

EFFECTS OF CPU 86017 (CHLOROBENZYL TETRAHYDROBERBERINE CHLORIDE) AND ITS ENANTIOMERS ON THYROTOXICOSIS-INDUCED OVERACTIVE ENDOTHELIN-1 SYSTEM AND OXIDATIVE STRESS IN RAT TESTES

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ABSTRACT

Objectives. To study the effects of CPU 86017, a berberine derivative, and its four enantiomers on thyrotoxicosis-induced oxidative stress and the excessive endothelin-1 system in rat testes.

Methods. Adult male SD rats were given high-dose L-thyroxin (0.2 mg/kg subcutaneously) once daily for 10 days to develop thyrotoxicosis. Subsets of the rats were treated with CPU 86017 or its four enantiomers (SR, SS, RS, and RR) once daily from day 6 to day 10. The alterations of redox, nitric oxide synthase, and endothelin-1 system in testes were examined by spectrophotometry and reverse transcriptase-polymerase chain reaction assay.

Results. After 10 days of high-dose L-thyroxin administration, increased mRNA expression of prepro-endothelin-1 and endothelin-converting enzyme was observed in the rat testes, accompanied by an elevated inducible nitric oxide synthase activity and oxidative stress. CPU 86017 and its enantiomer SR significantly improved these abnormalities.

Conclusions. High-dose L-thyroxin results in an overactive endothelin-1 system and oxidative stress in adult rat testis. CPU 86017 and its enantiomer SR suppressed the excessive ET-1 system by improving oxidative stress, and SR exhibited more potent efficacy than CPU 86017 and other enantiomers. *UROLOGY* **68**: 455–461, 2006. © 2006 Elsevier Inc.

Increasing evidence has suggested that an overactive endothelin-1 (ET-1) system, closely related to oxidative stress, plays an important role in the pathologic processes of many diseases.^{1–3} It has been confirmed that there is crosstalk between reactive oxygen species (ROS) and ET-1 under pathologic conditions. ROS and ET-1 interact with each other to form the ET-ROS pathway.¹ The overactive ET-1 system and oxidative stress are also present in some diseases that correlate with testicular dysfunction, including male hypogonad-

ism,⁴ erectile dysfunction,⁵ and diabetes-induced germ cell degeneration and apoptosis.⁶ The involvement of ET-1 and ROS in those diseases mentioned suggests that the ET-ROS pathway most likely takes part (at least partially) in the pathologic processes of testicular dysfunction, and its potential harm to the testis should not be ignored. Thyroxin increases ROS production by activating the respiratory chain of mitochondria,⁷ and the subsequent oxidative stress is the major reason for the tissue damage in thyrotoxicosis.⁸ A series of controlled clinical and experimental studies has indicated that male patients with thyrotoxicosis have a significant decrease in the sperm number and percentage of sperm forward progressive motility.^{9–11} Similar results were found in animals. In rabbits treated with high-dose thyroxin, sperm output and motility decreased significantly.¹² That a high level of thyroxin would increase the risk of testicular dysfunction makes sense, but additional understanding of the underlying mechanisms was

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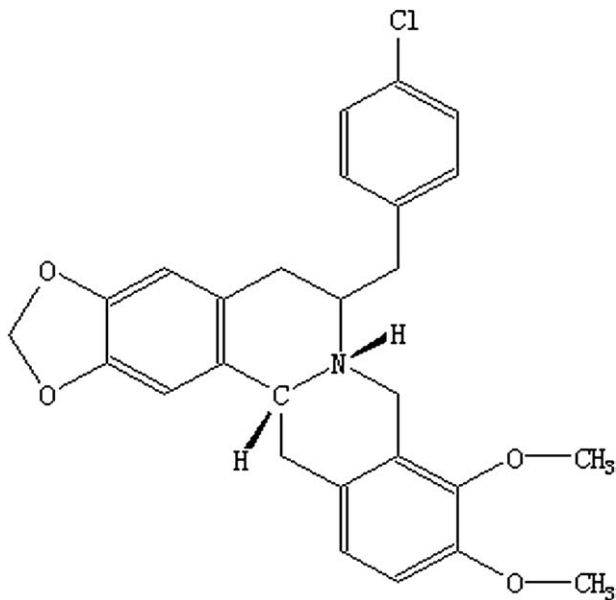


FIGURE 1. Chemical structure of CPU 86017.

not reported. Considering the role of the ET-ROS pathway in diseases relating to the testis, we hypothesized that the ET-1 system and ROS are also involved in thyrotoxicosis-induced testicular impairment and probably serve as important targets of drug therapy for testicular dysfunction.

