RIBOFLAVIN-DEFICIENT AND *TRICHINELLA SPIRALIS*-INDUCED STRESSES ON PLASMA CORTICOSTERONE ASSOCIATED WITH SPERMATOGENESIS IN MALE WISTAR RATS

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Abstract. The objective of this study was to investigate the effects of riboflavin-deficient and *Trichinella spiralis*-induced stresses on corticosterone associated with spermatogenesis in male Wistar rats. Rats were allocated into 4 groups: Group 1: control ; group 2: riboflavin-deficient diet; group 3: *T. spiralis* infection; group 4: riboflavin deficient diet with *T. spiralis* infection. This experiment lasted for 12 weeks. Plasma corticosterone was significantly enhanced when exposed to acute riboflavin deficiency and/or *T. spiralis* infection stress. When the rats were chronically subjected to such stresses, *T. spiralis per se* had prolonged effects, in a marked increase in corticosterone. *T. spiralis per se* tended to impact on such sperm characteristics as sperm motility, sperm count and daily sperm production, even defected seminiferous tubules. It was proposed that the *Trichinella spiralis*-induced stress probably had adverse effects on the level of adrenocortical-testicular axis whenever their habitats on muscle fibers were evident. However, riboflavin-deficient-induced stress had little implication in the adrenocortical-testicular axis.

INTRODUCTION

Riboflavin deficiency has been reported in many tropical countries (Bates, 1987). It appeared that the prevalence of riboflavin deficiency in the Thai population was almost 40% at subclinical level. It is still a problem in Thai society. Trichinellosis outbreaks caused by *Trichinella spiralis* have occurred in Thailand, especially in northern and southern Thailand, almost every year since 1962 (Khamboonruang, 1991). Infection is usually acquired via consumption of a local dish, "Larb", traditionally served during the northern Thai New Year and at wedding ceremonies. Infectious diseases and malnutrition are the most common health problems in developing countries. The situation may be more severe if these two conditions occur simultaneously. The interaction of infection and malnutrition may be considered cyclic insofar as one condition is capable of accentuating the other. Malnutrition increases host susceptibility to infection, while infection plays a role in malnutrition, particularly in borderline cases (Migasena, 1984).

Tumkiratiwong *et al* (2003) found that such erythrocyte antioxidant enzymes as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were significantly lowered in riboflavin-deficient and *T. spiralis*-infected rats. It was suggested that such occurrences were probably due to increased free radicals, especially reactive oxygen species (ROS), induced in the body. Whenever an imbalance between antioxidants and pro-oxidants in the body exists, it is said that oxidative stress has evolved. A variety of stressors have been reported to suppress male reproductive function (Nelson, 2000). Reproductive suppression in response to stress occurs on several physiological and behavioral levels and can have potentially severe effects on both humans and animals. Stress causes certain neurotransmitters to trigger the release of

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corticotropin-releasing hormone (CRH) from neurons in the paraventricular nucleus (PVN) of the hypothalamus (Welsh *et al,* 1999). CRH then stimulates adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary of the brain and finally enhances the release of adrenocortical glucocorticoids. It can inhibit reproduction in several ways. First, glucocorticoids can suppress gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) via negative feedback (Welsh *et al,* 1999). Glucocorticoids have also been reported to inhibit the formation of proteins necessary for producing hormone receptors, steroidogenic enzymes, and several intracellular signaling molecules (Rivier and Rivest, 1991). Therefore, the objective of this experiment is to monitor whether chronic riboflavin-deficient and *T. spiralis*-infected stress could contribute to the stressors that suppress the male reproductive system.

MATERIALS AND METHODS

Materials

Two types of feed were fed to the rats; one was commercial feed as a control diet, while the other, a riboflavin-deficient diet based on the formula of Hoppel and Tandler (1975) and modified by Adelekan and Thurnham (1986) was composed of the ingredients shown in Table 1

Animals

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

Parasite infection

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

Table 1
Ingredients of the riboflavin-deficient diet.

Ingredients	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 (grams) composed of $(Al_2SO_4)_3.K_2SO_4.24H_2O$ 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 (grams/kilogram mixture) composed of 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.

Experimental protocols

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

At weeks 4, 8 and 12 of the experiment, blood was drawn from the conjunctival artery into heparinized capillary tubes to measure plasma corticosterone.

