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Original Article

Application of Riboflavin-Deficient Rat Erythrocytes in the *in Vitro* Assay of Faba Bean Toxicity

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Abstract

Background: Faba bean toxicity (favism) is a common condition observed in our region.

Objective: To develop a short and simple technique involving the use of riboflavin-deficient rats to test *in vivo* faba bean toxicity.

Methods/Results: Sprague Dawley rats were maintained on a riboflavin-deficient diet and their vitamin B_2 status was monitored by the assay of erythrocyte glutathione reductase (EGR). Their blood was found to be sensitive enough as early as 5 weeks of eating the deficient diet. Initially RBC preparations from the riboflavin-deficient rats were used to test the toxicity of different concentrations of divicine (0.0-13.5 μM). It was found that an increase in divicine concentration resulted in an appreciable drop in reduced glutathione (GSH) of RBC. The assay procedure was then used to test the effectiveness of certain detoxification treatments of faba beans. A high correlation (r= 0.9) was noted between residual vicine (the main favism factor) concentration and the toxicity index (TI) of treated samples indicating the suitability of the above procedure for toxicity assay.

Conclusion: It is recommended to use blood from Sprague Dawley rats that have been fed a riboflavin-deficient diet for about 5 weeks or longer, in the bioassay of toxicity of faba beans and their isolated favism principles
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Keywords • Faba bean toxicity • riboflavin deficiency • rat erythrocytes • vicine • divicine • convicine • favism

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Introduction

Anti-nutritional substances in broad beans which cause the hemolytic disease or favism in glucose-6-phosphate-dehydrogenase (G-6-PD) deficient subjects, are pyrimidine aglycones of vicine and convicine (divicine and isouramil, respec-

tively) and other such compounds, yet to be characterized, which are released in the intestine by microbial β-glycosidase.[5,6] These aglycones enter the red blood cells (RBC) and then a number of oxidative reactions take place resulting in premature destruction of RBC, unless challenged by reducing agents such as reduced glutathione (GSH). Unfortunately, the level of GSH in G-6-PD deficient RBC's is already low and even unstable, and therefore, would be the limiting factor in the mechanism of red cell protection. The underlying reason is that because of G-6-PD deficiency, the cell cannot produce enough NADPH via the hexose-monophosphate shunt and consequently regeneration of GSH from the oxidized form GSSG is impaired. This eventually leads to manifestations of the disease favism.[9] The regeneration reaction depicted by Sauberlich *et al,*[10] also requires the enzyme glutathione reductase which, in turn, needs a riboflavin-derived coenzyme (FAD) for its activity.

Investigations on the disease and related toxicity aspects, in general, and the search for a means of detoxification of faba beans, in particular, have been hampered by the lack of suitable experimental animals. Efforts have been made to mimic human favism in laboratory animals. In their search for an animal model, D'Aquino and coworkers[3] depleted rats of protein, vitamin E or riboflavin and found that vitamin B_2-deficiency causes no change in GSH concentration but diminishes the erythrocyte glutathione reductase (EGR) activity to 10% of its normal level. Hence, the GSH of RBC from such rats dropped drastically when challenged by isouramil. So they suggested that riboflavin-deprived RBC from rats be used as a replacement for RBC from G-6-PD deficient human subjects to test the response of GSH to such factors as isouramil.

Further work by D'Aquino *et al,*[4] has focused on the induction of a double deficiency of riboflavin and tocopherol in the rats in order to make them more sensitive so that their blood could be employed to test for GSH response as affected by favism factors and also to evaluate the hemolytic activity of these as well as others simultaneously present in the test extract. Their procedure, however, involved feeding rats first a tocopherol-deficient diet for three months followed by two months of a tocopherol- and riboflavin-free diets.

Later, Yannai and Marquardt[13] sensitized experimental rats to mimic favism subjects, by pretreatment of the animals with buthionine-sulfoximine, and then tested the toxic effects of divicine by *in vivo* experiments using these animals.