CPU 86017 (chlorobenzyltetrahydroberberine chloride) is a berberine derivative (Fig. 1) and has two chiral centers: 7N and 13aC. Previous studies have demonstrated a potent antioxidant effect *in vivo* and *in vitro*¹³ and suppressed the overactive ET-ROS pathway in hypoxic pulmonary hypertension.¹⁴ To improve the solubility and availability of CPU 86017, its four enantiomers (7S,13aR-CPU 86017 [SR], 7S,13aS-CPU 86017 [SS], 7R,13aS-CPU 86017 [RS], and 7R,13aR-CPU 86017 [RR]) have been yielded through chiral separation. In the present study, we used high-dose L-thyroxin (0.2 mg/kg/day) to develop thyrotoxicosis in adult male rats. We intended to determine whether the ET-ROS pathway takes part in the testicular impairment induced by high-dose L-thyroxin and whether CPU 86017 and its four enantiomers could reduce the abnormalities of the ET-ROS pathway.

MATERIAL AND METHODS

ANIMALS AND TREATMENTS

CPU 86017 and its four enantiomers (SR, SS, RS, and RR) were obtained from the New Drug Center of China Pharmaceutical University. Fifty-six adult male SD rats weighing approximately 220 g were randomly divided into the following groups: control, thyrotoxicosis model, CPU 86017, SR, SS, RS, and RR (n = 8). Each rat was fed 15 g of chow daily and given free access to water. L-Thyroxin (Sigma), CPU 86017, and its enantiomers were suspended in 0.5% carboxymethylcellulose-sodium solution for administration. The rats of the thyrotoxicosis model (CPU 86017, SR, SS, RS, and RR groups)

were subcutaneously injected with L-thyroxin (0.2 mg/kg) once daily for 10 days. In addition, the rats in the CPU 86017, SR, SS, RS, and RR groups were treated, respectively, with CPU 86017 and the four enantiomers (4 mg/kg subcutaneously) once daily from day 6 to day 10. The control group was given vehicle. All procedures performed on the animals were conducted in accordance with the Animal Regulations of Jiangsu Province.

BIOCHEMISTRY ASSAYS

On the 11th day, the rats were killed by dislocation of the cervical vertebra, and the testes were removed and rinsed with 0.9% NaCl solution. One testis of each rat was used for biochemistry assay, and the other was stored in liquid nitrogen for reverse transcriptase-polymerase chain reaction assay.

The testes were homogenized in nine times the volume of ice-cold 0.9% NaCl solution, and the homogenates were centrifuged at 4000 rpm at 4°C for 10 minutes. Aliquots of the supernatants were collected for assays of xanthine oxidase (XOD), catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-px), constitutive nitric oxide synthase (cNOS), and inducible nitric oxide synthase (iNOS) activity and malondialdehyde (MDA) content. The protein content of the aliquots was determined using the Coomassie brilliant blue method with a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Previously reported methods were used for the XOD,¹⁵ CAT, SOD, and GSH-px,¹⁶ cNOS, and iNOS,¹⁷ and MDA¹⁸ assays.

In brief, XOD activity was reflected by a blue product produced by nitro-blue tetrazolium and superoxide that derived from the reaction of XOD and xanthine. CAT activity was determined from its ability to decompose hydrogen peroxide, and a yellow product produced by hydrogen peroxide and ammonium molybdate reflected CAT activity. The SOD activity assay was based on its inhibition on a red product derived from the reaction of hydroxylamine and superoxide. GSH-px activity was determined by the absorbance of a yellow product formed in the reaction between reduced glutathione and di-thiobisnitrobenzoic acid. NOS activity was determined by nicotinamide adenine dinucleotide phosphate oxidation, using L-arginine as a substrate. MDA content was determined using the thiobarbituric acid method.

