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Research Article
Study on the Regulation of Hypothalamic-Pituitary-Adrenal Axis (HPA Axis) in Rats with Kidney-Yin Deficiency Syndrome by the Raw and Salt-Water Processing of *Phellodendri chinensis* Cortex

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Abstract

Background and Objective: *Phellodendri chinensis* Cortex (PC) is a common medicine used in the clinic to treat Kidney-Yin deficiency syndrome (KYDS) in traditional Chinese medicine and has been proven to have clear efficacy in improving KYDS. Moreover, after salt-water processing of *Phellodendri chinensis* Cortex (SPC), enhancing its effect of nourishing Yin and tonifying the kidney. This study aims to better understand the efficacy mechanism of PC processed by salt-water in improving Kidney-Yin deficiency syndrome rats. **Materials and Methods:** In this study, a rat KYDS model was prepared by short-term high-dose gavage of hydrocortisone and pharmacodynamic experiments with multidimensional indicators such as macroscopic signs, HPA axis-related hormone levels, histological lesions, inflammatory factors and immune function, combined with ELISA, RT-PCR, western-blot, immunohistochemical staining, were used to investigate the effects of the raw and salt-water processing of PC. **Results:** After treatment of different processed products of PC, the symptoms of KYDS were relieved to different degrees in rats of each dosing group, among which the best treatment effect was the closest in the group of SPC and the positive control group, the anti-inflammatory, immunomodulatory and regulatory effects on hypothalamus and adrenal tissue were enhanced after the salt-water processing of PC. The salt-water processing of PC improved the inhibition of HPA axis function in rats with exogenous high-dose hydrocortisone. **Conclusion:** Both the raw product and the salt-water processing product of PC improved the inhibition of HPA axis function in rats with exogenous high dose of hydrocortisone, the therapeutic effect of PC on KYDS can be enhanced by salt-water processing.

Key words: *Phellodendri chinensis* Cortex, salt-water processing, Kidney-Yin deficiency, HPA axis

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phellodendri chinensis Cortex (PC), also known as Huangbai in Chinese, is the dried bark of the *Phellodendron chinense* Schneid (family Rutaceae) and has been used for thousands of years in traditional Chinese medicine clinical practice[1]. The PC is known for its ability to clear heat and dry dampness, purge fire, relieve steaming and remove toxins[2], making it effective in treating various afflictions[3] such as diarrhea, dysentery, jaundice, dark urine, abnormal vaginal discharge pudendal itching and seminal emission[4]. In Chinese Material Medica (CMM) theory, CMM needs to be processed into Chinese herbal decoction pieces before they can be used clinically. This processing allows them to reduce toxicity and increase efficacy, changing their medicinal properties to meet the needs of clinical treatment better. The salt-water processing method is a common method of CMM processing. According to the theory of CMM processing is currently only described phenomenologically and there is not enough literature to clarify the corresponding principles. In this essay, the theory of salt-water processing will be analyzed using PC as a representative medicine. In the traditional theory and experience of CMM, PC is often processed with salt water (SPC). Compared to raw PC (RPC), SPC has the ability to lead the drug into the kidney and has an enhanced effect of nourishing the yin and purging the fire of the kidney. To confirm these traditional theories of CMM processing, Hypothalamic-Pituitary-Adrenal axis (HPA axis) regulation experiments were carried out in rats with Kidney-Yin deficiency syndrome (KYDS) in this study.

Modern research of traditional Chinese medicine shows that the changes in clinical symptoms of Kidney-Yin deficiency are closely related to the dysfunction of the HPA axis[5,6]. The HPA axis is one of the important neuroendocrine function axes composed of the hypothalamus, pituitary gland and adrenal gland to maintain the basic life activities of the human body. The paraventricular nucleus of the hypothalamus secretes adrenocorticotropin-releasing hormone, which reaches the adenohypophysis through the pituitary portal system, binds with Corticotropin-Releasing Hormone Receptor-1 (CRHR-1) on the membrane of Adrenocorticotropic Hormone (ACTH), promotes the secretion of ACTH by the adenohypophysis and acts on adrenal cortical cells. This promotes the proliferation of adrenal cortex and the synthesis and secretion of cortisol in the fasciculata. Cortisol participates in the body's metabolism, immunity and growth and development process and plays an important role in maintaining the body's homeostasis[7,8].

