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The Traditional Chinese Medicine, *Monascus*-Fermented Rice, Prevents Zn Deficiency-Induced Testis and Sperms Injury

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ABSTRACT

Monascus purpureus is a fungus used in rice fermentation. *Monascus*-fermented rice is called red-mold rice (RMR) in Taiwan since the fermentation products contain a considerable amount of red pigments. The protective effects of RMR on oxidative stress in the testis and sperm were evaluated in this study. Zn deficiency was induced in rats by feeding them with a Zn-deficient diet for 12 weeks while the control rats were fed with a normal diet. The Zn-deficient rats were then divided into 6 groups, designated as Zn-deficient (ZD), Zn-compensated (ZC), 1R (151 mg RMR/kg), 5R (755 mg RMR/kg), 1R + ZC (1RZ), and 5R + ZC (5RZ). The animals were administered with the mentioned diet (RMR/Zn) for 8 weeks. In the ZD rats, no spermatid cell formation was observed in the seminiferous tubular epithelia, and testis necrosis/atrophy was apparent. However, 5R and 5RZ administration improved testicular antioxidant enzyme activity, elevated serum testosterone levels, and increased sperm number in ZD rats; this treatment also exhibited inhibitory effects on caspase activities and reactive oxygen species (ROS) levels in the ZD rats, suggesting that RMR attenuated ZD-induced oxidative stress and testis apoptosis. The preventive activity against ZD-induced reproductive damage was more apparent in the 1RZ and 5RZ groups than in the ZC group, suggesting that RMR can serve as a supplement in adjuvant therapy for diseases associated with Zn deficiency.

Key words: red mold rice (RMR), Zn deficiency, antioxidant enzymes activity, testosterone, sperm

INTRODUCTION

Brain and testis contain significant levels of essential microelements including zinc (Zn), which is a cofactor of superoxide dismutase (SOD) and metalloenzymes in a variety of animal species[(1)]. Regulatory effects of Zn on spermatogenesis, spermatozoa maturation, motility, and fertilizing capacity have been reported previously[(2,3)]. In addition, Zn deficiency (ZD) leads to reduced testosterone production[(4)], impairment of sex glands[(5)], and decrease in the seminal volume[(6,7)]. Moreover, prostate growth depends on testosterone levels and is thus inhibited by ZD induction[(8)]. On the other hand, because Zn level in the body decreases with age[(4)], and meat contains Zn in abundance, Zn-deficient patients are usually found among the elderly or vegetarians.

Monascus is a type of fungus used in rice fermentation. *Monascus*-fermented rice is known as red-mold rice (RMR) and has been used as a traditional Chinese medicine for curing digestive problems and vascular dysfunction for many centuries in Asia[(9,10)]. In our previous study, we showed that RMR contains various antioxidants, i.e. dimerumic acid, tannin, and phenol, and the antioxidative capacity of RMR ethanol extracts, which contain 1,1-diphenyl-2-pichrylhydrazyl (DPPH) with radical scavenging activity and reducing power, has also been reported[(11)]. The fact that RMR stimulates antioxidant enzymes in the brains of ZD rats has been demonstrated in our recent study[(12)]. Therefore, we hypothesized that RMR may protect against the oxidative stress-induced injury caused by Zn deficiency via stimulating antioxidant enzymes, thus resulting in improved sperm activity and quality (motility and quantity) in ZD rats.

MATERIALS AND METHODS

I. *Materials and Chemicals*

SOD assay kit was purchased from Randox Laboratories

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Ltd. (Antrim, UK). Glutathione (GSH), glutathione reductase (GR), glutathione disulfide (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), and nitroblue tetrazolium (NBT) were purchased from Sigma (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4) and hydrogen peroxide (H_2O_2) were obtained from Merck (Darmstadt, Germany).

II. *Sample Preparation*

RMR is obtained from *Monascus purpureus* NTU 568-fermented rice. *M. purpureus* NTU 568 strain was maintained on a potato dextrose agar slant at 4°C and transferred monthly. The RMR was prepared using solid-state culture on *Monascus*-fermented long-grain rice (*Oryza sativa*) purchased from local supermarket in Taiwan. Briefly, rice (500 g) was soaked in deionized water for 1 h and then excess water was removed. The rice was autoclaved in an autoclave (HL-341 model, Gemmy Corp, Taipei, Taiwan) for 20 min at 121°C. After cooling, the rice was inoculated with a 5% (v/w) spore suspension. The inoculated rice was cultivated at 30°C for 10 days. During the culturing stage, 100 mL of water was added to the rice daily from the second to the fifth day. At the end of cultivation, the crushed and dried product with the mold was used for the experiments.