PREPRO-ET-1, ECE, AND iNOS mRNA EXPRESSION

The extraction of RNA was done as previously reported.¹⁴ In brief, the whole testis was homogenized in TRIzol as fully as possible, and the homogenates were centrifuged (5000 rpm for 10 minutes at 4°C). The supernatants were collected and treated with chloroform and hydroxybenzene to remove protein, and RNA was precipitated in isopropanol and washed by ethanol. After reverse transcription, the cDNA was stored at -70°C before use.

The amount of amplified cDNA of prepro-ET-1, endothelin-converting enzyme (ECE), and iNOS was compared with that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers of prepro-ET-1 were sense, 5'-AGCAATAGCATCAAGGCATC-3'; and antisense, 5'-TCAGACACGAACACTCCCTA-3'. The primers of ECE were sense, 5'-CGTAGCGATAGTCTTAGCAC-3'; and antisense, 5'-GTGCCACACCAAACTACAG-3'. The primers of iNOS were sense, 5'-CTTCAGGTATGCGGTATTGG-3'; and antisense, 5'-CATGGTGAACACGTTCTTGG-3'. The primers of GAPDH were sense, 5'-GCTGGGGCTCACCTGAAGG-3'; and antisense, 5'-GGATGACCTTGCCCA-CAGCC-3'. The polymerase chain reaction experiment was performed in 25 μ L final volume with the following profile: predenaturation for 5 minutes at 94°C, denaturation for 40

seconds at 94°C, annealing for 40 seconds at 64°C for prepro-ET-1, 54°C for ECE, 57°C for iNOS, and 63°C for GAPDH, with extension for 1 minute at 72°C. The number of cycles for prepro-ET-1, ECE, iNOS, and GAPDH was 30, 30, 40, and 34, respectively, and a final extension step at 72°C for 10 minutes was performed after the last cycle. The polymerase chain reaction products were separated by gelose electrophoresis and stained with ethidium bromide before semiquantitative analysis (GDS8000, Syngene, England).

STATISTICAL ANALYSIS

Data are expressed as the mean \pm SD and were analyzed by one-way analysis of variance. Differences between the two groups were analyzed by Student-Newman-Keuls or Dunnett's test as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

SUPPRESSION ON OXIDATIVE STRESS IN TESTES

High-dose L-thyroxin resulted in oxidative stress in the testis, showing a redox disturbance. In the thyrotoxicosis model group, XOD activity increased by 39.9% ($P < 0.01$) compared with the control group. SR, RS, and CPU 86017 significantly decreased it by 22.2%, 27.2%, and 23.6% ($P < 0.05$), respectively, against the thyrotoxicosis model group (Fig. 2A).

CAT activity decreased in the thyrotoxicosis model group by 18.7% ($P < 0.05$) compared with the control group. SR and CPU 86017 increased it by 10.4% and 12.2% ($P < 0.05$), respectively, compared with the thyrotoxicosis model group (Fig. 2B).

L-Thyroxin sharply decreased SOD activity by 21.6% ($P < 0.01$) compared with the control group. Significant increases (19.4%, $P < 0.01$; 18.0%, $P < 0.05$; and 18.2%, $P < 0.05$, respectively) were achieved after SR, RS, and CPU 86017 administration compared with the thyrotoxicosis model group (Fig. 2C).

GSH-px activity decreased by 31.0% ($P < 0.01$) in the thyrotoxicosis model group compared with controls and increased by 22.8% and 21.6% ($P < 0.05$) in the SR and CPU 86017 groups compared with the thyrotoxicosis model group (Fig. 2D).

MDA content increased significantly in the thyrotoxicosis model group (82.8%, $P < 0.01$) compared with the control group and decreased by 36.0% ($P < 0.01$), 35.4% ($P < 0.05$), and 28.8% ($P < 0.05$), respectively, after SR, RR, and CPU 86017 treatment compared with the thyrotoxicosis model group (Fig. 2E).

