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Structure-activity relationships of preventive effects of flavonoids in alloxan-induced diabetes mellitus in rats*

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ABSTRACT

The effects of flavonoids quercetin (Q) and chrysin (Ch) were evaluated in rats with alloxan-induced diabetes mellitus. A single dose of 60 mg alloxan (A) per kg body weight was injected to rats fasting for at least 16 h. Q or Ch in amounts of 50 and 100 mg/kg body weight were administered orally to the control non-treated and A treated rats (10 rats per group) for 3 days prior and 7 days after A injection. Glycaemia, glycosuria, total antioxidant status (TAS) and activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined. Both Q and Ch prevented serum glucose elevation due to A, but the effect of Ch was weaker, particularly at the higher dose. The beneficial effects of flavonoids on glycosuria and antioxidant status differed between Q and Ch, being generally greater in case of Q, and depended on the dose. It is suggested that the protective effect of flavonoids under study is partly related to their antioxidative/chelatory properties and partly to the alteration of renal glucose absorption.

KEY WORDS: flavonoids, quercetin, chrysin, alloxan-induced diabetes, preventive effect, oxidative stress, rats

INTRODUCTION

Increasing evidence from both experimental and clinical studies suggests that oxidative stress plays a major role in the pathogenesis of diabetes mellitus. Free

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radicals are formed in diabetes by glucose oxidation, non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins (Mehta et al., 2006). Abnormally high levels of free radicals and the simultaneous decline of antioxidant defence mechanisms can lead to damage of cellular organelles and enzymes, increased lipid peroxidation, and development of insulin resistance. These consequences of oxidative stress can promote the development of complications of diabetes mellitus. Recent experimental and clinical studies have uncovered new insights into the role of oxidative stress in diabetic complications, suggesting a different and innovative approach to a possible "causal" antioxidant therapy, e.g., flavonoids (Ghosh and Konishi, 2007).

Flavonoids (more than 8000 known substances) constitute the largest and the most important group of polyphenolic compounds in plants. They are widely distributed in many frequently consumed beverages and food products of plant origin such as fruit, vegetables, wine, tea and cocoa (Ross and Kasum, 2002). It is now widely accepted that dietary polyphenolics have beneficial effect in protecting the body against chronic diseases, such as cancer, cardiovascular diseases, and diabetes mellitus (Knekt et al., 2002).

The mechanism of many of the protective actions of flavonoids remains little known. One of common denominators is antioxidant activity. Flavonoids can exert their antioxidant activity by various mechanisms, e.g., by scavenging or quenching of free radicals, by chelating of metal ions, or by inhibiting enzymatic systems responsible for free radical generation (Firuzi et al., 2005).

Experimental diabetes mellitus in animals and possibly applying to human disease too, is one of the well defined conditions which include free radical damage. It is known that alloxan administration causes severe necrosis of pancreatic β-cells (Szkudelski, 2001). It has been suggested that alloxan induces the production of H_2O_2 and of some free radicals such as $O_2^{\bullet -}$ (superoxide) and $OH^{\bullet}$ which first damage and later lead about the death of the cells (Soto et al., 1994). Therefore, the above model was considered adequate for the study of a pathology, such as diabetes mellitus.

The aim of this study was to evaluate the effect of the flavonoids quercetin and chrysin on the alloxan-induced diabetes mellitus.

MATERIAL AND METHODS

Chemical agents

Alloxan as well as quercetin and chrysin were purchased from Sigma Chemical Company (St. Louis, MO).

Animals

Experiments were performed in 100 male Wistar rats (150-180 g body weight (BW)) obtained from our animal facility. All aspects of animal care complied with the ethical guidelines and technical requirements approved by the Institutional Animal Ethics Committee.

Animals were housed individually in glass-bottomed metabolic cages in an environmentally controlled animal facility (22±1°C, humidity 60±5%, 12 h light:12 h dark cycle) with free access to a standard commercial diet and water *ad libitum*. Weight gain, feed and water intake, urine output were determined daily in morning hours.