Previous studies have shown that PC can increase body weight, thymus index, spleen index, 24 hrs urine volume, cAMP content and cAMP/cGMP ratio, while reducing serum FT3, FT4, cGMP content and urine 17-OHCS content in rats with Kidney-Yin deficiency with hyperthyroidism[9]. These effects nourish yin and reduce fire, with SPC being more effective than RPC. Pharmacodynamics experiments were conducted using ELISA, RT-PCR, western-blot, immunohistochemical staining and other techniques to study the therapeutic effect of RPC and SPC. This comprehensive approach aims to better understand the efficacy mechanism of PC processed by salt-water in improving Kidney-Yin deficiency syndrome rats.

MATERIALS AND METHODS

Study area: The study was carried out from October 2021 to October 2022, in the Teaching and Research Office of the Department of Medicine at Liaoning University of Traditional Chinese Medicine.

Preparation of RPC and SPC and their gavage solution: *Phellodendri chinensis* Cortex (about 1000 g) was collected from Ya'an City, Sichuan Province and identified as the dried bark of *Phellodendron chinense* Schneid, family Rutaceae, according to the 2020 edition of the Chinese Pharmacopoeia. Voucher specimens have been deposited in the herbarium of CMM Processing Technology Innovation Center of Liaoning Province, Liaoning University of Traditional Chinese Medicine. To prepare RPC, an appropriate amount of *Phellodendri chinensis* Cortex was washed, moistened thoroughly, cut into shreds, dried and obtained. To prepare SPC, an appropriate amount of RPC was mixed well with salt-water (using 2 g salt for every 100 g of RPC) in an appropriate container and moistened for 2 hrs, so that the auxiliary salt-water was absorbed by RPC. The mixture was then fried in a roasting container at 150-160°C for 5-6 min, cooled and obtained.

In order to prepare the gavage solution for rats, RPC and SPC were individually decocted twice with 10 times the volume of water or 60 min each round and the resulting filtrates were combined separately. The gavage concentration of RPC and SPC in the low dose and high dose groups was 0.11 and 0.96 g/mL, respectively, whereas the gavage concentration of Liuwei Dihuang pill (positive control group) was 0.02 g/mL[6].

Animals: A total of 70 healthy adult male SD rats aged 6-8 weeks, weighing 180~220 g, were provided by Liaoning Changsheng Biotechnology Co., LTD., with the certificate NO. SCXK (Liao) 2021-0001. The rats were adaptively reared for 7 days in the laboratory with 30-50% humidity and a room temperature of 25°C with free access to diet and water.

Establishment of KYDS animal model and method of drug administration: A total of 70 male rats were randomly divided into seven groups with ten rats in each group, which were: Blank control group, model control group, positive control group, low dose group of RPC, high dose group of RPC, low dose group of SPC and high dose group of SPC.

The rats were given 1 mL/100 g of prepared gavage solution once daily for each experimental group. The blank control group and the model control group were given the same dose of saline for ten days. On the eleventh day of gavage administration, all groups except the blank control group were given hydrocortisone at a concentration of 50 mg/L in a dose of 1 mL/100 g. The blank control group was given the same dose of physiological saline and continued to be gavaged for four days[6].

Measurement of body temperature and organ indices of the thymus, spleen and kidney of rats in each experimental group: Before the experiment, the animals were weighed and their anal temperature was measured. During the experiment, the body weight was measured once every two days and the anal temperature was measured once every five days. At the same time, the body weight of each group was recorded before death. After anesthesia, the thymus, spleen and kidney tissues were quickly removed and cleaned twice with normal saline in an ice bath. After the water in the above organs was drained with filter paper, the weight was measured and the organ index of each organ was calculated.

ELISA assay: Rats were administered with moulding and 24 hrs later, they were anaesthetized with intraperitoneal injection of uratan solution (20 g/100 mL). The rats were dissected about 2 cm along the abdominal midline and blood was collected through the abdominal aorta using a medical blood collection tube. The blood was left to stand for 30 min at room temperature, centrifuged at 3500 rpm for 15 min and the supernatant was collected. The corresponding indexes of cAMP, cGMP, CRH, ACTH, corticosterone (Cor.), IL-2, IL-6, IL-10, TNF-α, INF-γ and T in the rat's blood were measured according to the instructions in the ELISA kit.