III. *Animal and Diets*

Male Wistar rats (4-week old; 90.7 ± 10.6 g) were obtained from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Animals were acclimatized for 1 week prior to use. They were randomly divided into seven treatment groups (12 rats/group) and provided with food and water ad libitum. Animals were subjected to 12 h light/dark cycle with maintained relative humidity of 60% and temperature at 25°C (Protocol complied with guidelines described in the "Animal Protection Law", amended on Jan. 17, 2001 Hua-Zong-(1)-Yi-Tzi-9000007530, Council of Agriculture, Executive Yuan, Taiwan, ROC). Experimental diets were provided in accordance with the dietary formula, containing 200.0 g egg white, 631.1 g dextrose, 100.0 g corn oil, 30.0 g fiber, 9.9 g calcium carbonate, 3.2 g calcium phosphate, 0.002 g cobalt chloride, 0.01 g cupric sulphate, 0.9 g ferric citrate, 3.4 g magnesium sulphate, 0.009 g manganese sulphate, 0.026 g potassium iodide, 5.55 g sodium chloride, 0.004 g biotin, 0.02 g vitamin B_{12}, 0.016 g calcium pantothenate, 1.5 g choline chloride, 0.25 g chlortetracycline, 0.0005 g folic acid, 0.0003 g menadione, 0.25 g niacin, 0.004 g pyridoxine HCl, 0.006 g riboflavin, 0.01 g thiamin HCl, 10,000 IU retinyl palmitate, 1,250 IU ergocalciferol, and 110 IU tocopheryl acetate per kg of diet[13]. Rats were fed with the daily diet containing 60 mg Zn/kg control feedstuff in the normal group whereas approximately 0.3 mg Zn/kg ZD feedstuff was fed in the ZD rats. Rats were induced Zn deficiency by a Zn-deficient diet for 12 weeks, and normal diet was fed to control rats. The Zn-deficient rats were divided into six groups, including Zn-deficient (ZD), Zn-compensative (ZC), 1R (151 mg RMR/kg), 5R (755 mg RMR/kg), 1R + ZC (1RZ), and 5R + ZC (5RZ). The animals were administered with samples (RMR/Zn) for 8 weeks. In addition, Zn gluconate (1.1 mg Zn/kg) was administered in the ZC, 1RZ, and 5RZ groups. The Zn level of RMR was 25.9 mg/kg. Thus, the Zn administrating levels were 1.1 mg, 0.004 mg, 0.02 mg, 1.104 mg, and 1.12 mg/kg in the ZC, 1R, 5R, 1RZ, and 5RZ groups, respectively.

IV. *Histopathologic Study and Sperm Observation*

Testis and prostate were first trimmed into 2 mm thickness, and fixed with buffered formaldehyde for 24 h. The fixed tissues were further processed including embedded in paraffin, sectioned and rehydrated. The injury of Zn deficiency induction was evaluated by histological examination with hematoxylin and eosin stain[14]. The epididymis was sheared and added 2 mL phosphate buffered saline (PBS), and subsequently, the spermatozoa motility (%) and concentration were calculated. The spermatozoa shape was evaluated by referring to the gist from spermatozoa acrosome, head and axial filament observations[15].

V. *Sex Hormone Assay*

Blood were centrifuged at 1,000 ×*g* for 10 min and serum was collected and frozen at -20°C until analysis. Serum progesterone and testosterone were assayed using commercial kits from Cayman Chemical Company (Ann Arbor, MI, USA).

VI. *Assays for Antioxidant Enzymes and ROS Level*

The testis and prostate tissues were homogenized in ice-cold 20 mM Tris-HCl (pH 7.4, 1 : 10, w/v) and the homogenates were centrifuged at 2,500 ×*g* for 30 min at 4°C. Glutathione peroxidase (GPx) activity was measured as previously described[16]. Briefly, 0.1 mL of homogenate was mixed with 0.8 mL of 100 mM potassium phosphate buffer (1 mM EDTA, 1 mM NaN_3, 0.2 mM NADPH, 1 U/mL GR, and 1 mM GSH, pH 7.0) and incubated for 5 min at room temperature. Thereafter, the reaction was initiated after adding 0.1 mL of 2.5 mM hydrogen peroxide (H_2O_2). GPx activity was calculated by the change of the absorbance at 340 nm for 5 min. In another reaction containing 0.1 M phosphate buffer, 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM GSSG, and 0.1 mM NADPH, pH 7.0, 0.1 mL of homogenate was added for glutathione reductase (GR) activity assay. The decrease of absorbance at 340 nm after 3 min incubation was measured[17]. Catalase (CAT) activity was determined according to the method of Aebi [18]. Fifty microliters of homogenate was mixed with 950 μL of 0.02 M H_2O_2 and incubated at room temperature for 2 min. CAT activity was calculated by the change of the absorbance at 240 nm for 3 min. The assay of SOD activity was accomplished using a commercial kit (Randox Laboratories Ltd, UK). The level of ROS was assayed with nitroblue tetrazolium (NBT). NBT is reduced to form blue-black formazan by ROS and dissolved

