ANTIOXIDANT ENZYME LEVELS IN THE ERYTHROCYTES OF RIBOFLAVIN-DEFICIENT AND *TRICHINELLA SPIRALIS*-INFECTED RATS

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Abstract. The erythrocyte antioxidant enzyme levels of catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) of riboflavin-deficient and *Trichinella spiralis*-infected rats were investigated. The rats were deprived of riboflavin at the 8th week of the experiment. At that time, the erythrocyte glutathione reductase activity coefficient (EGR AC), as an indicator of riboflavin status, was ≥ 1.30 in rats fed a riboflavin-deficient diet and *T. spiralis*-infected rats fed a riboflavin-deficient diet showed no biochemical sign of riboflavin deficiency. At the 12th week of the experiment, the levels of catalase, SOD and GSH-Px were significantly lower in the riboflavin-deficient, *T. spiralis*-infected, and combined riboflavin-deficient and *T. spiralis*-infected, rats, compared to the control group. This may have been due to an increase in free oxygen radicals caused by riboflavin deficiency and parasitic infection.

INTRODUCTION

Riboflavin deficiency is present in many tropical countries (Bates, 1987). The high rate of vitamin B-2 deficiency in Thailand is well known, as confirmed by studies undertaken in children and their mothers in a well-baby clinic (Changbumrung *et al*, 1984), preschool children in the northeast of Thailand (Vudhivai *et al*, 1986), new-borns and their mothers from the northeast (Vudhivai *et al*, 1989), vegetarians (Vudhivai *et al*, 1991), elderly (Pongpaew *et al*, 1991), construction site workers (Pongpaew *et al*, 1993) and Thai road sweepers in Bangkok (Vudhivai *et al*, 1997). Therefore, it remains a public health problem in Thailand. Outbreaks of trichinellosis caused by *T. spiralis*, especially in northern and southern Thailand, are quite common, and have occurred almost every year since 1962 (Khamboonruang, 1991). The infection is usually acquired through the consumption of a local dish, known as "Larb", containing raw meat from wild animals and whole blood without cooking**,** traditionally served during the northern Thai New Year and wedding ceremonies. Infectious diseases and malnutrition are the most common health problems in the developing countries, and the situation may be more severe if these two conditions occur simultaneously, *eg* riboflavin deficiency and trichinellosis. The interaction of infection and malnutrition may be considered cyclic, insofar as one condition is capable of accentuating the other. Not only may nutrition increase host susceptibility to infection, but infection may also contribute to malnutrition, particularly in borderline cases (Migasena, 1984).

The importance of reactive oxygen species (ROS) in the host defenses against parasitic infections is well known (Adelekan and Thurnham, 1998). They are synthesized during the normal cellular metabolism, especially by activated phagocytes, and also by some anti-parasitic drugs (Callahan *et al*, 1988). In the erythrocyte, antioxidant enzymes and antioxidant substances provide protection against oxidative damage. Superoxide dismutase (*EC* 1.15.1.1; SOD) cataly-

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ses the dismutation of superoxide anions (O_2^-) to H_2O_2 and O_2, while catalase (*EC* 1.11.1.6) and glutathione peroxidase (*EC* 1.11.1.9; GSH-Px) break H_2O_2 down into H_2O and O_2 (Fairfield *et al*, 1988). The antioxidant defense enzymes in *Plasmodium*-infected and uninfected erythrocytes have been investigated in several studies (Suthipark *et al*, 1982; Seth *et al*, 1985; Stocker *et al*, 1985). The role of riboflavin in the GSH-Px and SOD activities in riboflavin-deficient rats infected with *P. berghei* malaria has been reported, and it was concluded that riboflavin deficiency has no marked effect on erythrocyte GSH-Px and SOD activity but malaria had a marked effect on erythrocyte GSH-Px (Adelekan and Thurnham, 1998). The larval stage of *T. spiralis* was found to be susceptible to leukocyte-generated free oxygen radicals (Bass and Szejda, 1979). The migratory larvae of *T. spiralis* were more susceptible to free radical damage than adult- or muscle- stage larvae because of deficiencies in the levels of protective enzymes, such as SOD and GSH-Px (Kazura and Meshnick, 1984). Callahan *et al* (1988) reported that newborn larvae, adult and muscle larvae of *T. spiralis* contained SOD 6.8, 19.2 and 30.3 U/mg, respectively; catalase, 0, 0 and 0 U/mg, respectively, and GSH-Px, 0, 16 and 36 mU/mg, respectively. However, the role of riboflavin in this complex chain of events has not yet been studied.