In the present work a shorter and simpler technique involving the use of riboflavin-deficient rats with minor modifications was applied to test *in vivo* faba bean toxicity.

Materials and Methods

Experimental Animals

Male albino Sprague Dawley rats having an initial weight of 35-50 g were selected and divided into three groups of 20 each. They were housed individually in an air-conditioned rat room with proper light and humidity. One group served as the control and was fed a diet providing all daily requirements for nutrients throughout the experimental period. The second group was initially fed a control diet for one week and then fed a riboflavin-deficient diet starting the second week. The third group was given the control diet for two weeks and was then switched to a riboflavin-deficient diet starting the third week. This was done in order to have two groups of sensitized rats in succession. Food and water were provided *ad libitum* throughout the experiment.

Diets

The control diet was composed of 68% sucrose, 18% vitamin-free casein, 10% vegetable oil and 4% salt mixture. Vitamin mixture was added at the rate of 0.22 kg/10 kg diet.

Riboflavin-deficient diet was initially purchased from Nutritional Biochemicals Corp. but subsequently prepared in the laboratory as was the control diet, while using a riboflavin-free vitamin mixture obtained from the same supplier.

Faba Beans and Their Treatments

Faba beans (*Vicia faba L.*) were purchased from the market. They were subjected to various treatments with the aim of their detoxification[7].

Assay of Erythrocyte Glutathione Reductase (EGR)

EGR status of rats was monitored by weekly determination of activity coefficient (A/C) of blood collected from anesthetized rats by cardiac puncture.[12] Enzymes and other biochemicals were purchased from Sigma Chem. Co. The EGR assay was based on comparing the decrease in the absorbance of the coenzyme NADPH at 340 nm in the presence or absence of FAD and reported as:

$$\mathrm{A/C} = \frac{\downarrow \text{Absorb.of NADPH with FAD}}{\downarrow \text{Absorb.of NADPH without FAD}}$$

where "Absorb. of NADPH" is absorbance of NADPH measured at a wavelength of 340 nm.

In vitro *Bioassay of Toxicity of Extracts of Treated Faba Beans*

Blood samples were collected randomly from rats consuming control as well as riboflavin-deficient diets at weekly intervals. They were subjected to EGR assay to monitor the stage of EGR deficiency and hence GSH instability.

Afterward, pooled blood from EGR-deficient rats was used for *in vitro* bioassay of toxicity by monitoring the GSH content of erythrocytes. Separate samples of enzyme-hydrolyzed crude extracts from faba beans, which had been treated by various procedures for detoxification,[7] were incubated with the EGR deficient RBC. Their GSH recoveries were determined according to the method of Dacie and Lewis[2] and reported as toxicity index (TI).[7]

Vicine Reference Material and Divicine Preparation

Vicine was isolated from a 2-kg batch of broad beans according to the method of Brown and Roberts.[1] One-hundred milligrams of vicine were dissolved in 5 ml of 0.01 M Sorensen's phosphate buffer (pH=5.00) to serve as stock solution. One thousand micrograms of β-glycosidase (EC 3.2.1.21) were also dissolved in 5 ml of the above buffer. One milliliter of the vicine stock solution was mixed with 1 ml of the enzyme solution and incubated at 37 ^{o}C for 30 min. in the absence of air to effect complete hydrolysis. Similarly, 1 ml each of a 2-fold and a 4-fold dilutions of vicine stock solution were hydrolyzed as above and stored in deep-freeze until use.

In vitro *Bioassay of Toxicity of Divicine*

Triplicate 0.5-ml aliquots of divicine preparations (0.0, 4.5, 9.0 and μM/ml) were incubated in small test tubes with 0.5-ml portions of washed RBC (in saline buffer, 1:1) from riboflavin-deficient rats for 3 hours at 37 ^{o}C with constant shaking. At the end, their GSH contents were determined as above.

