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Haemato-protective influence of dietary fenugreek (*Trigonella foenum-graecum* L.) seeds is potentiated by onion (*Allium cepa* L.) in streptozotocin-induced diabetic rats

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ABSTRACT

We have recently reported the beneficial modulation of metabolic abnormalities and oxidative stress in diabetic rats by dietary fenugreek seeds and onion. This investigation evaluated the protective influence of dietary fenugreek seeds (100 g kg^{-1}) and onion (30 g kg^{-1}) on erythrocytes of streptozotocin-induced diabetic rats, through modulation of reduced haematological indices and antisickling potency. This study also evaluated the altered erythrocyte membrane lipid profile and beneficial countering of increased lipid peroxidation, osmotic fragility, along with reduced membrane fluidity and deformability, nitric oxide production and echinocyte formation. Dietary fenugreek seeds and onion appeared to counter the deformity and fragility of erythrocytes partially in diabetic rats by their antioxidant potential and hypocholesterolemic property. The antisickling potency of these spices was accomplished by a substantial decrease in echinocyte population and AGEs in diabetic rats. Further insight into the factors that might have reduced the fluidity of erythrocytes in diabetic rats revealed changes in the cholesterol: phospholipid ratio, fatty acid profile, and activities of membrane-bound enzymes. Dietary fenugreek seeds and onion offered a beneficial protective effect to the red blood cells, the effect being higher with fenugreek + onion. This is the first report on the hemato-protective influence of a nutraceutical food component in diabetic situation.

1. Introduction

There is increasing evidence to believe that oxidative stress is involved in the etiology of diabetic complications. Any decrease in the body's antioxidant defense system provokes the oxidative attack on lipids, proteins, and cytoskeleton of membranes. Hyperglycemia produces reactive oxygen species (ROS) as a result of glucose auto-oxidation and the development of advanced glycation end products (AGE); and is considered as a primary cause of diabetic vascular complications. A hallmark of the vascular disease is endothelial cell dysfunction characterized by altered nitric oxide (NO)-dependent phenomena such as vasodilatation, angiogenesis, and vascular maintenance [1]. The highly reactive NO is secreted by the endothelium and is a major modulator of endothelial function [2]. Abnormalities in the body's production of NO have also been implicated in high blood pressure, atherosclerosis, erectile dysfunction and stroke. Its elevated level leads to reactive nitrogen species, which cause damage to the body similar to ROS [3].

Erythrocytes are highly vulnerable to oxidative damage perhaps due to the high content of polyunsaturated fatty acid in their membrane. Thus, increased lipid peroxidation, besides alteration in the membrane lipid distribution resulting from the hyperlipidemic situation in diabetes, altered membrane fluidity, and activities of membrane-bound enzymes are to be expected during diabetic condition. Thereby, the structural and functional deformity might result when erythrocyte membrane lipid bilayer gets altered as indicated by increased osmotic fragility and modifications in erythrocyte morphology [4].

Dietary fenugreek (*Trigonella foenum-graecum*) seeds are well recognized to possess multiple health beneficial potential which understandably contributes to antidiabetic, hypocholesterolemic, and antioxidant influences of this spice [5]. Onion (*Allium cepa*) is well understood both by preclinical and clinical trials to have a wide range of health benefits, including antidiabetic, antithrombotic, and hypocholesterolemic effects as has been recently reviewed [6]. These two spices have also been recently demonstrated to ameliorate hyperglycemia, with associated metabolic abnormalities and oxidative stress in hepatic and cardiac tissues in streptozotocin-induced diabetic rats [7,8]. Information on the deleterious effect of diabetes on erythrocyte's structural and functional integrity is limited. In the absence of any information on the haemato-protective influences of dietary fenugreek

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seeds and onion in conditions of diabetes, it would be relevant to examine these spices, particularly for this nutraceutical effect. Since there is a possibility of synergy among these spices in their hypoglycemic and antioxidant action, since they work by different mechanisms, it is also relevant to evaluate their specific combination in this context. Hence, this study was designed to investigate the haemato-protective influence of dietary fenugreek seeds, as a provider of dietary fibre, and onion, a well-established insulinotropic and antioxidant agent, both individually and in combination in STZ-induced diabetic rats. The present study has particularly focussed on the levels of oxidative stress and structural integrity in erythrocytes under diabetic condition.

2. Materials and methods

2.1. Materials

All the chemicals were procured from Sigma-Aldrich Chemical Co., (St Louis, MO, USA) or SISCO Research Laboratories (Mumbai, India) and were of analytical grade and highest purity. Fenugreek seeds (*Trigonella foenum-graecum* L.) were purchased from the local market and powdered, and stored at 4 °C. Onion (*Allium cepa* L.) which was procured from the local market was chopped, freeze-dried and powdered, and stored at 4 °C. Casein (Food grade) was procured from Nimesh Corporation (Mumbai, India). Maize starch, cane sugar powder, and refined groundnut oil were purchased from the local market.