The objective of this investigation is to study the effect of riboflavin deficiency, *T. spiralis* infection and riboflavin deficiency combined with *T. spiralis* infection on changes in the levels of erythrocyte antioxidant enzymes in a Wistar rat model.

MATERIALS AND METHODS

Materials

Two types of feeds were fed to the rats; one was a commercial feed (the control diet) and the other was designed to cause riboflavin deficiency based on the formula of Hoppel and Tandler (1975) and modified by Adelekan and Thurnham (1986). It was composed of ingredients as shown in Table 1.

Animals

Twenty-eight post-weaning Wistar rats aged 6 weeks were bought from the National Laboratory Animal Center, Mahidol University. They were allowed to acclimatize to the dietary change for 3 days before they were weighed and randomly allocated into 4 groups; each group contained 7 rats. All the rats were individually housed in stainless steel metabolic cages where the controlled temperature ranged between 22º-24ºC, with a relative humidity between 55-60%. The light period was 12 hours/day.

Table 1
Composition of riboflavin deficient feeds.

Composition	Grams/kilogram diet
Acid washed casein	180
Sucrose	660
Fat (oil)	100
Salt mixture[a]	40
Vitamin mixture[b]	20
Total	1,000

[a]Salt mixture composition (grams) was $(Al_2SO_4)_3$. $K_2SO_4.24H_2O$ 0.21, $CaCO_3$ 309.83, $CaHPO_4.2H_2O$ 98.12, $CoCl_2.6H_2O$ 0.26,$CuSO_4.5H_2O$ 0.21, $FeC_6H_5O_7$. $5H_2O$ 6.00, $MgSO_4.H_2O$ 51.13, $MnSO_4.H_2O$ 4.13, KI 0.83, $K_2HPO_4.3H_2O$ 333.09, NaCl 173.00, NaF 0.26, $Na_2B_4O_7.10H_2O$ 0.26, $ZnCl_2$ 0.26.

[b] Vitamin mixture composition (grams/kilogram mixture) was ascorbic acid 45.0, inositol 5.00, choline chloride 75.00, P-aminobenzoic acid 5.00, nicotinamide 4.50, menadione 0.0025, pyridoxine HCl 1.00, thiamin HCl 1.00, calcium pantothenate 3.00, biotin 0.02, folate 0.09, cobalamin 0.00135, cholecalciferol 50,000 IU, retinyl palmitate 200,000 IU, dl-α-tocopherol 2,500 IU and dextrose, to make 1 kilogram of mixture.

Parasite infection

All animals allocated to groups 2 and 4 (according to the experimental protocols) were individually infected with muscle larvae of *Trichinella spiralis* (200 larvae/100 grams body weight) by the intraesophageal route.

Experimental protocols

Twenty-eight rats were fed *ad lib* and allocated into 4 groups, as follows: group 1 non-infected rats fed a commercial diet; group 2 non-infected rats fed a riboflavin-deficient diet; group 3 *T. spiralis*-infected rats fed a commercial diet; group 4 *T. spiralis* infected rats fed a riboflavin-deficient diet.