Histopathological observation of the adrenal gland and pituitary gland: After blood collection, the adrenal and pituitary tissues of the rats were immediately dissected, washed twice with normal saline in an ice bath and fixed with 4% paraformaldehyde solution. Hematoxylin and Eosin (H&E) staining was performed and the adrenal and pituitary gland histopathological changes were observed under a light microscope (Lot: CX31RTSF, Olympus Corporation, Japan) after paraffin embedding and sectioning.

Immunohistochemical assays for adrenal histones: Immunohistochemical staining was used to observe the expression of adrenal Bax, Bcl-2, caspase 3, Fas and FasL proteins. The tissue was fixed using (pH 7.0~7.6) 4% paraformaldehyde-0.1M PBS for 60 min, rinsed 2 min 4 times using PBS solution, dehydrated at 5°C, routinely paraffin-embedded and sectioned at 4 μm thickness on slides treated with anti-desquamation agent poly-lysine. The sections were retrieved and placed in an oven at 60°C and baked for 60 min to make the sections tightly adherent. The sections were routinely de-waxed and rinsed twice for 5 min in phosphate buffer (PBS 0.01 M, pH 7.4). The sections were mixed with 1 part of freshly prepared 3% H_2O_2 and 10 parts of steaming water for 5 min at room temperature to inactivate the endogenous enzymes. The sections were washed 3 times with steaming water for 2 min. For thermal repair of antigen, the sections were immersed in 0.01 M citrate (pH 6.0), heated to boiling in a wave oven and then disconnected. The sections were cooled and washed 2 times with PBS (pH 7.2-7.6) and left at room temperature for about 5 min. The sections were washed 3 times with PBS (pH 7.2-7.6). About 5% BSA blocking solution was added dropwise for about 20 min at room temperature and excess was shaken off the sections without washing. The primary antibody was appropriately diluted and added dropwise (labeled at 37°C for 1 hr or overnight at 4°C). The sections were washed 3 times with PBS (pH 7.2~7.6). Biotinylated goat anti-mouse IgG was added dropwise at 30°C for 20 min and the sections were washed for 5 min with PBS (pH 7.2~7.6) 4 times. The DAB colour development was performed by taking 1 mL of distilled water, adding one drop each of reagents A, B and C to the DAB kit, mixing well and adding to the cut pieces. The pieces were reacted at 37°C for 20 min and then washed with distilled water. The slices were dehydrated, cleared and sealed with resin glue.

RT-PCR assay for CRH mRNA expression in the hypothalamus: As 24 hrs after the last administration, the rats were decapitated in an ice bath and the hypothalamus was quickly removed and placed in a frozen

Table 1: Table of primer sequence

Primer name	Primer sequences	Primer length (bp)
CRH	Upstream: 5'-GATCTCACCTTCCACCTTCTG-3'	1196
	Downstream: 5'-CGCAACATTTCATTTCCCGATA-3'	
β-actin	Upstream: 5'-CCTGTGGCATCCATGAAACTAC-3'	223
	Downstream: 5'-CTTCTGCATCCTGTCAGCGAT-3'	

storage tube and immediately fixed in liquid nitrogen. Total RNA was extracted with an RNAex kit (Lot: AG21102, Guangzhou Aikerui Medical Biotechnology Co., Ltd., Guangzhou Province, China) and the reverse transcription was performed in the 10 μL reverse transcription reaction system. The reverse transcription product was 3 μL and added into the 7 μL amplification reaction system (the upper and lower primers were 1 μL each, PCR reaction was performed in reverse transcription kit, 5 μL). The PCR reaction was performed with 95°C for 10 min denaturation, 60°C for 1 min annealing, 60°C 1 min extension, 40 cycles. The primer design was shown in Table 1.

Western blot assay was conducted to determine the expression of CRHR1 and CRHR2 proteins in the hypothalamus: After euthanizing the rats, the hypothalamic tissue was quickly removed, washed with saline in a 4°C ice bath, blotted dry on filter paper and weighed. The tissue was then lysed using pre-chilled RIPA lysis solution with PMSF protease inhibitor at a ratio of 500 μL of prepared RIPA per 50 mg of tissue. The lysate was added to the pre-cooled grinding tank, ground in a tissue grinder (2000 rpm, 1 min) and then lysed on ice for 30 min. The lysate was then centrifuged (4°C, 12000 rpm, 30 min) and the supernatant was collected. Protein quantification was performed using the Bradford method[6].

