powdered. The extraction was prepared in ethanol-water (2:1). The extract was transferred to vials and kept at 4°C.

Cell Culture: The fetus fibroblast cells were obtained from Pasteur Institute of Iran (NIH-3T3D4). Cells were maintained in Dulbecco´s Modified Eagle´s Medium (DMEM) supplement with 10% foetal calf serum, 2mM L-Glutamine, 50U/ml sodium penicillium, 50µg/ml streptomycin. The cells were cultured at 37°C in a humidified atmosphere and 5% CO_2. The oxidative stress markers and intracellular antioxidant could be assayed in any kind of cell and for this purpose; the fetus fibroblast cells were chosen.

Experimental Design: The fetus fibroblast cells were seeded in a 24 well plates. After cells reached confluence, a stock of medium with 35.5mM glucose (a) was prepared. The medium was replaced for seven days with this medium. The medium was renewed two times in this period. Next, the medium was divided into two groups: 35.5mM glucose (a_1) and 35.5mM glucose+1000ppm of plant extraction (a_2). The plates were incubated for 24 hrs. For determining oxidative stress, lipid per-oxidation, CUPRAC assay and the glutathione content were investigated.

Determination Lipid Per-Oxidation: The formation of thiobarbituric acid in cells was measured for lipid per-oxidation according to the method of Sicinska *et al*. [10]. Briefly, the cells were rinsed three times with PBS. Then, they were lysed by sonication and repeated freeze and thawing for three times. The cell solution mixed with 20% trichloroacetic acid. Samples were centrifuged. Thiobarbituric acid was added to the supernatant and the samples were heated. The absorbance of the supernatant was measured at 532 nm.

Determination Total Antioxidant Level : The CUPRAC assay was applied for measuring total antioxidants capacity according to the method of Apak *et al.* [11] with some modification. The cells were lysed in138mM Tris buffer (pH 7.6) containing0.1% Triton X-100, 1 mM Na-EDTA and 1mM phenylmethylsulfonyl fluoride. According to our pervious study, to apply this mixture had been shown to protect the antioxidants more than other lysing methods. Then, the cell solution was incubated with $Cucl_2$ + Neocuproine + NH_4Ac. The absorbance of the supernatant was measured at 450 nm.

Determination Glutathione Content: The glutathione content was applied according to the previous method with some modification. The cells were rinsed three times with PBS. The cell solution mixed with 20% trichloroacetic acid. Samples were centrifuged. The supernatant was mixed with 4 vol. of Tris. Then, 1mM DTNB was added to the sample and incubated for 30 minutes. The absorbance was read at 412 nm.

Measurement of Protein Content : In order to eliminate variances from the cell culture, results of individual wells have to be normalized with respect to protein concentration. The protein in cells was measured by Bradford method. Bovine serum albumin was determined as the standard.

RESULTS

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS). The data is expressed as the mean ± SD. In this study evaluation was made by comparing between two groups (a_1 and a_2) by analysis of *t*-test and the p-value <0.05 was considered as statistically significant. The results of experiments are shown in Table (1). The cytoprotective effect of plant extraction against hyperglycemia was determined by oxidative stress markers. The level of CUPRAC assay,

Table 1: Effects of plant extraction on the levels of oxidative stress markers

Kind of stocks	Lipid per-oxidation	Glutathione content	Total antioxidant
(a_1) 35.5mM glucose	0.018±0.004	0.006±0.0005	0.077±0.01
(a_2) 35.5mM glucose+1000ppm of plant	0.015±0.0009	0.014±0.001	0.15±0.03

Each value represents the mean ±SD per group.

glutathione content and inhibition of lipid peroxidation were analyzed with respect to OD measured at wavelength with spectrophotometer. These oxidative stress markers were changed and the values were significant difference in extract treated cells (P<0.05). These changes in level of glutathione content was markedly observed (p=0.002).

DISCUSSION

The present finding indicates that *Morus alba* leaves increase the ability of cells to develop resistance to hyperglycemia.

The effects of hyperglycemia are often irreversible and lead to progressive cell dysfunction. Hyperglycemia cause via six pathways ROS formation [11]. ROS are highly unstable capable damaging molecules such as DNA, protein and lipid. It is important mediators for several changes, including apoptosis. Major complications of diabetes mellitus have been associated with increased oxidative stress [13]. The clinical state of diabetes is accompanied by elevated blood levels of cholesterol, triglyceride, free fatty acids and LDL [12]. These components should be protected from oxidation. The oxidation of LDL is believed to play an important role in the development of atherosclerosis and oxidative stress is invoked as a pathogenic mechanism for atherosclerosis [14]. Moreover, the alteration in antioxidant level also is one of the possible links between hyperglycemia and cardiovascular disease [15]. These changes should be prevented by endogenous and exogenous antioxidants. People with diabetes are at two to fourfold greater risk of cardiovascular disease [16]. It also impairs the endogens antioxidants defense system in many ways during diabetes [17]. The ROS scavenging capacity through the antioxidant system becomes insufficient in diabetes [18]. Therefore, the antioxidant therapy should be continued in addition to other conventional treatment in diabetic patients.

To elucidate the possible link between the antioxidant activity of this plant and resistance to hyperglycemia effects, CUPRAC assay, glutathione level and lipid peroxidation endpoints were employed. The results obtained from oxidative stress markers (Table 1) suggest that the antioxidant characteristics of this plant may mediate the resistance to hyperglycemia-induced damage of cells. The glutathione content and total antioxidant capacity (CUPRAC assay) markedly increased. Glutathione concentration decreased in the liver, kidney, pancreas, plasma, red blood cells, nerve and precataractous lens of chemically induced diabetic animals [18]. The degree of lipid peroxidation was established by measuring thiobarbituric acid reactive substance (TBARS) concentration. There is an increase in TBARS in diabetes. In the cell, TBARS level moderately decreased following treatment with plant extraction (Table 1). It was reduced by 20% compared with that of the control group (cells without plant treatment).

Collectively, *Morus alba* leaves could be used as lowering glucose and a natural antioxidant sources. Oxidative damages should be prevented or limited by dietary antioxidants. Therefore, regular consuming this plant could also decrease susceptibility of these people to oxidative damage, improve resistance to hyperglycemic and delay diabetic complications. However, further investigation especially *in vivo* studies and human clinical trials are recommended.

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