EFFECTS ON NOS ACTIVITY IN TESTES

L-Thyroxin decreased cNOS activity by 30.8% ($P < 0.01$) compared with the controls. SR, RR, and CPU 86017 treatment restored it by 27.4% ($P < 0.01$), 13.2% ($P < 0.05$), and 18.0% ($P < 0.01$), respectively, compared with the thyrotoxicosis model group (Fig. 3A).

iNOS activity was significantly increased by 25.0%

($P < 0.01$) in the thyrotoxicosis model group compared with the control group. After SR, RS, and CPU 86017 treatment, it was decreased by 11.8% ($P < 0.05$), 10.5% ($P < 0.05$), and 10.0% ($P < 0.05$), respectively, compared with the model group (Fig. 3B).

INCREASE OF PREPRO-ET-1, ECE, AND iNOS mRNA EXPRESSION

Prepro-ET-1 mRNA expression in the thyrotoxicosis model group increased nearly 50% compared with the control group ($P < 0.01$) and was significantly decreased by SR ($P < 0.01$), RS ($P < 0.05$), and CPU 86017 ($P < 0.01$) (Fig. 4A).

mRNA expression of ECE in the thyrotoxicosis model group was increased by nearly 45% compared with the control group and decreased significantly after SR ($P < 0.05$), and CPU 86017 ($P < 0.05$) treatment (Fig. 4B).

L-Thyroxin increased iNOS mRNA expression by 90% compared with controls. SR ($P < 0.01$) and CPU 86017 ($P < 0.05$) decreased it significantly (Fig. 4C).

COMMENT

ET-1 is a 21-aminoacid peptide that is derived from prepro-ET-1 through two proteolytic reactions catalyzed by endopeptidase and ECE.¹⁹ ET_A and ET_B receptors mediate different functions of ET-1 (eg, vasoconstriction and mitogen activation),²⁰ and both of the two subtypes have been found in rat and human testes.²¹ Recent studies have confirmed the ET-ROS pathway by revealing the close correlation between ET-1 and ROS. Cheng *et al.*²² reported that ROS increase the ET-1 level in endothelial cells through the Ras/Raf/ERK signaling pathway, and Dong *et al.*²³ found that ET-1 increases ROS production by activating nicotinamide adenine dinucleotide phosphate oxidase in human umbilical vein endothelial cells. The ET-ROS pathway plays a role in various diseases.^{1,24} In the present study, a disturbed redox balance in rat testis was observed after high-dose L-thyroxin (0.2 mg/kg/day) treatment, accompanied by a significant increase in mRNA expression of prepro-ET-1 and ECE, indicating an overactive ET-ROS pathway with this pathologic condition. The redox status disturbance was induced by high-dose L-thyroxin, through its ability to increase energy consumption and facilitate electron leakage in the mitochondrial respiratory chain. As expected, an upregulation of the ET-1 system was observed. The beneficial effects of CPU 86017 and the enantiomers can be mainly attributed to their antioxidant activity by which an indirect suppression of the ET-1 system was achieved. A normal level of the ET-1 system may be very important for maintaining testicular function, because ET-1 has various functions in the rat and human testes. Studies