The proteins were separated by electrophoresis on 10% SDS-PAGE separator gel and 5% SDS-PAGE concentrate gel at a constant pressure of 90 V until bromophenol blue reached the bottom of the gel. The proteins were then transferred to PVDF membranes, which were soaked in methanol for 10s to activate them and electrophoresed at a constant current of 350 mA for 90 min. After electrophoresis, the PVDF membranes were rinsed in TBST for 5 min at room temperature on a shaker and then 5% skimmed milk powder solution prepared in TBST was added to block the membranes for 1 hr at room temperature on a shaker (Lot: SL-62508, Haimen Kylin-Bell Lab Instruments Co., Ltd., Jiangsu Province, China).

The CRHR1 antibody (1:1000), Rabbit Anti-CRHR2 (1:1000) and the internal reference antibody Anti-GAPDH Rabbit (1:1000) were added and incubated overnight at 4°C in a shaker. The next day, the membranes were rinsed 3 times for 5 min each time at room temperature in TBST and then the secondary antibody HRP-coupled Goat Anti-Rabbit igG (1:5000) was added and incubated for 1 hr at room temperature on a shaker. The membranes were then rinsed 5 times for 5 min each time on a TBST shaker. The ECL luminescence solution (1:1) was prepared, placed in a dark box and then dropped on the membrane. The membrane was imaged using a machine exposure and the expression was analyzed using ImageJ software (version 1.42 National Institutes of Health, Bethesda, Maryland, USA). The GAPDH was used as the internal reference and the relative expression was calculated.

Ethical consideration: The experimental protocol and procedures were approved by Animal Ethics Committee of the Affiliated Hospital of Liaoning University of TCM, with the certificate of approval No. 2019YS(DW)-029-01. Mice were obtained from the Liaoning Changsheng Biotechnology Co., ltd., with the certificate No. SCXK (Liao) 2021-0001.

Statistical analysis: Statistical analysis was performed using SPSS 16.0 software. Data are expressed as ($x \pm s$) and One-way Analysis of Variance (ANOVA) with LSD test was used for multiple comparisons between groups. A p-value of less than 0.05 was considered statistically significant.

RESULTS

The rats in the control group exhibited significantly larger body size, dry and smooth fur and good mental condition compared to the rats in the model and dosing groups. The rats in the model and dosing groups gradually developed symptoms of Kidney-Yin deficiency, such as decreased body weight, increased anal temperature, hair loss, emotional irritability, piling up and arching of the back, dry stools, yellow and red urine and significantly reduced water intake and activity.

Weight change results of rats in each experimental group: Figure 1 shows the trend of body weight change in each group within 15 days. The body weight of rats in the control group exhibited an overall increasing trend. Before the modeling, the body weight of rats in each group increased

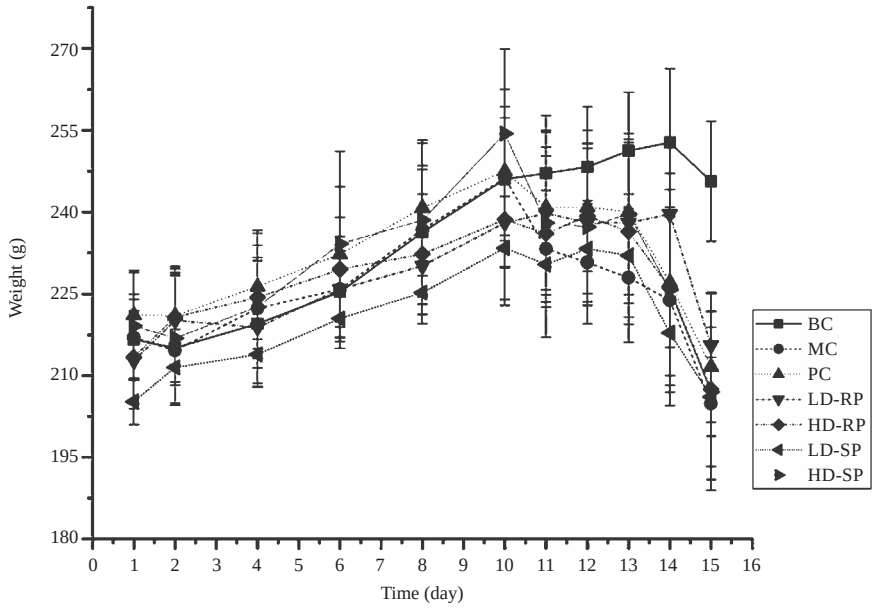

Fig. 1: Trend of body weight change in each group in 15 days

BC: Blank control group, MC: Model control group, PC: Positive control group, LD-RP: Low dose group of RPC, HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