Induction and treatment of diabetes

Diabetes was induced by single injection of alloxan in freshly prepared 10 mM/l sodium citrate, pH 4.5, 60 mg/kg BW to rats fasting for at least 16 h through the tail vein. Blood glucose levels were measured daily, 3 day prior and 7 days after alloxan administration. Development of diabetes mellitus was proven by sustained hyperglycaemia and glycosuria (diabetic rats had glycaemia >16 mM/l).

Rats were treated with flavonoids quercetin and chrysin for ten consecutive days, 3 days prior and 7 days after alloxan administration. One dose was given 1 h immediately before saline/alloxan injection.

Experimental design

The rats were randomly divided into 10 groups. Each group included 10 animals: group I (C): control animals which received vehicles used for flavonoids (carboxymethyl cellulose, 1%, orally) and for alloxan (saline solution, intravenous injection (iv), in the tail vein); groups II (Q50) and III (Q100): animals treated with quercetin in doses 50 and 100 mg/kg BW, respectively, groups IV (Ch50) and V (Ch100): animals treated with chrysin in doses 50 and 100 mg/kg BW, respectively, group VI (A): animals treated with alloxan (60 mg/kg BW, by a single iv; diabetic control animals). The rats developed diabetes within 2 days after alloxan injection as evidenced by sustained hyperglycaemia and glycosuria.

Groups VII-X A animals treated with alloxan plus flavonoids - quercetin or chrysin - at the same doses and schedule as groups II-V together with alloxan (60 mg/kg BW, by a single iv).

Biochemical evaluation

In order to determine plasma and urine glucose levels, respectively, blood and urine samples were collected daily in morning hours. Blood from tail vein was col-

lected and centrifuged at 1000 *g* for 10 min, and the glucose level was determined by the glucose oxidase-peroxidase enzymatic method (Lab test Set for Glucose, BioLaTest, Lachema, Czech Republic). Twenty-four h urine samples were collected in metabolic cage urine separator bottles containing 10 ml 0.33 mM/l perchloric acid. One ml urine was taken for glucose and ketones analyses with enzymatic (spectrophotometric) method similarly as in blood.

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in erythrocytes and total antioxidant status (TAS) of plasma, respectively, were evaluated by commercially available kits (Randox Laboratory, UK).

Statistical analysis

The data are presented as mean±SEM. Statistical comparisons were made by one-way analysis of variance (ANOVA) and followed by Student-Neuman-Keuls as the post hoc test. Data were considered statistically significant if P values were lower than 0.05.

RESULTS

The effects of quercetin and chrysin on blood glucose levels of control and alloxan-treated animals are summarized in Figures 1 and 2. Additional data as weight gain, feed and water intake, urine volume, glucose index, assimilating coefficient, and mortality are presented in Table 1.

Table 1. Body weight gain, feed and water intake, output of urine, glucose index (GI; glucose in food/glycaemia), assimilating coefficient (AQ; glucose in food/glucose output in urine) of rats treated with quercetin and chrysin

Treatment group	Body weight gain, g	Food intake g	Water intake ml	Volume of urine ml	GI	AQ	Mortality %	Diabetic rats, %
C	32 ± 3	19.6 ± 4	26.8 ± 6	14.8 ± 2	1.61	0.99	0	0
Q50	29 ± 4	21.3 ± 4	27.7 ± 5	16.1 ± 2	1.69	0.99	0	0
Q100	34 ± 4	20.6 ± 5	25.9 ± 6	17.0 ± 3	1.66	0.98	0	0
Ch50	31 ± 5	22.3 ± 6	28.6 ± 8	18.4 ± 3	1.69	0.97	0	0
Ch100	29 ± 6	21.9 ± 5	33.2 ± 8	20.3 ± 5	1.61	0.94	0	0
A	-2 ± 5	32.6 ± 7	96.0 ± 16	92.9 ± 9	0.58	0.46	40	100
A + Q50	27 ± 6	22.2 ± 4	28.1 ± 4	19.3 ± 4	1.35	0.95	0	0
A + Q100	21 ± 5	24.6 ± 3	33.7 ± 5	24.6 ± 4	1.92	0.86	0	0
A + Ch50	18 ± 7	26.8 ± 6	52.2 ± 7	38.1 ± 5	1.15	0.78	20	10
A + Ch100	12 ± 8	31.7 ± 5	68.1 ± 9	61.4 ± 6	0.84	0.62	20	80