Ethical aspects

This study was approved by the Ethics Committee of the Department of Zoology, Kasetsart University, Bangkok, Thailand.

Corticosterone determination

The corticosterone plasma level was measured by ^{125}I radioimmunoassay (RIA) without sample extraction. The corticosterone concentration was analyzed by Coat-A-Count® Rat Corticosterone (TKRC1) kit assay (Diagnostic

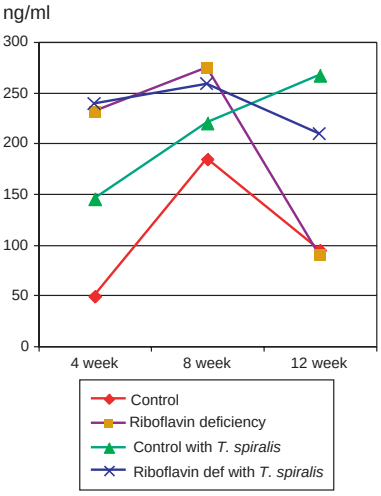
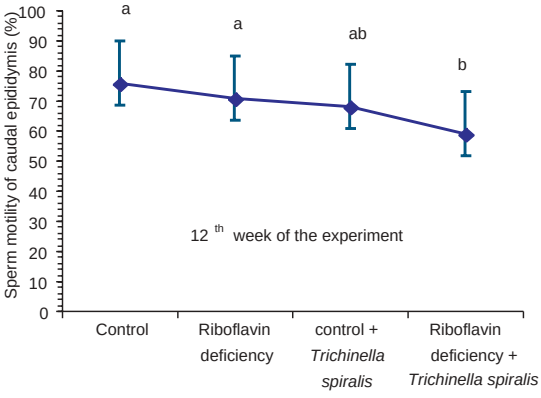
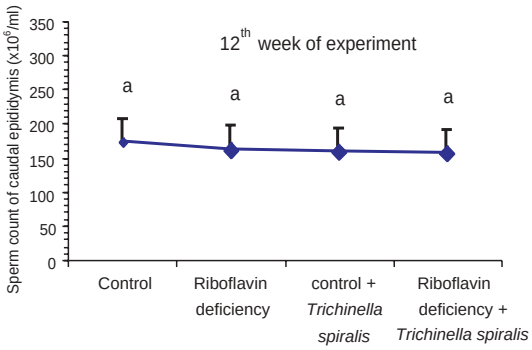


Fig 1–Corticosterone concentration (ng/ml) in 4 groups of Wistar rats during weeks 4, 8 and 12 of the experiment. Each point represents the median of corticosterone of 7 Wistar rats per group.



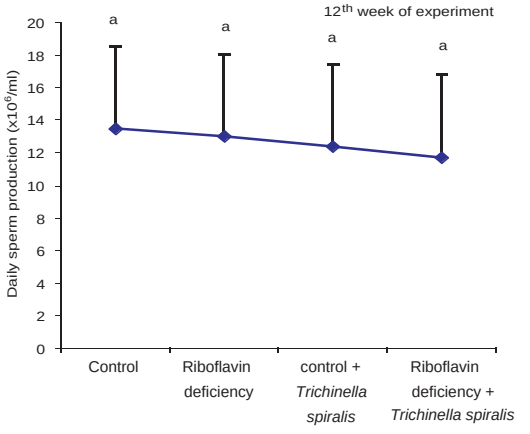
[a,b]; the small different letters appearing on the above each point demonstrate the significant difference among treatments during week 12 of experiment, at $p<0.01$.

Fig 2–Sperm motility of the caudal epididymis (%) among the 4 groups of Wistar rats at the 12th week of the experiment. Each point represents the medians (ranges) of 7 Wistar rats per group.



[a,b]; the small different letters appearing on the above each point demonstrate the significant difference among treatments during week 4 of experiment, at $p<0.05$.

Fig 3–Sperm count of the caudal epididymis ($x10^6$/ml) in the 4 groups of Wistar rats at the 12th week of the experiment. Each point represents the medians (ranges) of 7 Wistar rats per group.



[a,b]; the small different letters appearing on the above each point demonstrate the significant difference among treatments during week 12 of experiment, at $p<0.01$.

Fig 4–Daily sperm production ($x10^6$/ml) in the 4 groups of Wistar rats at the 12th week of the experiment. Each point represents the medians (ranges) of 7 Wistar rats per group.