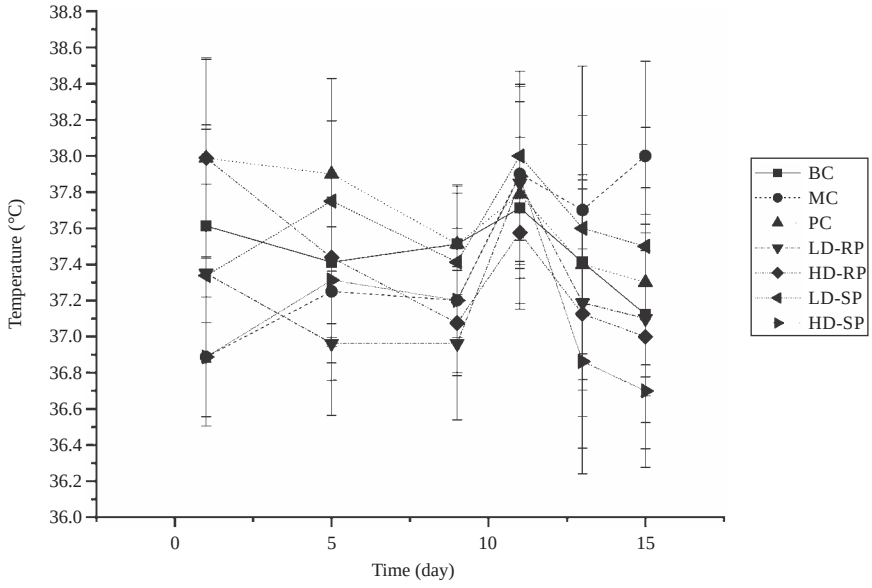

Fig. 2: Trend of anal temperature change of rats in each group in 15 days

BC: Blank control group, MC: Model control group, PC: Positive control group, LD-RP: Low dose group of RPC, HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

steadily for the first 10 days. On the 11th day, the first day of hydrocortisone modeling, the body weight of rats in each group showed an overall decreasing trend. The model control group exhibited the most significant decrease, followed by the SPC group and the RPC group, while the positive control group showed the least decrease.

Anal temperature change results of rats in each experimental group: Figure 2 shows the trend of anal temperature change of rats in each group within 15 days. Throughout the experiment, the change in anal temperature of rats in the control group was not significant. In the first 10 days, the anal temperature of rats in the high dose group

