ABSTRACT. Non-enzymatic glycosylation of proteins is the major cause of diabetic complications, such as cardiovascular disorders, retinopathy, nephropathy and neuropathy. It seems that protein glycosylation can be inhibited effectively by antioxidants. Several flavonoids, such as rutin, kaempferol, quercetin, apigenin, naringin, morin and biochanin A were selected to determine their antioxidant effects on in vitro insulin, hemoglobin and albumin glycosylation. The optimal glucose concentration and incubation time were obtained for each protein. Then, the inhibition percentage of protein glycosylation was measured in the presence of three different concentrations (0.5, 5, 10 μg/ml) of each flavonoids by a colorimetric method. The results demonstrated that biochanin A, the best inhibitor of insulin and hemoglobin glycosylation, inhibits their glycosylation 100% and 60%, respectively. Glycosylation of albumin was inhibited 100% by both biochanin A and apigenin. Therefore, it seems probable that plants containing flavonoids may have preventive effects in diabetic complications. [Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <getinfo@haworthpressinc.com> Website: <http://www.HaworthPress.com> © 2002 by The Haworth Press, Inc. All rights reserved.]

KEYWORDS. Flavonoids, protein glycosylation, antioxidants, albumin, insulin, hemoglobin
INTRODUCTION

Diabetes mellitus, the most common endocrine disease is the fourth leading cause of death in the United States, with a prevalence of 3-10%.\textsuperscript{1,2} High morbidity and mortality of diabetes are mainly due to its micro- and macrovascular complications which lead to adverse effects on the kidneys, eyes and cardiovascular and central nervous systems.\textsuperscript{3,4}

Evidence from clinical experience and animal model studies indicates that diabetic hyperglycemia may be the major factor contributing to the progressive secondary complications.\textsuperscript{4,5} Non-enzymatic glycosylation of body proteins such as hemoglobin, due to prolonged hyperglycemia, is an important consequence of such a pathophysiologic mechanism.\textsuperscript{3-7} Such glycosylation modifies the structural and functional properties of a number of proteins, including membrane lipoproteins, erythrocyte membrane proteins, platelet and lens \(\alpha\)-crystallin proteins human serum proteins, such as albumin, hemoglobin, collagen, myelin, as well as insulin.\textsuperscript{6-17}

Antioxidant inhibition of protein glycosylation is considered a possible preventive mechanism. Plant polyphenolic flavonoids, widely distributed in vegetables and other plant foods, have been studied for their capacity to scavenge free hydroxyl and peroxyl radicals.\textsuperscript{21-25} These dietary sources of antioxidants contribute over 4,000 chemically unique substances from all parts of vascular plants including fruits, vegetables, nuts, seeds, stems and flowers, as well as from tea and wine.\textsuperscript{26} The inhibitory effects of rutin, quercetin and kaempferol on hemoglobin glycosylation have been shown, \textit{in vitro}.\textsuperscript{27} The aim of this study was to measure the inhibitory effects of seven flavonoids, including rutin, quercetin, kaempferol, morin, apigenin, naringin and biochanin A, on glycosylation of albumin, hemoglobin and insulin, \textit{in vitro}.

MATERIALS AND METHODS

Preparation of Protein Solutions

\textbf{Hemoglobin}

The method is the same as our previous report on antioxidant effect of flavonoids.\textsuperscript{27} Blood from normal volunteers was drawn using EDTA as an anticoagulant. The red blood cells were washed thrice with 0.14 M NaCl solution. Then one vol. of red blood cells suspension was lysed with two vol. of 0.01 M phosphate buffer (pH 7.4) and 0.5 vol. \(\text{CCl}_4\). After lysing, the hemolysate was freed from debris by centrifugation. The upper layer was separated and hemoglobin concentration was measured by the Drabkin method.\textsuperscript{18,19}

\textbf{Albumin}

Human albumin (Sigma 5 g) was diluted with 0.01 M phosphate buffer (pH 7.4) to 100 ml.

\textbf{Insulin}

Regular bovine insulin (100 IU/ml) manufactured by Lilly was prepared by adding to 0.5 ml of 0.01 M phosphate buffer (pH 7.4).

Determining the Optimal Conditions for Protein Glycosylation

Glucose solutions (0, 4, 10, 20, 30, 40 and 50 mg/ml) were prepared with 0.01 M phosphate buffer (pH 7.4) and gentamycin (20 mg/100 ml). The optimal concentration of glucose and incubation time were determined separately for each protein solution. The protein solutions were incubated for periods of 24, 48, 72 and 96 hours. After incubation, they were washed twice with one ml of \(\text{CCl}_3\)COOH (20%) by centrifuging at 3000 rpm for 10 min. The sediments obtained were heated at 100°C for one hour in the hot water bath with one ml of 0.01 M phosphate buffer (pH 7.4) and 0.5 ml of 0.3 N oxalic acid. Cooled down to room temperature (25°C) and washed with 0.5 ml of \(\text{CCl}_3\)COOH (40%) by centrifugation at 3000 rpm for 10 min. The supernatant was separated and heated at 40°C for 30 min with 0.5 ml of 0.05 M thioobarbituric acid. The end product was measured at 443 nm by a colorimetric method.\textsuperscript{29}

Preparation of Flavonoid Solutions

Stock solutions (1 mg/ml) of the flavonoids (rutin, quercetin and kaempferol from Merck, morin, naringin and apigenin from Sigma, and biochanin A from Aldrich) were prepared with ethanol, and then the required concentrations (10, 100 and 200 \(\mu\)g/ml) were prepared in DMSO.