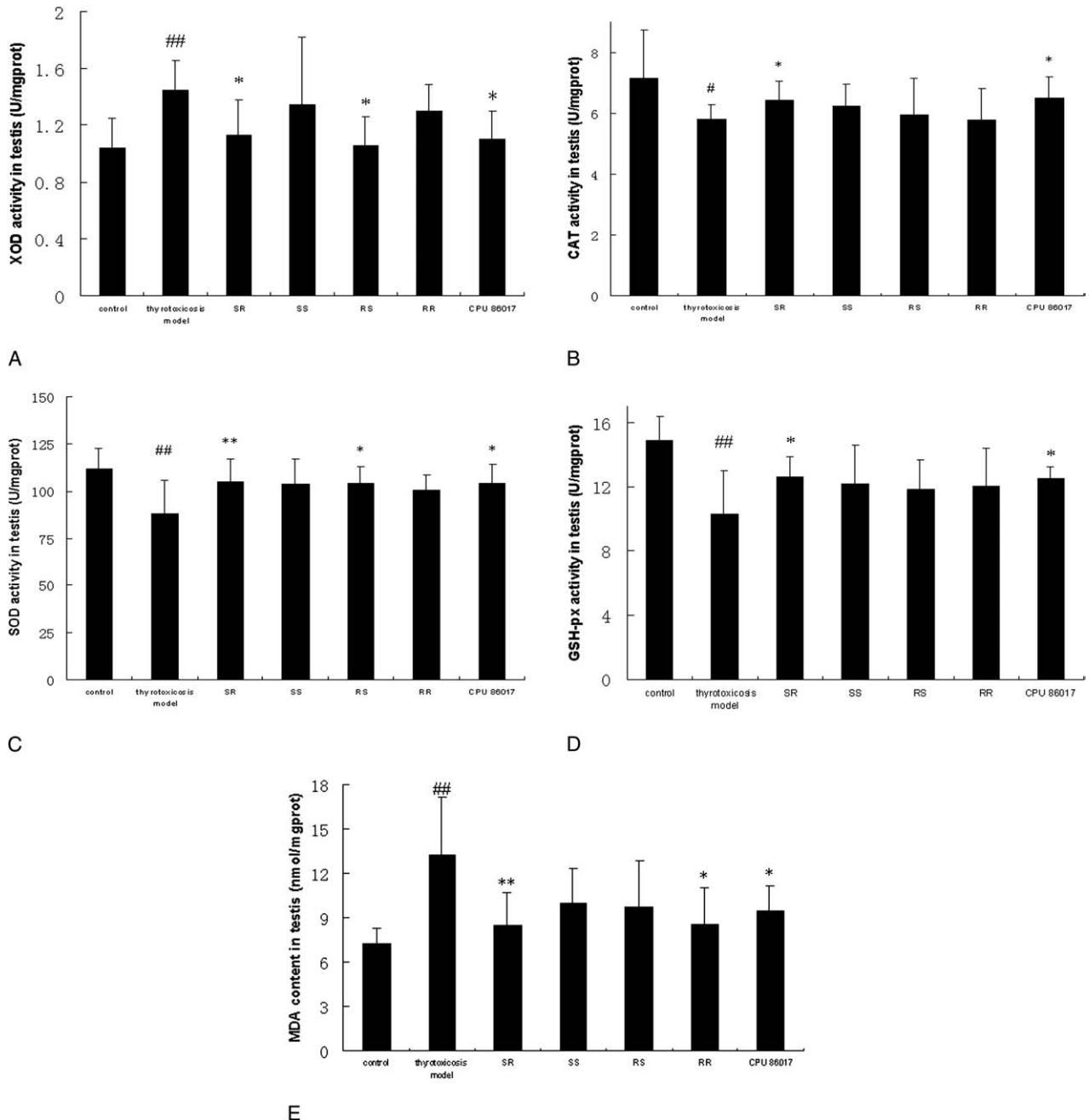
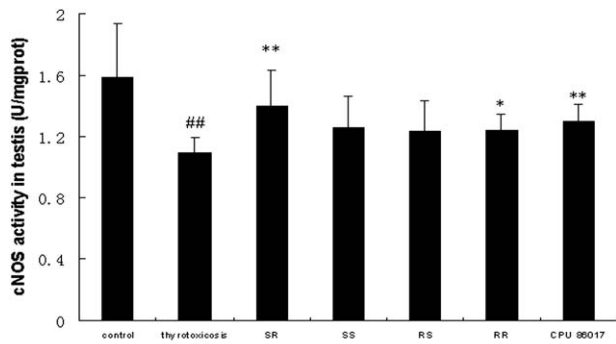
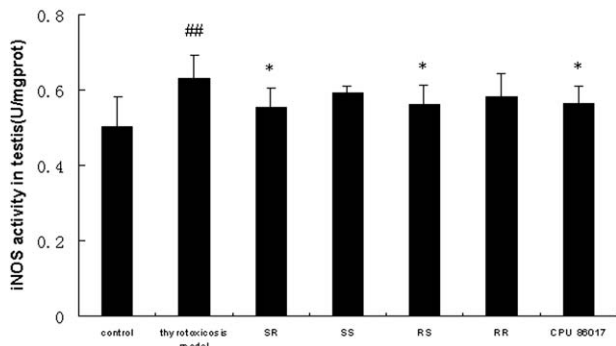