Estimation of Vicine in Faba bean Extracts

Extracts were prepared from treated faba bean samples and their residual vicine contents were estimated according to Jamalian *et al*.[8]

Statistical Analysis

Standard errors, where applicable, were determined according to Snedecor and Cochran.[11] Regression equations were calculated and plotted using the COSTAT program. Statistical significance was accepted at $p<0.01$ for the data in Table 1 and Fig. 2 and at $p<0.001$ for the data represented in Fig. 3.

Table 1. Erythrocyte glutathione reductase activity of riboflavin-deficient and control rat s

Experimental condition	No. of rats examined	Activity coefficient[(b)]
Control	9	0.92±0.07[(c)]
Riboflavin-deficient	36	1.56±0.14[(d)]

(a) Determination were made using blood from rats collected 6 weeks after the start of the experiment
(b) Activity coefficient: (A/C) (for definition see text)
(c) Data represent Mean±SEM
(d) Significantly different from control ($p< 0.01$)

Results and Discussion

Figure 1 demonstrates the status of the rats, the stages at which riboflavin-deficient diet was fed and the initial stage of exhibition of EGR deficiency. As seen, the rats in groups 2 and 3 exhibited a reduced rate of growth after about one week of eating deficient diet and their mean weights stayed more or less constant until the end of the experimental period.

The results of EGR activity (after six weeks) of rats fed the control as well as those eating the riboflavin-deficient diet are reported in Table 1. As shown, the rats consuming the control diet had on the average a lower A/C value (0.92±0.07) than those on the deficient diet (1.56±0.14). Tilloston and Sauberlich[12] have claimed that no quantitative A/C values can be ascribed with respect to the degree of riboflavin deficiency in the rat, yet in their 77-day experiment they obtained a mean A/C of 1.25±0.07 for control rats and varying A/C values (depending on the length of depletion) for riboflavin-depleted rats (2.37±0.12 to 2.66±0.13 for 1-9 days and 42-52 days of depletion, respectively). Obviously, these data are greater than those obtained in the present investigation. The discrepancy, however, would be explained in the light of the following:

a. Strains of rats (Sprague Dawley *vs*. Charles River)
b. Initial weight of rats (35-50 g *vs.* 50-60 g)

Despite the differences of the magnitude of the present A/C values, compared to those of Tilloston and Sauberlich[12] it is obvious that the rats were definitely riboflavin-deficient as they exhibited the clinical signs and lesions (loss of hair luster and silkiness, sticky appearance of hair, loss of hair, lesions of the ears, mouth, shoulder and back as well as hair pigmentation [not reported by Tilloston and Sauberlich]) which were also reported by the above authors.

Incubation of erythrocytes from riboflavin-deficient rats (in triplicate) with different concentra-

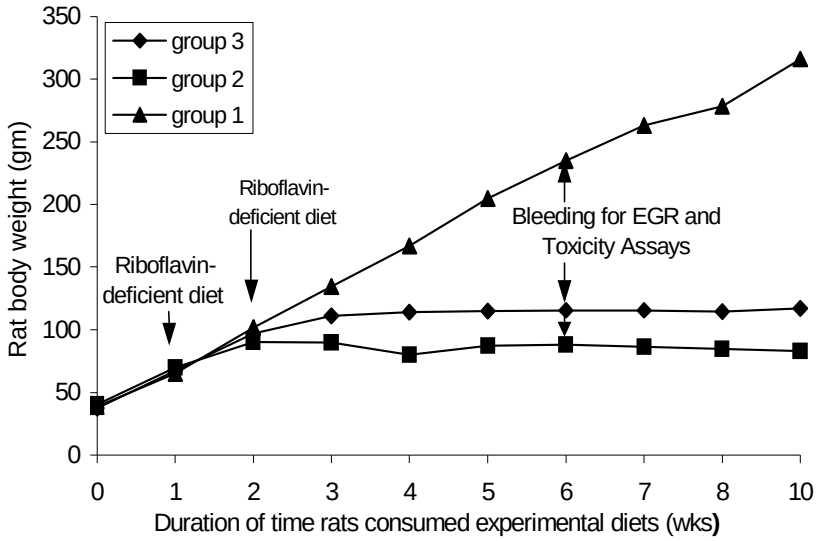

Figure 1: Growth pattern and EGR status of control and riboflavin-deficient rats. EGR: erythrocyte glutathione reductase; Group 1= control; Group 2 = fed control diet for 1 week followed by riboflavin-deficient diet; Group 3 = fed control died for 2 weeks then switched to riboflavin-deficient diet.