Prior to the start of the experiment, and at the end of the 4th , 8th and 12th weeks of the experiment, blood was drawn from the eye vein into heparinized capillary tubes for determination of riboflavin status, by measuring red cell glutathione reductase activity (Glatzle *et al*, 1968). The activity was determined in the venous hemolysate (red cells 20 µl per distilled water 0.4 ml) as the ratio of activity with and without exogenous flavin adenine dinucleotide (FAD) in terms of erythrocyte glutathione reductase activity coefficient (EGR AC). An EGR AC greater than or equal 1.20 was considered as biochemically deficient riboflavin status (Tillotson and Baker, 1972). At the 12th week of the experiment, blood was drawn from the eye vein directly into heparinized tubes and was centrifuged to collect red cells to determine of the three antioxidant enzymes: catalase, SOD and GSH-Px.

Analytical methods

Catalase catalyzes the breakdown of H_2O_2 into 2 moles of water and 1 mole of oxygen. The rate of decomposition of H_2O_2 by catalase is measured spectrophotometrically at a wavelength of 230 nm. Ethanol is added to stabilize the hemolysate by breaking down the "complex II" of catalase and H_2O_2. SOD was measured with a commercially available Ransod kit (Randox Laboratories, Ltd, UK). SOD is thought to accelerate the dismutation of the toxic superoxide radicals (O_2^-) produced during oxidative energy processes, to hydrogen peroxide and molecular oxygen. This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol) -5-phenyltetrazolium chloride (I.N.T) to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. GSH-Px was measured by a Ransel kit (Randox Laboratories, Ltd, UK). Its function is to catalyze the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG) by using t-butyl hydroperoxide as the substrate for the assay of the enzyme. The rate of formation of GSSG is measured by means of the glutathione reductase reaction. The oxidation of NADPH is measured at 340 nm.

Statistical analysis

Since all the data of each parameter investigated - EGR AC, catalase, SOD and GSH-Px - were not normally distributed, the Kruskall-Wallis one-way ANOVA was applied and the mean of two independent groups was then calculated by the Student independent *t* test.

RESULTS

Erythrocyte glutathione reductase activation coefficient (EGR AC)

EGR AC was determined among the 4 groups at the beginning, 4th, 8th and 12th weeks of the experiment. The *T. spiralis*-infected group did not show ariboflavinosis **(**vitamin B-2 deficiency**)** at the 12th week of the experiment, compared with the control group. However, the EGR AC values had progressively increased, both in rats fed a riboflavin-deficient diet and in *T. spiralis*-infected rats fed a riboflavin-deficient diet with a depletion in riboflavin at the 8th week onward. The EGR AC values were significantly different from the control groups ($p<0.05$) (Fig 1).

Erythrocyte catalase activity

From Fig 2, the level of red cell catalase activity in the rats given a riboflavin-deficient diet, and *Trichinella spiralis*-infected rats and *Trichinella spiralis*-infected rats fed a riboflavin-deficient diet, had significantly lowered compared

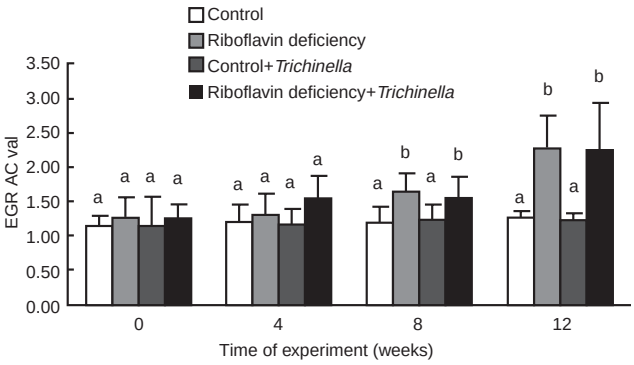

Fig 1–Erythrocyte glutathione reductase activation coefficient (EGR AC) in the 4 groups of Wistar rats during 0, 4, 8 and 12 weeks of the experiment. Mean±SD are demonstrated by vertical bars for 7 weight-matched rats per group. Significantly different means among treatments are represented by different letters ($p<0.05$).