Journal of Food and Drug Analysis, Vol. 19, No. 2, 2011

in dimethyl sulfoxide. Therefore, it displays high absorbance level of NBT-formazan when ROS is produced in abundance. In the measurement of ROS, 100 μL of homogenates was added to 96-well plates and 10 mg/mL of NBT was added. Absorbance at 570 nm was measured after 2-h reaction[19].

VII. *Assay for Fe, Ca, Mg, and Zn Concentrations*

Whole prostate and testis were minced finely with a razor blade, and the minced tissue was solubilized in 25% tetramethylammonium hydroxide. The prostate and testis were mixed with 1 mL of HCl solution, diluted to 10 mL with distilled water, and directly nebulized in an air/acetylene flame using the optimal instrumental parameters. The analyte addition technique was used for the determination. Analytical calibration solutions for Fe, Zn, Ca, and Mg were prepared by suitable dilution of stock standard solutions of $Fe(NO_3)_3$, $ZnCl_2$, $Ca(NO_3)_2$, and $Mg(NO_3)_2$ (Merck). The levels of Fe, Zn, Ca, and Mg were determined by atomic absorption spectrophotometry (AAS) in an air/acetylene flame (Z-8200 model, Hitachi Corp., Tokyo, Japan) using an aqueous standard calibration curve[12].

VIII. *Assay for Caspase-3, -8, and -9*

The caspase-3, -8, and -9 activities of testicular homogenate were determined using kits from BioVision Inc. (Mountain View, CA, USA). Testicular homogenate (50 μL) was mixed with 50 μL of 2X reaction buffer (contained 10 mM dithiothreitol), and then 5 μL substrate (4 mM) was added in and incubated at 37°C for 1-2 h. The optical density was measured at 405 nm by ELISA reader.

IX. *Statistical Analysis*

Data was expressed as means ± SD. The software of ANOVA was used to evaluate the difference among multiple groups. If significant difference ($p < 0.05$) was observed, Duncan's multiple range test was used to compare the means of two specific groups.

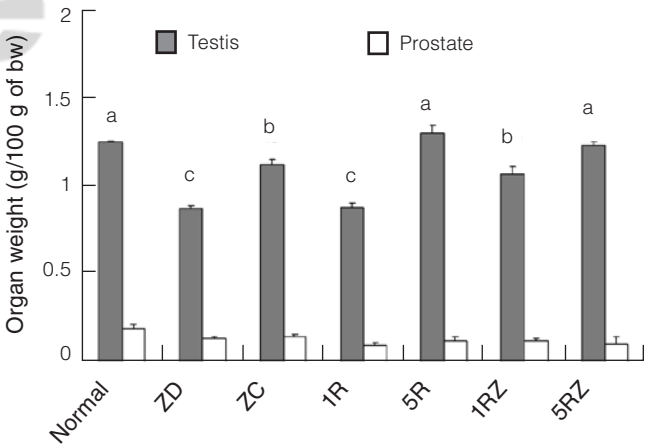

Figure 1. The changes in the testis and prostate weights of ZD rats. Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). Different letters showed significant difference ($p < 0.05$).

RESULTS

I. *Organ Weights*

Growth retardation and organ atrophy have been found in ZD rats[20,21]. Furthermore, ZD increase testicular Ca and Mg levels, and this finding is correlated with testis injury[22]. The testis and prostate of ZD rats weighed significantly less than those of normal rats in this study, revealing that the ZD resulted in dystrophy and growth inhibition. However, RMR administration for 12 weeks improved weights of the testis in the 5R, 1RZ, and 5RZ groups, and this effect was not observed in the prostates of ZD rats (Figure 1). The testicular