FIGURE 2. High-dose L-thyroxine administration (0.2 mg/kg subcutaneously) resulted in disturbed redox status in rat testes. Improvement to differing extents observed after treatment with CPU 86017 and its four enantiomers (4 mg/kg subcutaneously). #P <0.05 versus control, ##P <0.01 versus control, *P <0.05 versus thyrotoxicosis model, **P <0.01 versus thyrotoxicosis model (n = 8). (A) XOD activity in thyrotoxicosis model group increased by 39.9% compared with control group (P <0.01). SR, RS, and CPU 86017 significantly decreased it by 22.2%, 27.2%, and 23.6%, respectively, compared with thyrotoxicosis model (P <0.05). (B) CAT activity decreased in thyrotoxicosis model group by 18.7% compared with control group (P <0.05). SR and CPU 86017 effectively increased it by 10.4% and 12.2%, respectively, compared with thyrotoxicosis model (P <0.05). (C) SOD activity decreased in thyrotoxicosis model group by 21.6% compared with control. SR (P <0.01), RS (P <0.05), and CPU 86017 (P <0.05) significantly increased it by 19.4%, 18.0%, and 18.2%, respectively, compared with thyrotoxicosis model. (D) GSH-px activity decreased by 31.0% in thyrotoxicosis model group compared with control and increased by 22.8% (P <0.05) and 21.6% (P <0.05) with SR and CPU 86017 compared with thyrotoxicosis model group. (E) MDA content increased significantly in thyrotoxicosis model group by 82.8% compared with control and decreased by 36.0% (P <0.01), 35.4% (P <0.05), and 28.8% (P <0.05), respectively, with SR, RR, and CPU 86017 compared with thyrotoxicosis model group.



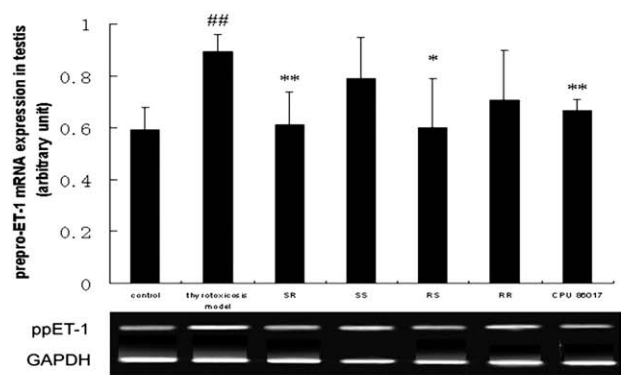
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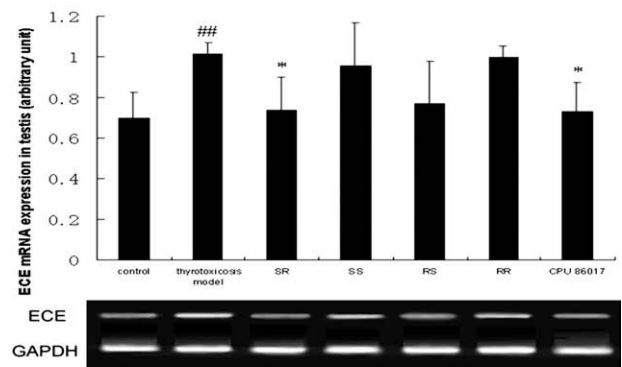
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FIGURE 3. High-dose L-thyroxin (0.2 mg/kg subcutaneously) resulted in increased iNOS and decreased cNOS activity in rat testes. CPU 86017 and its enantiomers (4 mg/kg subcutaneously) improved it to varying extents. ##P <0.01 versus control, *P <0.05 versus thyrotoxicosis model, and **P <0.01 versus thyrotoxicosis model (n = 8). (A) L-Thyroxin decreased cNOS activity by 30.8% compared with control. SR, RR, and CPU 86017 increased it by 27.4% (P <0.01), 13.2% (P <0.05), and 18.0% (P <0.01), respectively, compared with thyrotoxicosis model group. (B) iNOS activity increased significantly by 25.0% in thyrotoxicosis model group compared with control and decreased by 11.8% (P <0.05), 10.5% (P <0.05), and 10.0% (P <0.05), respectively, with SR, RS, and CPU 86017.