On its own quercetin as well as chrysin had no effect on plasma glucose concentrations of normoglycaemic animals. On the other hand, the alloxan-treated animals exhibited consistently hyperglycaemia. The simultaneous treatment with quercetin (in both doses used) and alloxan significantly reduced the increase in glucose concentration in plasma induced by alloxan at any time in the glucose experiment ($P<0.001$). A slow increase in plasma glucose was observed in animals treated with alloxan+quercetin in dose 50 mg/kg BW but it was not significant when compared with control animals.

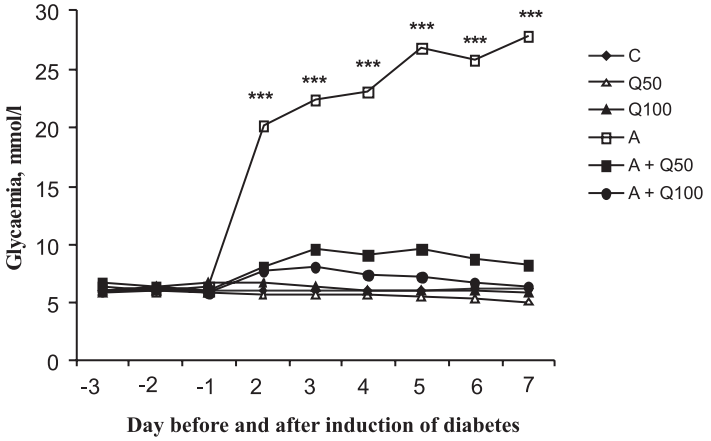


Figure 1. Effect of quercetin on the concentration of plasma glucose during the development of alloxan-induced diabetes mellitus. Each point represents the mean value; n=10 for each point. C-control animals, Q50-animals receiving quercetin in dose 50 mg/kg BW; Q100-animals receiving quercetin in dose 100 mg/kg BW; A-alloxan treated animals; A+Q50-animals treated with alloxan and quercetin in dose 50 mg/kg BW; A+Q100-animals treated with alloxan and quercetin in dose 100 mg/kg BW; ***P<0.001 alloxan-treated animals vs all groups

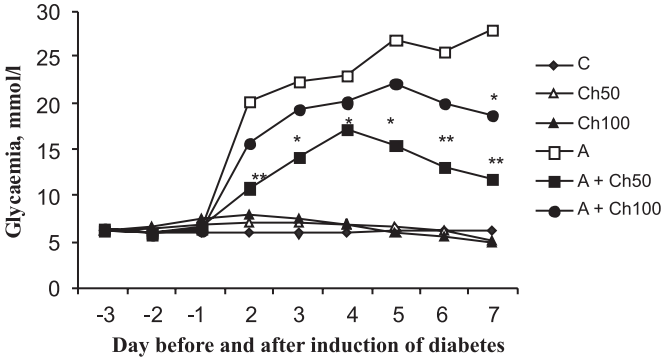


Figure 2. Effect of chrysin on the concentration of plasma glucose during the development of alloxan-induced diabetes mellitus. Each point represents the mean value 6 SEM; n=10 for each point. Abbreviations as in Figure 1. * P<0.5; **P<0.01 vs alloxan-treated animals

Quercetin in higher dose completely prevented elevation of glucose values in plasma. Chrysin in dose 50 mg/kg BW also significantly prevented alloxan-induced plasmatic glucose increase ($P<0.05$, $P<0.01$). However, antihyperglycaemic effect of chrysin in dose 100 mg/kg BW was surprisingly even less significant as compared to lower dose ($P<0.05$ at seventh day after alloxan administration).