Table 1. The variations of trace element in the testis and prostate of ZD rats

Groups	Testis				Prostate			
	Concentration (mg/kg)							
	Fe	Zn	Ca	Mg	Fe	Zn	Ca	Mg
Normal	34.6 ± 2.7	29.8 ± 2.4	14.2 ± 7.5	80.7 ± 30.7	30.2 ± 5.8	34.1 ± 6.1	114.0 ± 13.6	130.0 ± 31.0
ZD	55.0 ± 5.3*	22.4 ± 1.5*	32.3 ± 4.8*	97.3 ± 7.7*	33.8 ± 9.6	20.6 ± 5.3*	186.7 ± 18.5*	148.4 ± 28.3
ZC	49.0 ± 10.7	27.1 ± 2.8	25.5 ± 6.8	87.4 ± 8.4	27.7 ± 3.7	20.2 ± 9.2	160.3 ± 12.9	125.6 ± 18.0
1R	54.4 ± 10.1	20.7 ± 3.7	31.6 ± 7.3	94.5 ± 12.6	35.5 ± 6.4	21.7 ± 6.7	151.7 ± 14.2	134.4 ± 15.0
5R	55.7 ± 7.5	22.5 ± 2.5	32.6 ± 6.3	92.1 ± 6.1	25.2 ± 7.3	26.7 ± 7.7	129.1 ± 12.2#	135.3 ± 24.9
1RZ	53.1 ± 6.8	21.2 ± 5.6	45.2 ± 12.3	104.5 ± 8.3	26.7 ± 6.2	21.2 ± 6.0	133.0 ± 15.4#	150.2 ± 28.3
5RZ	41.0 ± 3.5#	24.2 ± 4.6	18.4 ± 6.9#	89.4 ± 7.5	28.8 ± 8.1	24.0 ± 5.3	130.9 ± 14.6#	132.8 ± 14.5

Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). *Significant difference from the normal group, $p < 0.05$. #Significant difference from the ZD group, $p < 0.05$.

Fe, Ca, and Mg levels considerably increased while the testicular Zn level decreased in the ZD rats. The prostatic Ca and Zn levels were increased and decreased, respectively in the ZD rats (Table 1). However, the testicular Fe and Ca levels recovered in the 5RZ group compared to the ZD group, but the Zn level did not increase. Finally, RMR with or without ZC administration failed to recover the Zn level, but it attenuated the Ca elevation caused by ZD in the prostate.

II. *Histology*

Atrophy in the testis and reduced spermatogenesis were examined[23]. The histological findings of testis are shown in Figure 2. Slight-to-severe necrosis and atrophy were observed in the testis of ZD rats, and no spermatids were produced in the seminiferous tubules. However, 1RZ and 5RZ administration increased spermatogenesis in the testis and repaired histological damage caused by ZD induction. Table 2 shows damage in the testis of ZD rats. The extent of injury to the testis was enhanced by 83.3% in the ZD group. However, the extents were reduced to 50.0% and 41.7% in the 5R and 5RZ groups, respectively. Damages to the testis were significantly revered in the 5RZ group, which was noted by reduced atrophy and enhanced spermatozoa formation. On the other hand, the prostates of the ZD rats were not damaged.

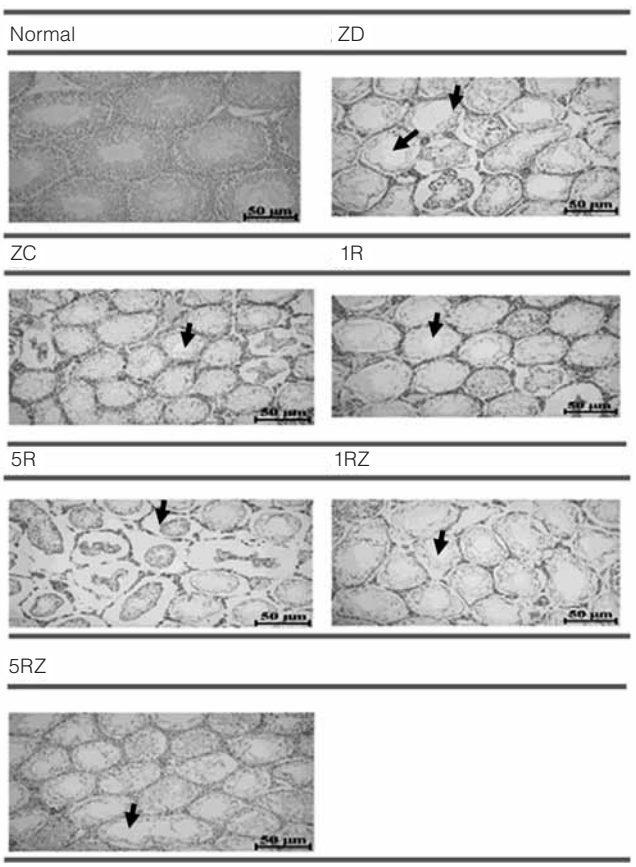

Figure 2. The effects of RMR and Zn on testis damage from ZD induction (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC).