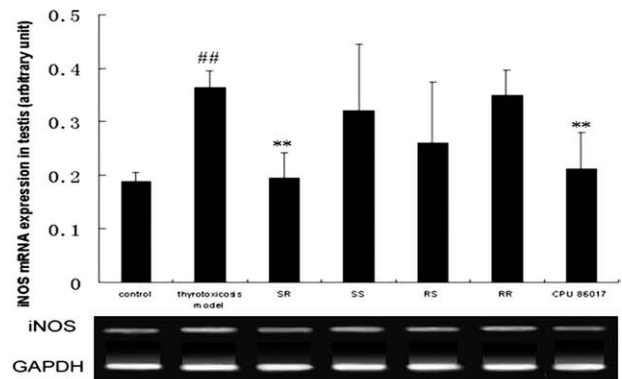
of transgenic rats overexpressing ET-1 have indicated that the blood flow in testicular capillaries is decreased, because of its most potent effect of vasoconstriction.²⁵ Additionally, the mitogenic effect of ET-1 promotes cell proliferation, leading to testicular microvessel remodeling.²⁶ The increased vessel wall thickness and decreased blood flow in the testis may result in the stress of hypoxia and ischemia, which is harmful to spermatogenesis and testosterone production. In normal conditions, Sertoli cells have a cyclic expression of ECE and pulsatile production of ET-1, which controls the rhythmic contractility of the seminiferous tubule wall to provide energy for sperm transport.²⁷ The overactive ET-1 system most likely disturbs the rhythmic ET-1 production and frustrates spermatozoa transport from the testis to the epididymis. Although the



A



B



C

FIGURE 4. mRNA expression of prepro-ET-1, ECE, and iNOS increased significantly after high-dose L-thyroxin administration and decreased with CPU 86017 and its enantiomers (4 mg/kg subcutaneously). ##P <0.01 versus control, *P <0.05 versus thyrotoxicosis model, and **P <0.01 versus thyrotoxicosis model (n = 8). (A) mRNA expression of prepro-ET-1 in thyrotoxicosis model group increased nearly 50% compared with control and was significantly decreased by SR, RS, and CPU 86017. (B) mRNA expression of ECE in thyrotoxicosis model group was increased nearly 45% compared with control group, and decreased significantly by SR, and CPU 86017. (C) mRNA expression of iNOS in thyrotoxicosis model group was increased by nearly 90% compared with control. SR and CPU 86017 significantly decreased it.

reports on male infertility caused by hyperthyroidism are very few, well-controlled clinical studies have indicated that hyperthyroidism results in alterations in sex steroid hormone metabolism, as well as spermatogenesis and fertility. Sperm motility is mainly affected. However, all these abnormalities are reversed after restoration of euthyroidism.²⁸ In clinics, patients with hyperthyroidism or thyrotoxicosis are usually treated with thyroid inhibitors, by which the triiodothyronine and thyroxine levels can be normalized and thus, testicular impairment may be subsequently relieved. However, the significant damage to male reproductive function may need a sustained elevation of thyroid hormones for a long period. These factors account for the few clinical reports on hyperthyroidism-induced male infertility. Despite this, the possibility that hyperthyroidism is a risk factor for male infertility and that ET-ROS plays a role in testicular dysfunction should not be excluded. The ET-ROS pathway is a likely new target of drug therapy for testicular diseases.

In this study, iNOS activity increased markedly and cNOS activity decreased after high-dose L-thyroxin treatment. Nitric oxide is a vasodilator produced in the endothelium and serves as an antagonist against ET-1. The decreased cNOS activity in the testes results in reduced nitric oxide production, which may facilitate the decrease in blood flow in testicular vessels, aggravating the ischemia. However, iNOS activation mainly happens under pathologic conditions, and most of the nitric oxide produced by iNOS is involved in producing peroxynitrite (ONOO⁻),²⁹ which serves as a more toxic free radical exacerbating oxidative stress. Thus, improvement of the NOS system in the testes by CPU 86017 and SR is beneficial for relieving the oxidative stress.

CONCLUSIONS

The results of our study have shown that high-dose L-thyroxin induces an excessive ET-ROS pathway in the adult rat testis. CPU 86017 improved the disturbed redox and ET-1 system, and SR possessed more potent efficacy than the other four enantiomers. It could be that hypogonadism in middle-aged and elderly populations is due to an upregulation of the ET-1 system and oxidative stress in the testis.

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