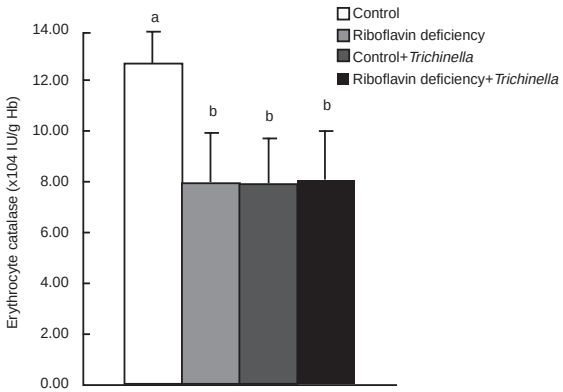

Fig 2–Red cell catalase activity in the 4 groups of Wistar rats at the 12th week of the experiment. Each vertical bar represents the mean ± SD for seven rats. The different letters appearing on vertical bars demonstrate the significant difference among the means, at $p<0.05$.

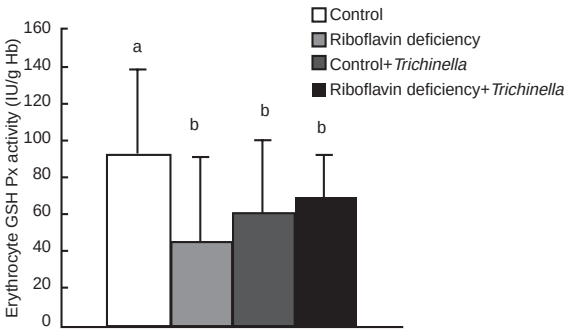

Fig 4–Red cell GSH-Px activity in the 4 groups of Wistar rats at the 12th week of the experiment. Each vertical bar represents the mean ± SD for seven rats. The different letters appearing on vertical bars demonstrate the significant difference among the means, at $p<0.05$.

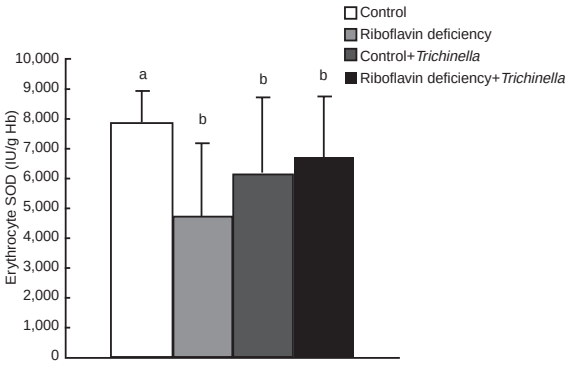

Fig 3–Red cell SOD activity in the 4 groups of Wistar rats at the 12th week of the experiment. Each vertical bar represents the mean ± SD for seven rats. The different letters appearing on vertical bars demonstrate the significant difference among the means, at $p<0.05$.

with the rats from the control group ($p<0.05$). However, among the 3 experimental groups, no differences in catalase activity were observed.

Erythrocyte SOD activity

The level of erythrocyte SOD activity decreased significantly ($p<0.05$) in the rats given a riboflavin-deficient diet, the *T. spiralis*-infected rats and the riboflavin-deficient-*T. spiralis*-infected rats, compared with the control group (Fig 3). In addition, no significant differences in erythrocyte SOD activity were detected among the 3 experimental groups.

Erythrocyte GSH-Px activity

From Fig 4, it can be seen that the level of red cell GSH-Px activity was statistically significantly lowered in rats under a riboflavin-deficient diet, *T. spiralis*-infected rats and riboflavin-deficient-*T. spiralis*-infected rats, when compared with the control of group of rats. It was also found that the level of red cell GSH-Px activity tended to be higher in riboflavin-deficient-*Trichinella*-infected rats than in both the riboflavin-deficient and *T. spiralis*-infected rats, but there was no significant difference among the 3 treated groups.