III. *Sperm Activity*

Increased ROS production is associated with the existence of overall abnormal sperm morphology, which can be explained by the fact that ROS disrupted the cell membrane and thus altered the sperm morphology. The preventive effect of RMR against generation of abnormally shaped sperm in ZD rats was evaluated by examining the morphologies of spermatozoa acrosomes, heads, and axonemes. The results indicated that RMR and Zn administration reduced the number of abnormal epididymal spermatozoa in the ZD rats (Table 3). In addition, ZD generated high levels of ROS in the sperm (Figure 3), thereby degrading sperm qualities such as motility and concentration. Sperm numbers decreased in

Table 2. The effect of RMR on testis and prostate damage induced by Zn deficiency

Groups	Testis		Prostate	
	Damaged animal number (%)	Atrophy, Seminiferous tubule	Damaged animal number	Necrosis, Inflammation
Normal	0 (0.0)	NSL	0	NSL
ZD	10 (83.3)	3.9 ± 0.2^{b}	0	NSL
ZC	8 (66.7)	3.6 ± 0.4^{b}	0	NSL
1R	7 (58.3)	3.5 ± 0.4^{b}	0	NSL
5R	6 (50.0)	3.4 ± 0.2^{b}	0	NSL
1RZ	8 (66.7)	3.7 ± 0.3^{b}	0	NSL
5RZ	5 (41.7)	3.1 ± 0.2^{a}	0	NSL

NSL: no significant lesions. Degree of lesions was graded from one to five depending on severity: 1 = minimal (< 1%); 2 = slight (1-25%); 3 = moderate (26-50%); 4 = moderate/severe (51-75%); 5 = severe/high (76-100%). Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). Mean values within the same column bearing different superscript letters differ significantly (a, b) ($p < 0.05$).

Table 3. The effect of RMR on sperm activity in ZD rats

Groups	Motility (%)	Abnormal shape (%)	Concentration (numbers × 10^8/mL)
Normal	15 - 76	2 - 5	1.31 - 3.87
ZD	9 - 11	24 - 43	0.11 - 0.90
ZC	3 - 18	14 - 32	0.82 - 1.32
1R	7 - 14	12 - 24	0.47 - 1.02
5R	1 - 18	14 - 17	1.11 - 1.36
1RZ	0 - 20	23 - 31	0.77 - 1.08
5RZ	2 - 32	17 - 21	1.84 - 2.77

Rats (n = 12) were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC).

Journal of Food and Drug Analysis, Vol. 19, No. 2, 2011

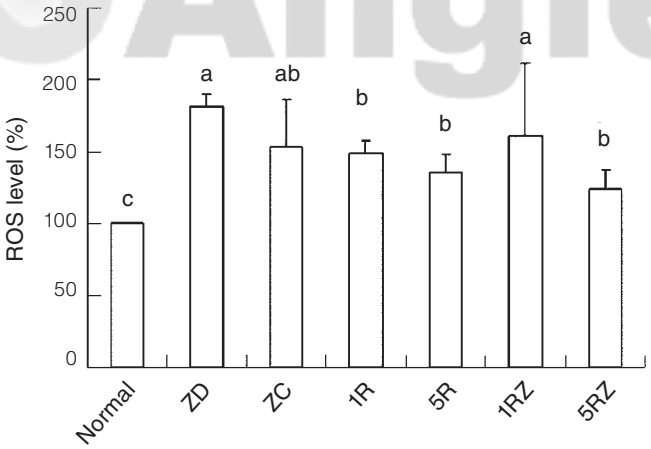

Figure 3. The inhibitory effect of RMR on ROS production in sperm of ZD rats. Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). Different letters showed significant difference ($p < 0.05$).

the ZD rats due to ZD-induced inhibition of both spermatogenesis and spermatozoa maturation. However, 5R and 5RZ administration markedly decreased the ROS level in the sperms of ZD rats. These findings suggested that RMR and Zn administrations attenuated the ROS levels, prevented oxidative damage in the sperm, and recovered spermatogenesis and spermatozoa maturation in the ZD rats.

IV. *Antioxidant Enzymes Activities*

RMR stimulates antioxidative activity in the brain of ZD rats[12]. ROS were excessively produced in the testis of ZD rats as compared to the normal group (Table 4). However, RMR and Zn administration reduced ROS levels in the testis of ZD rats. CAT, GR, GPx, and SOD activities decreased significantly in the testis of the ZD group compared to the normal group. In the 5RZ group, however, CAT, GR, GPx, and SOD activities were recovered, compared to those in the ZD group. The prostatic SOD activity also decreased in the ZD rats (Table 5). However, prostatic CAT, GR, and

Table 4. The effects of RMR on antioxidant enzymes activities in the testis of ZD rats.