2.2. Animals and experimental design

The animal study was carried out with due approval from the Institutional Animal Ethics Committee (CSIR-CFTRI, Mysore, India). Male Wistar rats (140–150 g body mass, $n = 80$) raised by the Experimental Animal Production Facility Unit of this Institute were housed individual cages under standard laboratory conditions with a 12/12 h light-dark cycles. All the animals had *ad libitum* access to food and water. Experimental diabetes was inducted by *i.p.* injection with 45 mg/kg STZ (Sigma-Aldrich, St Louis, MO, USA) dissolved in freshly prepared citrate buffer, pH 4.5. Animals with the fasting blood glucose level above 250 mg/dL were recruited as diabetic animals previously determined by Huggett and Nixon [9]. The animals were maintained on various experimental semisynthetic diets and water *ad libitum* for six weeks. Rats were divided into eight groups out of which four groups were diabetic (12 rats in each group), and the other four groups were non-diabetic (8 rats in each group). One group of diabetic animals ($n = 12$ per group) and a group of normal animals ($n = 8$ per group) were maintained on a semi-synthetic basal diet. The basal diet consisted of ($g\,kg^{-1}$): casein, 210; cane sugar, 100; corn starch, 540; NRC vitaminized starch, 10; Bernhardt-Tommarelli modified NRC salt mixture, 40, fat-soluble vitamins at the recommended levels and refined peanut oil, 100. The three experimental diets consisted of fenugreek seed powder ($100\,g\,kg^{-1}$), onion powder ($30\,g\,kg^{-1}$), and a combination of fenugreek seed ($100\,g\,kg^{-1}$) and onion powder ($30\,g\,kg^{-1}$), respectively, replacing an equivalent amount of starch in the basal semisynthetic diet. Thus, the eight animal groups were: (1) Normal control (C), (2) C + Fenugreek (10%), (3) C + Onion (3%), (4) C + Fenugreek (10%) + Onion (3%), (5) Diabetic control (D), (6) D + Fenugreek (10%), (7) D + Onion (3%), and (8) D + Fenugreek (10%) + Onion (3%). At the end of 6 weeks, the animals were sacrificed under euthanasia. Blood was collected in heparinised tubes by heart puncture and plasma was separated by centrifugation. Erythrocytes, hemolysate and their membranes were isolated according to the method of Dodge et al. [10]; stored at 4 °C until further analysis.

2.3. Measurement of lipid peroxidation

In vitro studies on H_2O_2-induced lipid peroxidation in erythrocyte and erythrocyte membrane isolated from experimental group were assayed according to the method of Rajasekaran et al. [11]. Lipid peroxides were assayed by measuring the malondialdehyde (MDA) concentration as thiobarbituric acid reactive substances (TBARS) spectrophotometrically according to the method described by Ohkawa et al. [12]. The percentage of maximal MDA release was calculated according to the following equation:

% Maximal release (MDA) = MDA release (3% H_2O_2)/ MDA release (0.75% H_2O_2 + Sodium azide

2.4. Advanced glycation-related fluorescence study

Advanced glycation-related fluorescence spectra in erythrocyte membrane were obtained from 400–500 nm with excitation at 370 nm in a spectrofluorometer (Shimadzu RF-5301 PC) according to Monnier and Cerami [13].

2.5. Haematological parameters, osmotic fragility and nitric oxide determination

Haematological parameters were quantified using an automated haematology analyzer (XP-100, Sysmex Corp., Japan). Erythrocyte osmotic fragility was determined according to the method described by Dacie and Lewis [14]. Nitric oxide (NO) concentration was determined using a commercially available kit (Catalog # K262, Biovision, Mountain View, CA, USA).

2.6. Erythrocyte antioxidant status and membrane bound enzymes

Catalase activity (CAT) was determined by the method of Aebi [15]. Superoxide dismutase (SOD) [16], glutathione peroxidase (GPx) [17], glutathione reductase (GR) [18] and glutathione-*S*-transferase (GST) activity [19] were assayed in the erythrocyte haemolysate. Ascorbic acid [20], total sulfhydryl (TSH) [21] and glutathione was estimated spectrophotometrically using Ellman's reagent [22]. Membrane-bound Na^+/K^+-ATPase was determined by the method of Jorgensen [23]. Mg^{2+}/Ca^{2+}-ATPase activity was measured independently by the method of Vajreswari et al. [24].

2.7. Measurement of anisotropy in erythrocyte membranes

Erythrocyte membrane fluidity was measured according to the method of Levin et al. [25]. The polarization of fluorescence was expressed in terms of the fluorescence anisotropy 'r' and calculated according to:

$$r = (I_{VV} - I_{VH} \times G)/(I_{VV} + I_{VH} \times G)$$

where, I_{VV} and I_{VH} are the components of emitted light intensity, parallel and perpendicular, respectively, with respect to the direction of polarization of the excitation light, and G is the correction factor ($G = I_{VV}/I_{VH}$) used to correct for unequal transmission in the optics. The anisotropy parameter $[(r_0/r)\text{-}1]^{-1}$ was calculated using the limiting anisotropy of 1,6-diphenyl-1, 3,5-hexatriene ($r_0 = 0{\cdot}362$).

2.8. Lipids and fatty acid profile

Erythrocyte membrane lipids were extracted by the procedure of Folch et al. [26]; total cholesterol [27] and phospholipid [28] was quantified. The fatty acid methyl esters were analyzed by gas chromatography (PerkinElmer, USA) using Elite-Wax (30 m × 9.25 mm) fused silica capillary column, a flame ionization detector (FID). The analysis was carried out in the isothermal condition. The operating conditions were as follows: column temperature: 240 °C, injection temperature: 250 °C and detector temperature: 260 °C. Nitrogen was used as the carrier gas. Individual fatty acids were identified by