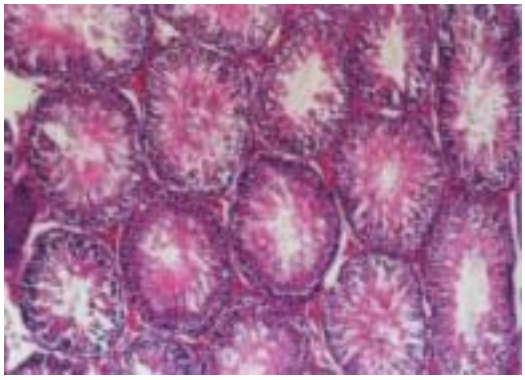
Products Corporation; DPC®, CA 90045).

Sperm characteristics

At the end of the experiment, sperm motility and sperm counts of the caudal epididymis were monitored by the method of Amann and Howards (1980) and daily sperm production (DSP) of the testes were determined by the method of Amann *et al* (1976).

Histology

At the end of the experiment (12th week of treatment) the testis specimens were excised and fixed in 10% v/v buffered neutral formalin solution, processed by paraffin technique. The tissue was cut to 6 μm in thickness using an AO



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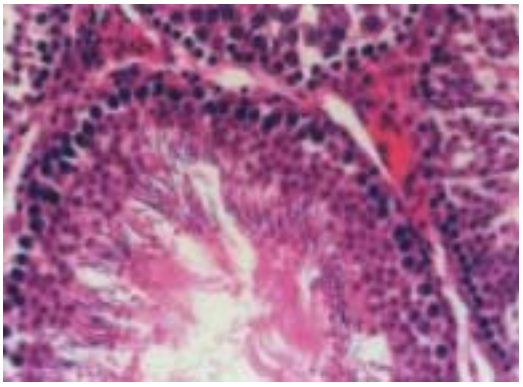
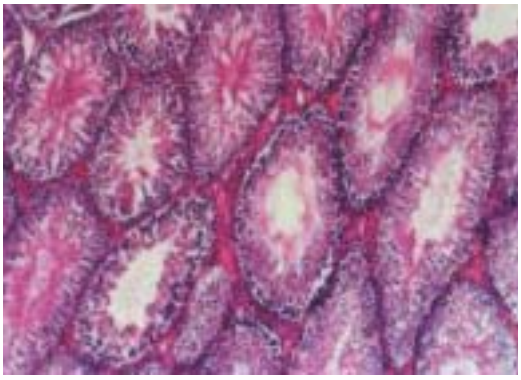


B

Fig 5.1–Photograph of testes at 12th week of experiment in control group showing normal features with successive stages of transformation of seminiferous epithelium to spermatozoa. A: H.E. x 100. B: H.E. x 400.



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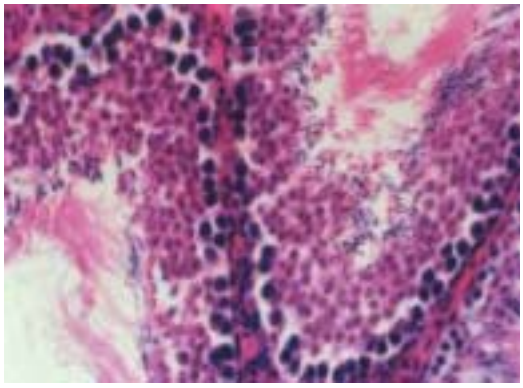

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Fig 5.2–Photograph of testes at 12th week of experiment in Wistar rat fed riboflavin- deficient diet showing a few irregular features of seminiferous tubules. A: H.E. x 100. B: H.E. x 400.

rotary microtome model 820. The sections were stained with hematoxylin and eosin (Luna, 1968).

Statistical analysis

Data were expressed as medians (range) and analyzed for significance by one-way anlysis of variance (ANOVA). Significant differences among the medians were tested by Mann-Whitney *U* test, at $p<0.05$.