Assay

One ml of albumin (50 mg/ml), 1 ml of hemoglobin (50 mg/ml), and 0.5 ml of regular insulin (100 IU/ml), in 0.5 ml of 0.01 M phosphate buffer...
buffer (pH 7.4) were incubated for 72 hours separately in the presence of different concentrations of the flavonoids and 1 ml of a solution containing glucose (30 mg/ml for insulin and albumin, 20 mg/ml for hemoglobin) and gentamycin 20 mg/100 ml in 0.01 M phosphate buffer (pH 7.4). Also, the control solutions containing proteins and glucose were incubated in the absence of flavonoids. Then the glycosylation degree in the presence of different products and also in their absence were measured by the colorimetric measurements.

RESULTS

The optimal time and glucose concentration were found for each protein solution are shown in Figures 1-3. The inhibition percentage of protein glycosylation was used to express the effect of the tested flavonoids (Tables 1-3). For each concentration of each of the proteins the experiments were repeated thrice, and the figures reported are means of three measurements. The Student’s t-test showed a significant difference (P < 0.05) between a test sample and its respective control in all cases. Comparison of the inhibitory potency of each flavonoid (in the maximal concentration) for non-enzymatic glycosylation of the proteins mentioned showed the following results:

- Rutin had its strongest inhibitory effect on insulin glycosylation (100%). The order of inhibition was insulin > albumin > hemoglobin.
- Kaempferol inhibitory effect on the production of glycated insulin was most pronounced (93%) when compared to the other two proteins; the order of inhibition was insulin > albumin > hemoglobin.
- Quercetin had the highest inhibitory effect (85%) on albumin glycosylation; the order of inhibition was albumin > insulin > hemoglobin.
- Apigenin showed the strongest inhibitory effect on albumin glycosylation (100%), the order of inhibition was albumin > insulin > hemoglobin.
- Naringin inhibited albumin glycosylation quite appreciably (97%). The order of inhibition was albumin > insulin > hemoglobin.
- Morin inhibited albumin glycosylation, with the highest inhibitory effect (at a concentration of 10 µg/ml) as 90%. The inhibitory potency order was albumin > insulin > hemoglobin.
- Finally, biochanin A showed the strongest inhibitory effect on albumin and insulin glycosylation (100%); the order of inhibitory potency was albumin, insulin > hemoglobin.

DISCUSSION

Despite insulin therapy, diabetic patients suffer from some chronic clinical complications due to high blood glucose which induces non-enzymatic glycosylation of natural proteins such as hemoglobin, lens proteins, biomembrane proteins, albumin, collagen and myelin.

In our previous study, the preventing effect on hemoglobin glycosylation at final concentrations of 0.5, 5 and 10 µg/ml was found to be 11%, 27% and 42% for rutin, 3%, 37% and 52% for quercetin, and 10%, 12% and 15% for kaempferol, respectively (27). In the present study, the antioxidants, i.e., flavonoids, were assayed for their potential inhibitory effects on oxidative glycosylation.

Since the glycosylation inhibitory potency was concentration-dependent, the comparisons were made at similar concentrations for each flavonoid. The potency increases as the concentration increases in the case of all the flavonoids studied. The wide differences observed among the flavonoids as regards the protein glycosylation inhibitory potency.

FIGURE 1. The glycosylation percentage of hemoglobin in different concentrations of glucose and incubation times (P < 0.05)
FIGURE 2. The glycosylation percentage of albumin in different concentrations of glucose and incubation times (P < 0.05)

FIGURE 3. The glycosylation percentage of insulin in different concentrations of glucose and incubation times (P < 0.05)

may be partly due to differences in the types and sites of the functional groups and the reactivity of the protein molecule. More generally, a protein's molecular structure may partly explain the differences. For example, at a concentration of 0.5 µg/ml, rutin showed the lowest inhibitory effect (6%) in the case of albumin, while the effect was quite

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration of tested compound (µg/ml)</th>
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<tbody>
<tr>
<td>Rutin</td>
<td>62 83 93</td>
</tr>
<tr>
<td>Quercetin</td>
<td>45 67 79</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>62 81 93</td>
</tr>
<tr>
<td>Morin</td>
<td>67 77 82</td>
</tr>
<tr>
<td>Apigenin</td>
<td>42 47 61</td>
</tr>
<tr>
<td>Naringin</td>
<td>64 72 72</td>
</tr>
<tr>
<td>Biochanin A</td>
<td>70 96 100</td>
</tr>
</tbody>
</table>

For quercetin, rutin and kaempferol see Ref. 27.
appreciable in the case of the other two proteins. The fact that it was the glycosylation of albumin which was most strongly inhibited (100%) by apigenin (10 µg/ml) could further indicate the possible role of the structure and chemical properties of the protein. On the other hand, the structure and antioxidant properties of the flavonoids themselves can also explain the differences. It is very interesting to note that biochanin A (10 µg/ml) with the strongest inhibitory effect on all the 3 proteins is an isoflavone with an antioxidant feature, while apigenin (10 µg/ml), a flavone, exhibits a comparable inhibitory antioxidant effect (100%) only on albumin glycosylation. The findings of the present study show that flavonoids inhibit glycosylation of albumin, hemoglobin and insulin in vitro. It is highly recommended that studies be conducted to assess a possible in vitro inhibitory effect in animal and human models, as well as to understand the exact mechanisms involved.

REFERENCES