Groups	CAT	GR	GPx	SOD	ROS
	nmol H_2O_2/min/mg protein	nmol NADPH/min/mg protein		U/mg protein	(%)
Normal	4.8 ± 0.1^{a}	125.6 ± 24.9^{a}	193.3 ± 29.0^{a}	6.0 ± 0.9^{a}	100.0 ± 10.0^{c}
ZD	3.3 ± 0.2^{b}	29.0 ± 10.9^{c}	58.3 ± 14.0^{b}	4.0 ± 0.2^{b}	290.8 ± 13.2^{a}
ZC	3.7 ± 0.6^{ab}	57.6 ± 15.2^{b}	67.0 ± 16.2^{b}	3.2 ± 0.6^{b}	229.9 ± 16.4^{b}
1R	3.8 ± 0.4^{ab}	67.3 ± 15.9^{b}	57.7 ± 28.4^{b}	4.5 ± 0.2^{ab}	225.4 ± 14.1^{b}
5R	3.5 ± 0.8^{ab}	77.1 ± 16.5^{ab}	163.0 ± 17.6^{a}	5.2 ± 0.1^{a}	202.1 ± 26.5^{b}
1RZ	4.2 ± 0.2^{a}	97.4 ± 13.3^{a}	173.9 ± 32.9^{a}	5.2 ± 0.5^{a}	236.0 ± 15.3^{b}
5RZ	4.4 ± 0.2^{a}	137.9 ± 20.5^{a}	203.2 ± 20.2^{a}	5.4 ± 0.8^{a}	178.3 ± 31.3^{b}

Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). CAT: catalase; GR: glutathione reductase; GPx: glutathione peroxidase; SOD: superoxide dismutase; ROS: reactive oxygen species. Mean values within the same column bearing different superscript letters differ significantly (a, b) ($p < 0.05$).

Table 5. The effects of RMR on antioxidant enzymes activities in the prostate of ZD rats

Groups	CAT	GR	GPx	SOD	ROS
	nmol H_2O_2/min/mg protein	nmol NADPH/min/mg protein		U/mg protein	(%)
Normal	18.5 ± 5.9^{b}	144.9 ± 15.8	184.6 ± 23.0	5.2 ± 1.2^{a}	100.0 ± 13.5^{a}
ZD	18.8 ± 5.1^{b}	124.3 ± 16.8	167.9 ± 13.6	3.2 ± 0.2^{b}	106.4 ± 6.3^{a}
ZC	19.1 ± 5.8^{b}	127.5 ± 26.4	178.0 ± 18.6	5.5 ± 1.2^{a}	57.5 ± 13.6^{b}
1R	33.0 ± 3.4^{a}	137.9 ± 15.8	177.8 ± 17.1	5.2 ± 0.9^{a}	67.4 ± 7.1^{b}
5R	39.4 ± 4.5^{a}	122.9 ± 25.9	184.5 ± 20.6	4.4 ± 1.1^{ab}	70.3 ± 8.6^{b}
1RZ	38.0 ± 7.2^{a}	143.3 ± 20.8	163.1 ± 17.4	6.3 ± 1.6^{a}	45.4 ± 7.0^{b}
5RZ	40.0 ± 7.6^{a}	158.4 ± 22.9	196.8 ± 23.8	7.6 ± 2.0^{a}	46.5 ± 8.5^{b}

Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). CAT: catalase; GR: glutathione reductase; GPx: glutathione peroxidase; SOD: superoxide dismutase; ROS: reactive oxygen species. Mean values within the same column bearing different superscript letters differ significantly (a, b) ($p < 0.05$).

GPx activities did not significantly decrease in any group. Administration of RMR and Zn in the 5RZ group effectively increased the CAT activity and recovered SOD activity in the prostate of ZD rats. Therefore, RMR and Zn administration markedly reduced prostatic ROS generation.