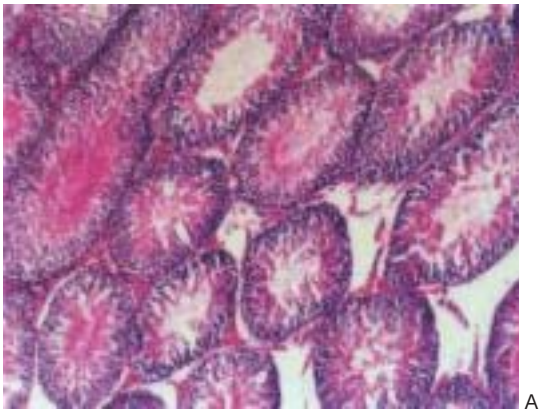
RESULTS

Corticosterone level

The corticosterone levels in the riboflavin-deficient rats tended to decrease from weeks 4 to 12 of the experiment, while the levels in the *T. spiralis*-infected rats tended to increase significantly from weeks 4 to 12 of the experiment. The corticosterone levels of the riboflavin-deficient rats with the *T. spiralis*-infected also increased compared with the control group (Fig 1).

Sperm motility

From Fig 2 it may be seen that, sperm motility of the caudal epididymis (%) in the rats fed the riboflavin-deficient diet or had *T. spiralis* infection, tended to be insignificantly reduced ($p>0.05$). However, rats treated with the riboflavin-deficient diet combined with *T. spiralis* infection had significantly lower sperm motility than the control, and riboflavin-deficient or *T. spiralis* infection, groups ($p<0.05$). In addition, no significant difference was found in the sperm motility for the *T. spiralis*-infected rats, compared with the combined riboflavin-deficient and *T. spiralis*-infected rats ($p>0.05$).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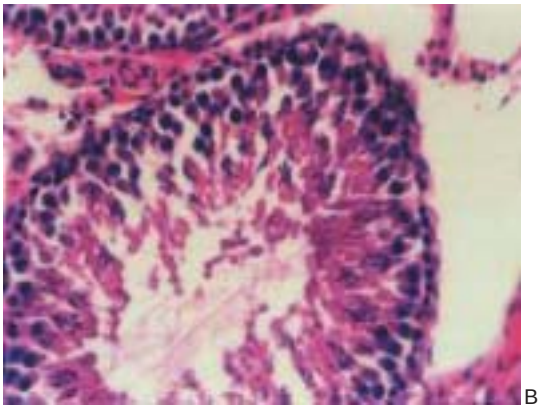
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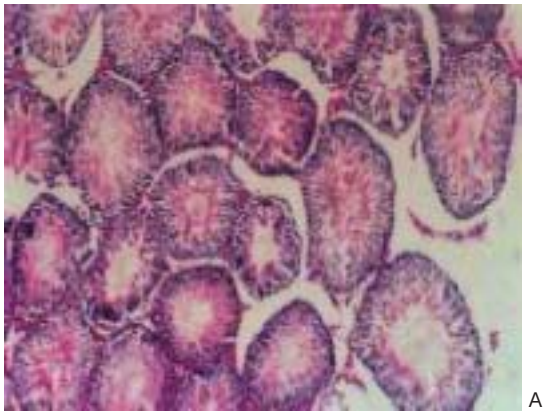
Fig 5.3–Photograph of testes at 12th week of experiment in Wistar rats infected with *T. spiralis,* showing irregular features with certain atrophy of the seminiferous tubules and large interstitium among the seminiferous tubules. Note: No mass of spermatozoa in the lumen of the seminiferous tubules (B). A: H.E. x 100. B: H.E. x 400.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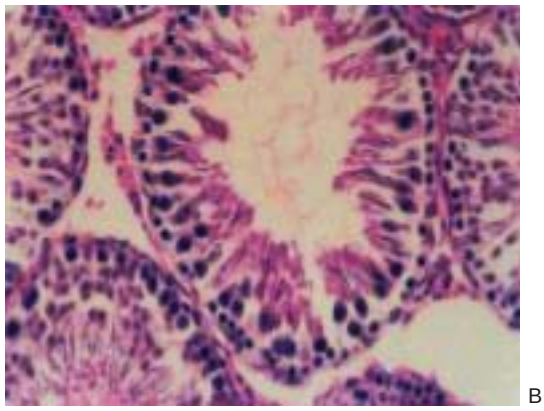
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Fig 5.4–Photograph of testes at 12th week of experiment in Wistar rat fed the riboflavin deficient diet with infected *Trichinella spiralis,* showing prominent atrophy features and large interstitium of the seminiferous tubules. Note: No mass of spermatozoa in the lumen of the seminiferous tubules (B). A: H.E. x 100. B: H.E. x 400.