The levels of antioxidant enzymes (SOD, GPx) and total antioxidant status (TAS) after pre-treatment with quercetin and chrysin are presented in Figures 3-5. In most of aloxan-treated diabetic animals the levels of antioxidant enzymes SOD and GPx decreased significantly ($P<0.001$). In A+Q50 group this decline was less pronouced ($P<0.05$). On the contrary, application of quercetin in dose of 50 mg/kg of BW evoked in non-diabetic animals rise of both SOD and GPx activities, respectively. In either dose applied, quercetine prevented steep decline of TAS in most of alloxan-treated diabetic rats. Interestingly, this protective effect was slightly higher in dose 50 than in 100 mg/kg of BW.

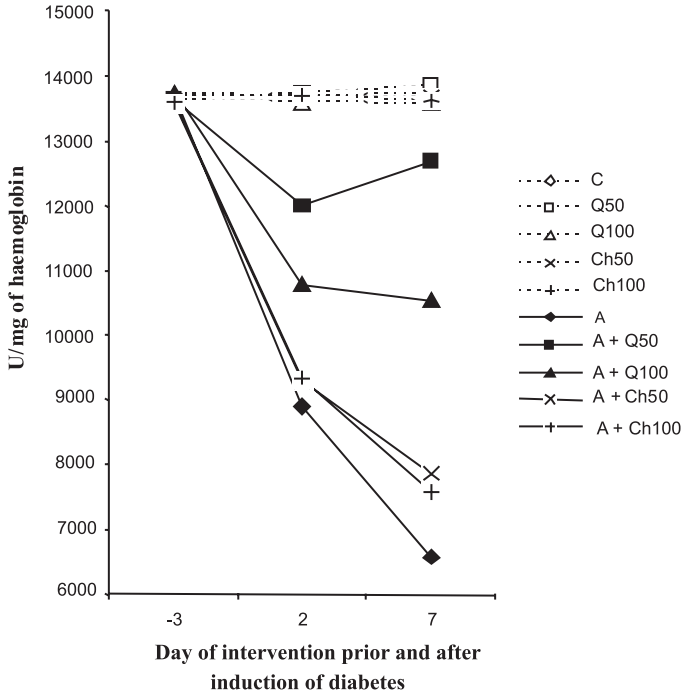


Figure 3. Effect of quercetin and chrysin on the activity of SOD in erythrocytes during the development of alloxan-induced diabetes mellitus. Each point represents the mean value; n=10 for each point. C - control animals, Q50 - animals receiving quercetin in dose 50 mg/kg BW; Q100 - animals receiving quercetin in dose 100 mg/kg BW; Ch50 - animals receiving chrysin in dose 50 mg/kg BW; Ch100 - animals receiving chrysin in dose 100 mg/kg BW; A - alloxan treated animals; A+Q50 - animals treated with alloxan and quercetin in dose 50 mg/kg BW; A+Q100 - animals treated with alloxan and quercetin in dose 100 mg/kg BW; A+Ch50 - animals treated with alloxan and chrysin in dose 50 mg/kg BW; A+Ch100 - animals treated with alloxan and chrysin in dose 100 mg/kg BW

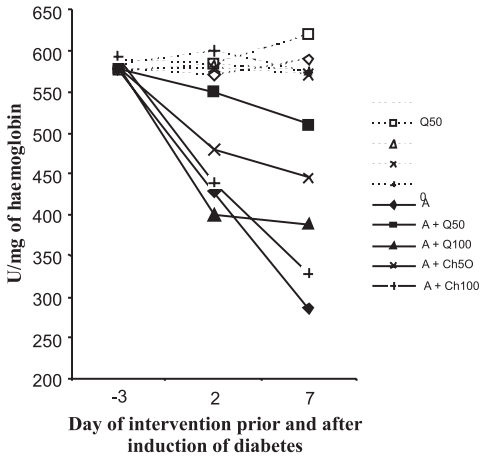


Figure 4. Effect of quercetin and chrysin on the activity of GPx in erythrocytes during the development of alloxan induced diabetes mellitus. Each point represents the mean value; n=10 for each point. Abbreviations as in Figure 3