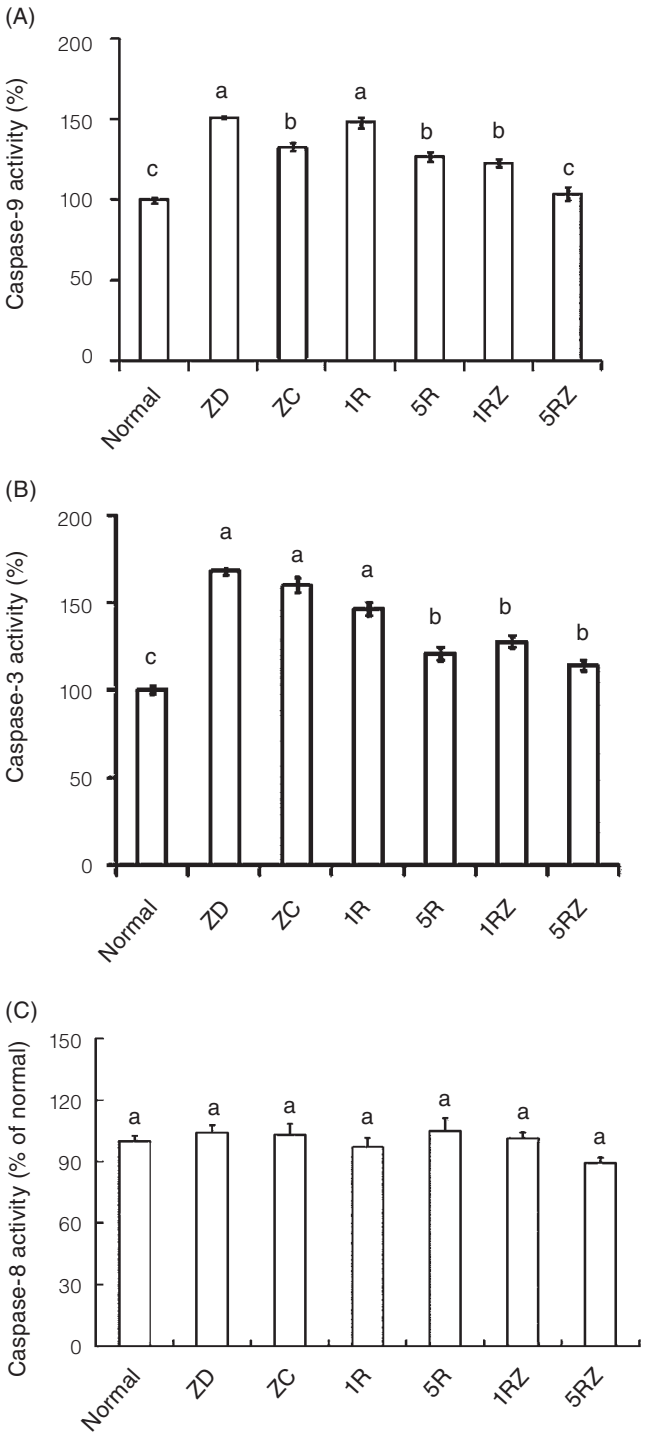

Figure 4. The inhibitory effects of RMR on caspase-3 (A), -9 (B), and -8 (C) in testis of ZD rats. Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). Different letters indicated significant difference ($p < 0.05$).

V. *Caspase-3, -8, and -9 Activities in The Testis*

ZD may generate apoptosis. Therefore, we further investigated the caspase activity in the testis of ZD rats. The caspase-3 and -9 activities in the testis of ZD rats are shown in Figure 4. The results show that the induction of ZD significantly increased caspase-3 and -9 activities significantly. However, RMR with or without ZC reduced these enzyme activities and thus prevented testicular apoptosis. In contrast, the caspase-8 activity did not significantly rise in the testis of ZD rats. These findings indicate that ZD induction resulted in testicular apoptosis via oxidative stress (mitochondrial pathway), and caspase activation. Therefore, RMR attenuated ROS production caused by ZD induction in the testis and protected against oxidative stress-induced apoptosis.