Sperm count

The sperm counts (x10^6 cells/ml) of the caudal epididymis were not significantly different among the riboflavin-deficient, *T. spiralis*-infected and both riboflavin-deficient and *T. spiralis* infected rats ($p>0.05$), but all treated groups had a trend of reduced sperm count (Fig 3).

Daily sperm production

The daily sperm production (x10^6 cells/ml) of the caudal epididymis was not significantly different among the riboflavin-deficient, *T. spiralis*-infected and both riboflavin-deficient and *T. spiralis* infected, rats ($p> 0.05$), but all-treated groups had a trend towards reduced daily sperm production (Fig 4).

Histopathology of testes

On histological examination, transverse sections of testes of the Wistar rats showed the presence of all stages of spermatogenesis in the seminiferous tubules and Leydig cells (LC) in the interstitial tissue in the control group (Fig 5.1). Some spermatozoa were found in the seminiferous tubules, but demonstrated some irregularly shaped seminiferous tubules among the representative rats given the riboflavin-deficient diet (Fig 5.2). However, no spermatozoa were found in the seminiferous tubules of the rats infected with *T. spiralis* and given a riboflavin-deficient diet

(Fig 5.3). In addition, the seminiferous tubules were small in size and irregular in shape at 12 weeks, especially in the rats given the riboflavin-deficient diet with *T. spiralis* infection; certain germ cells had also degenerated, and atrophy was more pronounced (Fig 5.4).

DISCUSSION

In our study, we tried to demonstrate the relationship between nutrition and parasitic infection, and the pathophysiology of the host. Many investigators have reported that diet supplemetation can prevent the establishment of helminthes, or cure diseases caused by helminths (van Houtert and Sykes, 1996). However, others began to associate differences in response to infection to components of the diet, *eg*, vitamin A and dietary protein (Gibson, 1963). Our result showed low levels of corticosterone in riboflavin-deficient rats from weeks 4 to 12 of the experiment. The levels in *T. spiralis*-infected rats, and riboflavin-deficient rats with *T. spiralis*-infected rats, tended to be significantly increased from weeks 4 to 12 of the experiment. The high level of corticosterone seemed to be induced by the *T. spiralis* infection. It has been reported that some cytokine concentrations are changed in helminth-infected animals (Horbury *et al*, 1995), and that cytokines are regulatory proteins with a wide range of physiological functions (Husband, 1995). These cytokines also depress basal energy expenditure, while they increase glucose oxidation, gluconeogenesis, lipolysis, and muscle protein degradation (Husband, 1995). Corticotropin-releasing factor (CRF), which stimulates corticosteroid hormone production, might be significantly affected by parasitism. Detailed quantitative information is lacking, however, and further study might be required.

Increases in free radicals, especially reactive oxygen species (ROS), induced in the host body of riboflavin-deficient and *T. spiralis*-infected rats, might be an other cause of male reproductive function impairment (Nelson, 2000), and it was found that erythrocyte antioxidant enzymes, catalase, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were significantly lowered in riboflavin-deficient and *T. spiralis*-infected rats (Tumkiratiwong *et al*, 2003). It was suggested that such occurrences are probably due to increased free radicals especially reactive oxygen species (ROS) induced in the body. However, the increased corticosterone levels are attributed to *T. spiralis*-induced stress rather than riboflavin-deficient-induced stress. It is suggested that the riboflavin-deficient status did not make the male Wistar rats experience stress as much as the *T. spiralis* infection during the 12th week of the experiment. Riboflavin deficient might cause lower oxidative stress than the parasitic infection.

In addition, the synchronization of infection and malnutrition in the present study did not cause these animals to endure much more stress, as expected. Therefore, one adverse condition could not attenuate the other to become more severe and vice versa. The *T. spiralis* infection *per se* rather than the riboflavin-deficient diet also had an adverse effect on spermatogenesis, in terms of sperm motility in the caudal epididymis. Nevertheless, the *T. spiralis* infection or the riboflavin-deficient diet had little impact on sperm count and daily sperm production, even when the factors were combined. This is probably due to the impact of such nematode infections on the male reproductive organs. In one respect, this mechanism is a consequence of the glucocorticoid group, such as corticosterone, which suppresses reproductive function. This accords with Nelson (2000) who stated that the glucocorticoids suppress spermatogenesis somewhat directly. However, nutrition, the host, and the parasite relationship, should become the focus of research in the future.

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