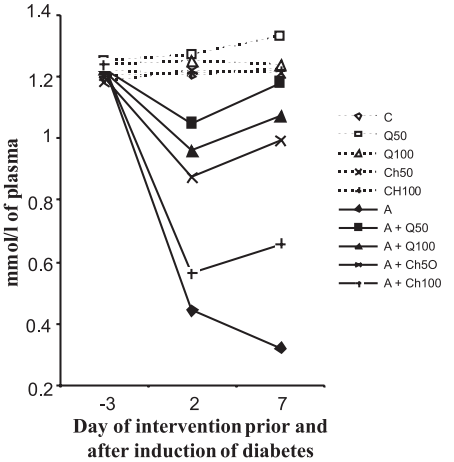


Figure 5. Effect of quercetin and chrysin on the total antioxidant status (TAS) of plasma during the development of alloxan-induced diabetes mellitus. Each point represents the mean value; n=10 for each point. Abbreviations as in Figure 3

Glycosuria was elevated significantly and regularly in all diabetic rats as compared to non-diabetic animals. Interestingly, in spite of normalized blood glucose levels, significantly high glycosuria was also observed in animals treated with quercetin, particularly at dose of 100 mg/kg BW. Higher levels of glucose in urine were regularly observed in diabetic rats treated with chrysin. Similar to blood glucose data, higher dose of chrysin showed much lesser effect on glycosuria than smaller one and practically did not differed from untreated diabetic control. However, glycosuria, was also increased in non-diabetic animals receiving chrysin in both doses and quercetin in dose 100 mg/kg BW (Figure 6).

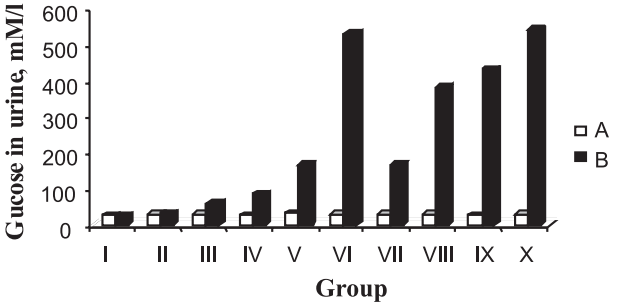


Figure 6. Effect of quercetin and chrysin on glucosuria in rats. A-three days before and B-seven days after alloxan administration. Groups: I-C; II-Q50; III-Q100; IV-Ch50; V-Ch100; VI-A; VII-A+Q50; VIIIA+Q100; IX-A+Ch50; X-A+Ch100

DISCUSSION

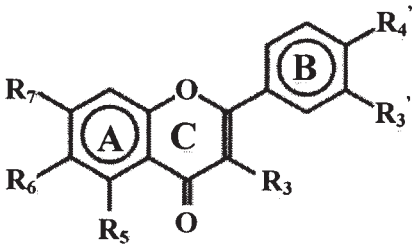
The possible sources of oxidative stress in the pathogenesis of diabetes and diabetic complications have been extensively studied for years both in animal models and in clinical setting. Certain studies have found increased lipid peroxides and/or ROS in different animal models of diabetes (Anjaneyulu et al., 2004; Mehta et al., 2006). However the results in clinical practice are not unambiguous and the usefulness of antioxidant therapy in diabetic patients is far from convincing (Newsholme et al., 2007).

Alloxan, a chemical diabetogen, in the presence of glutathione is reduced *via* the alloxan radical into dialuric acid. During this redox cycling process, reactive oxygen species are formed that destroy β-cells in islets of Langerhans. Moreover, it is suggested that transitional metals such as iron, zinc and copper may be involved in alloxan toxicity (Szkudelski, 2001).

Several works have demonstrated that flavonoids may reduce hyperglycaemia and exert protective effect against non-enzymatic glycation of proteins in animals (Anjaneyulu and Chopra, 2004; Ghosh and Konishi, 2007). The present study demonstrated that flavonoids quercetin, and partially also chrysin, prevented alloxan-induced hyperglycaemia in rats. Our results have further showed that protective effect of quercetin is greater than that of chrysin in terms of antioxidant defence as well.