VI. *Serum Sex Hormone Levels*

Serum testosterone levels decrease and progesterone

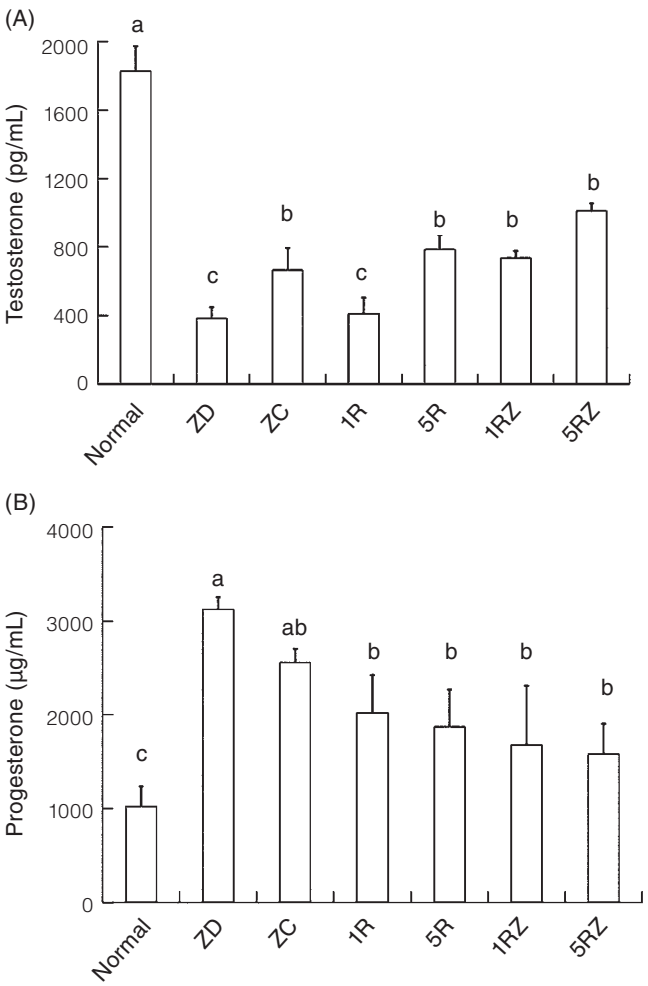

Figure 5. Effects of RMR on the testosterone and progesterone levels in serum of ZD rats. Each value is expressed as mean ± SD (n = 12). Rats were divided into normal, Zn deficiency (ZD), Zn compensation (ZC), 1X RMR (1R; 151 mg/kg), 5X RMR (5R; 755 mg/kg), 1RZ (1R + ZC), and 5RZ (5R + ZC). Different letters showed significant difference ($p < 0.05$).

Journal of Food and Drug Analysis, Vol. 19, No. 2, 2011

levels increase with age and in ZD patients[24]. Several traditional Chinese herbs such as *Ganoderma tsugae* and *Lycium barbarum* elevate testosterone production[25,26]. Testosterone levels increased in the 5R, 1RZ, and 5RZ groups compared to the levels in the ZD group (Figure 5A). Furthermore, the serum progesterone levels are shown in Figure 5B. These results indicate that the administration of RMR and Zn decreased the progesterone levels and increased the testosterone levels in the serum of ZD rats.

DISCUSSION

Testosterone affects spermatogenesis and regulates prostate growth[27], and studies have reported that the testosterone level is affected by serum Zn concentration[22,24]. Similarly, in our experiments, inducing ZD reduced the serum testosterone production in the ZD rats (Figure 5). ZD induction for 34 weeks induced testicular apoptosis[23]. Furthermore, low Zn concentrations in the serum and testis are harmful for spermatogenesis, spermatozoa maturation, and fertility[2,3]. Therefore, reduced spermatocyte levels were accompanied by testis atrophy and apoptosis in the ZD rats.

Suppression of the activities of antioxidant enzymes and ROS overproduction have been demonstrated in ZD rats[28]. Oxidative damage due to ROS is attenuated by antioxidative enzymes, including GPx, GR, GST, and CAT, which scavenge ROS[29,30]. There are 2 possible explanations for the antioxidative effects of RMR in the testis and prostate of ZD rats after RMR administration: (1) RMR directly scavenged ROS caused by ZD induction or (2) RMR indirectly inhibited oxidative stress by activating the antioxidative enzyme system.

Traditional Chinese herbal medicines have been reported to cure male infertility. Records show that these herbs effectively improve sperm motility from 34.0% to 46.0%, and significantly increase spermatozoa concentrations in infertile patients[31]. In addition, *Rubus coreanus* has been reported to increase sperm counts and motility in the New Zealand white rabbit[32]. Moreover, spermatogenesis and sperm counts are both elevated by food items, such as honey[33]. Antioxidants have been shown to improve human semen quality; this finding suggests that sperm activity is associated with antioxidation[34]. *L. barbarum*, *Cordyceps sinensis*, and soybean also affect testicular protection and sperm motility[35-37].

M. purpureus NTU 568-fermented rice reduced sperm dysfunction caused by Zn deficiency. In brief, the sperm-protective effect of RMR in the ZD rats can be attributed to its antioxidative activity in the testis. On the other hand, RMR increased the serum testosterone level, which suggests that RMR increases the serum Zn concentration. These results indicate that RMR facilitates a conversion between progesterone and testosterone. The RMR dose was calculated in accordance with Boyd's Formula of Body Surface Area, as recommended by the US Food and Drug Administration[38]. The recommended daily dose of a commercial *Monascus* product is 1.0 - 2.0 g for adults[39]. Therefore, the dose of 1 × RMR (151 mg/kg) used in this study was equivalent to 2 g for an adult 65 kg in weight and 170 cm in height. These dosages were used as a frame of reference for the dose formulation for an animal model. To conclude, RMR may serve as a supplement in adjuvant therapy for diseases associated with Zn deficiency.

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