tions of divicine (0.0-13.5 μM) resulted in a decrease in GSH with increase in the concentration of divicine in the mixture (Fig. 2).

The decrease in GSH level was dependent on the divicine dose ($p<0.01$). The pattern of decrease was very similar to that reported for isouramil (aglycone of convicine) by D'Aquino *et al*[4]. However, as expected, divicine seems much more potent than isouramil in causing disappearance of GSH.

Judging from the results presented in Table 1 and Fig. 2, it can be concluded that blood from Sprague Dawley rats which have been fed a riboflavin-deficient diet for approximately five weeks is sensitive enough to mimic blood from favism-prone

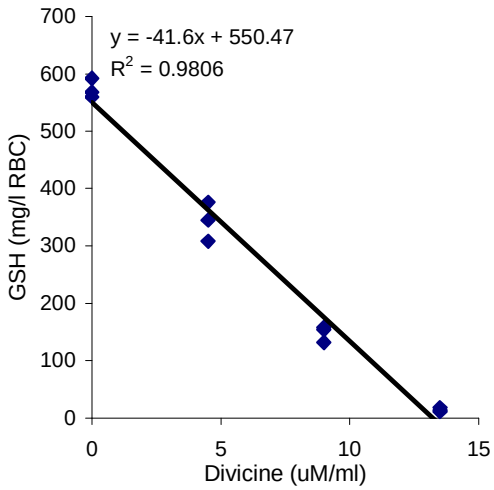

Figure 2: Effect of divicine concentration on GSH of riboflavin-deficient rat erythrocytes.

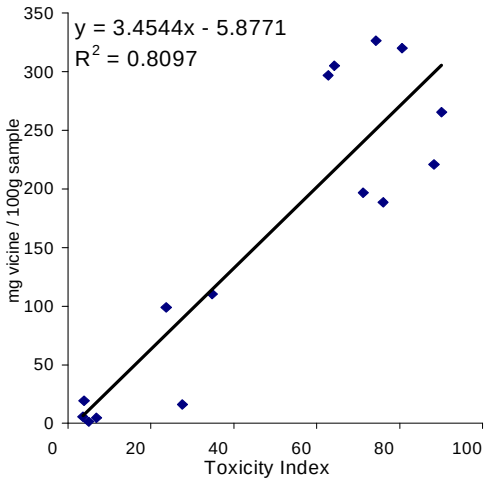

Figure 3: Toxicity indices of treated faba beans, determined *in vitro* using riboflavin deficient rat erythrocytes in relation to residual vicine content of samples.

subjects and so can replace the latter in the bioassay of favism-inducing principles of faba beans.

In the next stage, the bioassay technique was applied to actual faba bean extracts prepared from samples that had been subjected to a variety of treatments aimed at their detoxification.[7] Residual vicine contents of the extracts were also determined and their values were plotted against TI (Fig. 3).

A high correlation (r=0.9) was noted between the amount of vicine and the extent of toxicity which further indicates that riboflavin-deficient RBC (from as early as five weeks on deficient diet) could be used to test toxicity. Such results seem to indicate that vicine is indeed the dominant favism factor present in faba beans and, therefore, any treatment resulting in complete or nearly complete removal of vicine should remove, largely or totally, convicine and other favism factors as well.

In conclusion, it is recommended to use blood from Sprague Dawley rats that have been fed a riboflavin-deficient diet for about five weeks, or longer, in the bioassay of toxicity of faba beans and their isolated favism principles.

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