The observed antihyperglycaemic effect of quercetin is consistent with the previous experiments done in alloxan-induced diabetes mellitus in rats by Nuraliev and Avezov (1992), and streptozotocin (STZ)-induced diabetes mellitus by Coskun et al. (2005) who found that quercetin may even reverse the hyperglycaemia close to the normal levels. Present results showed that daily treatment with 50 and/or 100 mg/kg BW of quercetin prevented the steep onset of hyperglycaemia after alloxan application and maintained blood glucose values slightly above blood glucose of controls for the whole period of observation. Effects of chrysin were less prominent. Interestingly, effects of chrysin were even better in lower than in higher doses used in this study. These findings might be of interest since only little data are available about the effect of chrysin in chemically-induced experimental diabetes.

It is certainly tentative to speculate that such differences are mainly due to different chemical structure as suggested by previous works (Chen et al., 2002) (Figure 7). The structural requirement considered to be essential for effective radical scavenging by flavonoids is the presence of a 3',4'-dihydroxy, i.e. a o-dihydroxy group (catechol structure) in the B ring, possessing electron donating properties and being a radical target. Furthermore, the 3-OH moiety of the C ring is beneficial for the antioxidant activity of flavonoids, too. The C2-C3 double



Flavonoid	Substituents						
	3	5	7	2′	3′	4′	5′
Quercetin	OH	OH	OH	H	OH	OH	H
Chrysin	H	OH	OH	H	H	H	H

Figure 7. Flavonoid basic structure and chemical structure of the quercetin and chrysin

bond further enhances the radical-scavenging capacity (Firuzi et al., 2005). Moreover, presence of the catechol group in the B-ring, the 3-hydroxyl group in C-ring and that of 2,3-double bond in the C ring seems also important for Fe^{3+} reducing activity. On the other hand, the copper reducing activity seems to depend largely on the number of hydroxyl groups present in the flavonoid molecules (Mira et al., 2002).

The pharmacodynamic profile of quercetin has been well studied (Okamoto, 2005). Its ability to protect against oxidative stress-induced cellular damage (such as lipid peroxidation of membranes and subsequent membrane degradation) are commonly associated with free radical scavenging as well as its chelatory properties (Mira et al., 2002; Anjaneyulu and Chopra, 2004). As to the chrysin, the data are sparse. Furusawa et al. (2005) showed that chrysin had only moderate antioxidant effect and almost no metal chelatory properties.

Our results support the opinion that the most important criterion in protecting live cells from oxidative stress is the *ortho* arrangement of the two hydroxyl groups (free catechol grouping) in the B-ring and the 3-hydroxyl group in C-ring. Quercetin meets this criterion, whereas this feature is absent in chrysin.

Interesting effects were produced by both quercetin and chrysin in terms of glycosuria. In spite of slight decline of hyperglycaemic curve, high glycosuria persisted in all chrysin pre-treated and most of quercetin pre-treated alloxan-induced diabetic rats. Furthermore, moderate glycosuria was observed even in non-diabetic rats receiving only quercetin. This may be explained by previous findings that flavonoids or plant extract with high content of flavonoids may cause potent inhibition of renal glucose reabsorption through inhibition of the sodium-glucose symporters located in the proximal renal tubule (Maghrani et al., 2005). Studies with flavonoids are underway to further elucidate their mechanism of action. Further research also is

needed to determine which combinations might prove efficacious given the large number of persons taking nutriceuticals.

CONCLUSIONS

Our results show that oral administration of quercetin and chrysin has a beneficial effect on the alloxan-induces diabetes by reducing hyperglycaemia and improving the antioxidant status.

This study suggests that the induction of diabetes mellitus by alloxan in rats may be prevented by quercetin and partially by chrysin administration. We hypothetized that this effect may be result of antiradical/chelatory properties of flavonoids used. However, inhibition of renal glucose reabsorption may be also involved in hypoglycaemic effect of these flavonoids.

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