DISCUSSION

Riboflavin deficiency appeared at the 8th week of the experiment, with EGR AC values ≥ 1.20, both in normal rats fed the riboflavin-deficient diet and in combined *T. spiralis*-infection with the riboflavin-deficient diet, but ribofalvin deficiency was not shown in *T. spiralis* infected-rats. Therefore, it pointed out that *T. spiralis* did not cause erythrocyte riboflavin deficiency. This result was quite different from that of Adelekan and Thurnham (1998), who reported that malaria-infected rats tended to be more biochemically deficient in riboflavin than control rats, but this did

not reach statistical significance. This difference is due to Plasmodia, not *Trichinella*, duplicating in red blood cells and causing hemolysis of red blood cells. At the 12th week of the study, all of the activity of the erythrocyte antioxidant enzymes was monitored, including catalase, SOD and GSH-Px, and were significantly depressed both in rats deficient in riboflavin and in rats with *T. spiralis* infection, and also in the combined group of riboflavin deficiency and *T. spiralis* infection, as compared with the control group. The reduction of erythrocyte catalase, SOD and GSH-Px may be attributed to parasitic infection and malnutrition generating increased oxidative stress, which probably caused erythrocyte hemolysis. Hassen and Thurnham (1977) found increased red cell fragility in riboflavin-deficient rats and humans. Moreover, Kaikai and Thurnham (1983) found some evidence of increased red cell hemolysis in rats with *P. berghei* infection and riboflavin deficiency, but not in infected control animals. There was evidence that the level of SOD activity in the testes was reduced in cirrhotic rats because of an increase in the generation of reactive superoxide in the testis, while the decrease in GSH-Px activity might have been due to excessive production of free radicals, or enzyme inactivation might have decreased GSH-Px activity (Abul *et al*, 2002). In contrast, in parasitized erythrocytes such as *Plasmodium*, the efficiency of GSH-Px and SOD activity increased due to the possible activation of the existing enzymes during the intraerythrocytic growth phase of the parasite (Picard-Maureau *et al*, 1975; Eckman and Eaton, 1979). The depressed activity of catalase was attributed to either inactivation of the enzyme by metabolic products of the parasite (toxins) or the possible catabolism of this catalase during growth of the parasite (Picard-Maureau *et al*, 1975). Moreover, superoxide radical (O_2^-) production during parasite division may inhibit catalase (Kono and Fridovich, 1982). Therefore, increased oxidative stress in either malnutrition or parasitic infection was probably exacerbated by decreased availability of antioxidant enzymes.

Moreover, the result showed that the activity of the above three antioxidant enzymes in the riboflavin-deficient rats, concomitant with *T. spiralis* infection, tended to be less significantly increased than in the group fed only a riboflavin-deficient diet and the group with *T. spiralis* infection alone. *T. spiralis* itself may have to increasingly produce antioxidant enzymes, especially SOD and GSH-Px, to cope with increasing free oxygen radical production because of stress from both the parasitic infection and malnutrition. Many studies have reported the capacity of many parasites to produce antioxidant enzymes themselves. It is apparent that all the components of the mammalian cell oxidant defense system are also present in *P. berghei* (Seth *et al*, 1985). This oxidant defense system might protect *P. berghei* from free radical-mediated killing by the immune system of the host or by antimalarial drugs (Cox, 1983). All protozoan and helminthic parasites appear to have one or more antioxidant enzymes able to scavenge reactive oxygen species, and there is strong evidence that such enzymes play a crucial role in protecting against the host response (Callahan *et al*, 1988). GSH-Px and SOD may be responsible for the relatively high resistance of muscle larvae and adult *T. spiralis* to being killed (Callahan *et al*, 1988). In addition, different intracellular pathogens have different susceptibilities to oxygen radicals, probably because the parasites have evolved different mechanisms to evade the oxidative burst (Hughes, 1988).

ACKNOWLEDGEMENTS

This research was supported in part by the Ministry of University Affairs and the Faculty of Graduate Studies, Mahidol University, in the academic year 2002-2003. The authors also wish to express sincere thanks to all staff of the Department of Tropical Nutrition and Food Science and the Department of Helminthology, Faculty of Tropical Medicine, Mahidol University.

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