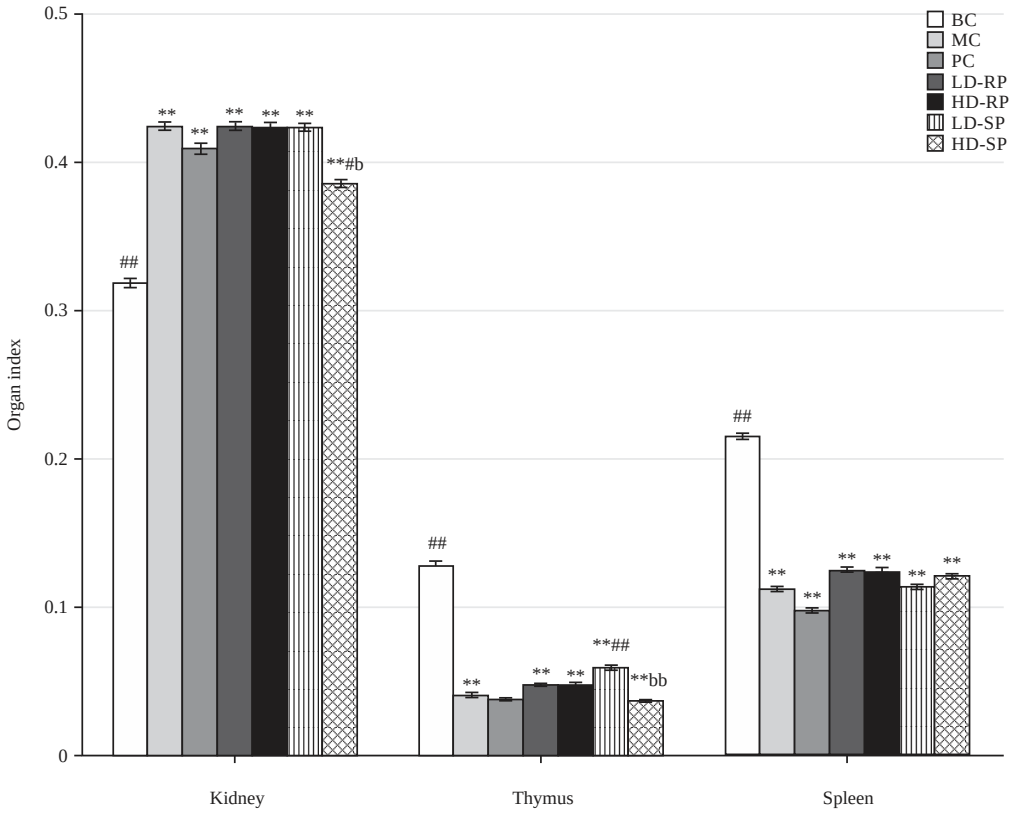

Fig. 3: Kidney, spleen and thymus organ indices of rats in each experimental group in 15 days

Compared with the blank control group, *p<0.05, **p< 0.01, compared with the model control group, #p<0.05, ##p<0.01, compared with the low dose group of SPC, ᵃp<0.05, ᵃᵃp<0.01, compared with the high dose group of SPC, ᵇp<0.05 and ᵇᵇp<0.01

of SPC, the low dose group of RPC and the positive control group gradually decreased, while the trend of anal temperature in the other groups was not significant. On the 11th day, the anal temperature of rats in all dosing groups increased and it decreased to varying degrees on the 12th to 15th day in all dosing groups except the model control group. The SPC group exhibited a greater decrease in anal temperature than the RPC group and the positive control group.

Organ indices results of rats in each experimental group: Figure 3 displays the organ indices of the kidney, spleen and thymus of rats in each administration group within 15 days. The thymus of rats in the model control group was observed to be atrophied and unshaped by the naked eye after dissection, while the thymus morphology of rats in each dosing group improved to varying degrees.

In terms of renal indices, compared to the blank control group, the renal organ index of other groups increased to varying degrees. The renal indices of the model control group were the highest (p<0.01), followed by the RPC group, low dose group of SPC and positive control group. The renal index of the high dose group of SPC was the lowest (p<0.05). The thymus index and spleen index of rats in each group decreased to varying degrees compared to the blank control group. The thymus index and spleen index of rats in the model control group were the lowest (p<0.01) and the thymus index of rats in the low dose group of SPC was the highest (p<0.05). There was no significant difference in the thymus index among the other groups.

Results of ELISA assay: Compared to the model control group, the contents of CRH, Cor. and ACTH in the serum of the other drug groups decreased to varying degrees. The content of CRH in the positive control group was the most significant (p<0.01), followed by the high dose group of SPC and the content of CRH in the RPC group was the lowest. The content of Cor. in the RPC group was the lowest and lower than that in the blank control group. The content of Cor. in the positive control group and SPC group was similar. The positive control group had the lowest ACTH content and the SPC group was lower than the RPC group under the same dose comparison.

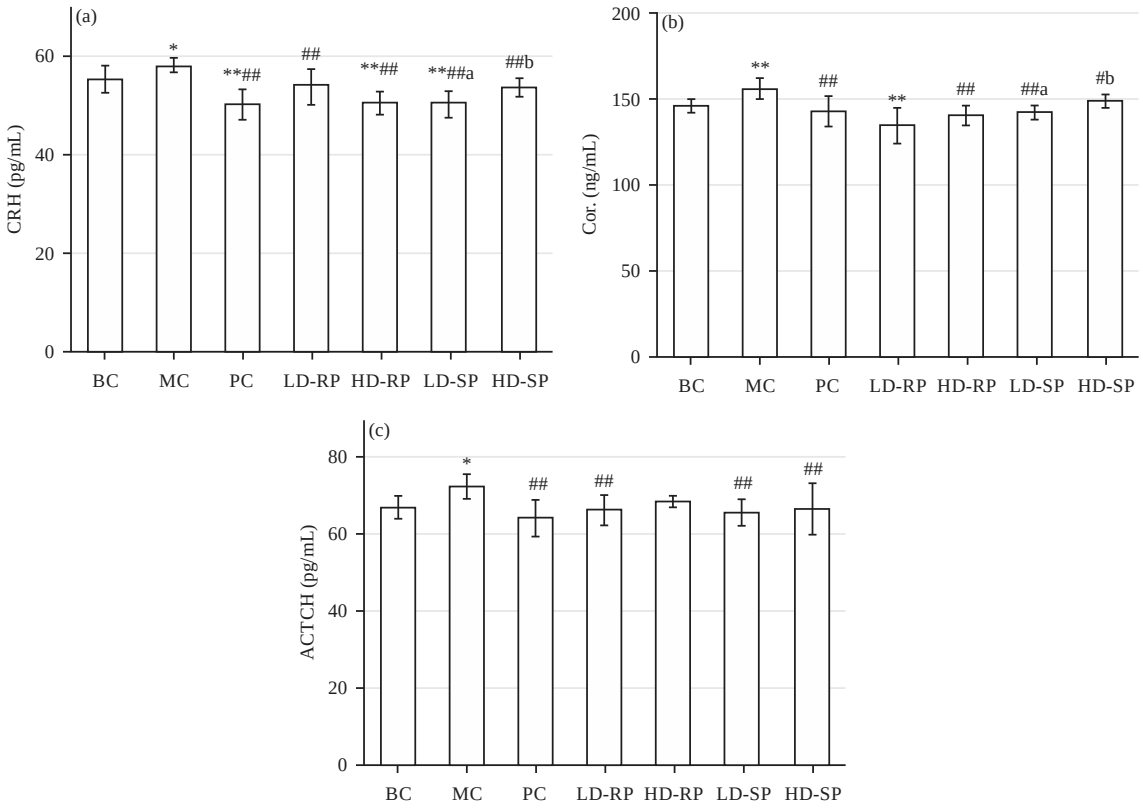

Fig. 4(a-c): The contents of CRH, Cor. and ACTH in the serum of rats in each experimental group

BC: Blank control group, MC: Model control group, PC: Positive control group, LD-RP: Low dose group of RPC, HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC, HD-SP: High dose group of SPC and A: CRH B: Cor. C: ACTH

The therapeutic effect of the SPC group was the closest to the positive control group and better than other drug administration groups. The changes in the above indexes further proved that PC enhanced its Yin-nourishing effect after salt-water processing. The contents of CRH, Cor. and ACTH in the serum of rats were shown in Fig. 4, respectively. The contents of CRH in the serum of rats in each experimental group is shown in the picture A, the contents of Cor. in the serum of rats in each experimental group is shown in the picture B, the contents of ACTH in the serum of rats in each experimental group is shown in the picture C.

Compared to the blank control group, the contents of IL-2 and IL-6 in the serum of rats in the model control group significantly increased ($p<0.05$), while the content of IL-10 significantly decreased ($p<0.05$). The contents of IL-2 and IL-6 in other drug administration groups decreased to varying degrees. Compared to the model control group, the contents of IL-2 and IL-6 in each drug administration group decreased to varying degrees. The content of IL-2 in the low dose group of RPC was the lowest ($p<0.01$). The content of IL-6 increased gradually in the positive control group ($p<0.01$), high dose group of RPC ($p<0.05$), high dose group of SPC ($p<0.05$) and low dose group of SPC ($p<0.05$) and the content of IL-6 was the lowest in the high dose group of SPC ($p<0.01$). The contents of the low dose group of RPC ($p<0.01$), low dose group of SPC ($p<0.05$), high dose group of SPC ($p<0.05$) and positive control group ($p<0.05$) gradually increased.

Compared to the blank control group, the contents of IFN-γ and TNF-α in the serum of rats in the model control group significantly increased ($p<0.01$), while the content of T was not significantly changed. The content of TNF-α in other groups significantly decreased ($p<0.01$) and the content of T in the positive control group and high dose group of SPC significantly increased ($p<0.01$). The contents of IFN-γ and TNF-α in the serum of rats in all drug administration groups decreased to varying degrees. The contents of IFN-γ were the lowest in the high dose group of SPC ($p<0.01$), while the contents of TNF-α in other drug administration groups significantly decreased ($p<0.01$) but the differences were not obvious. The content of T in the positive control group and high dose group of SPC significantly increased ($p<0.01$) and the content of T in the SPC group significantly increased under

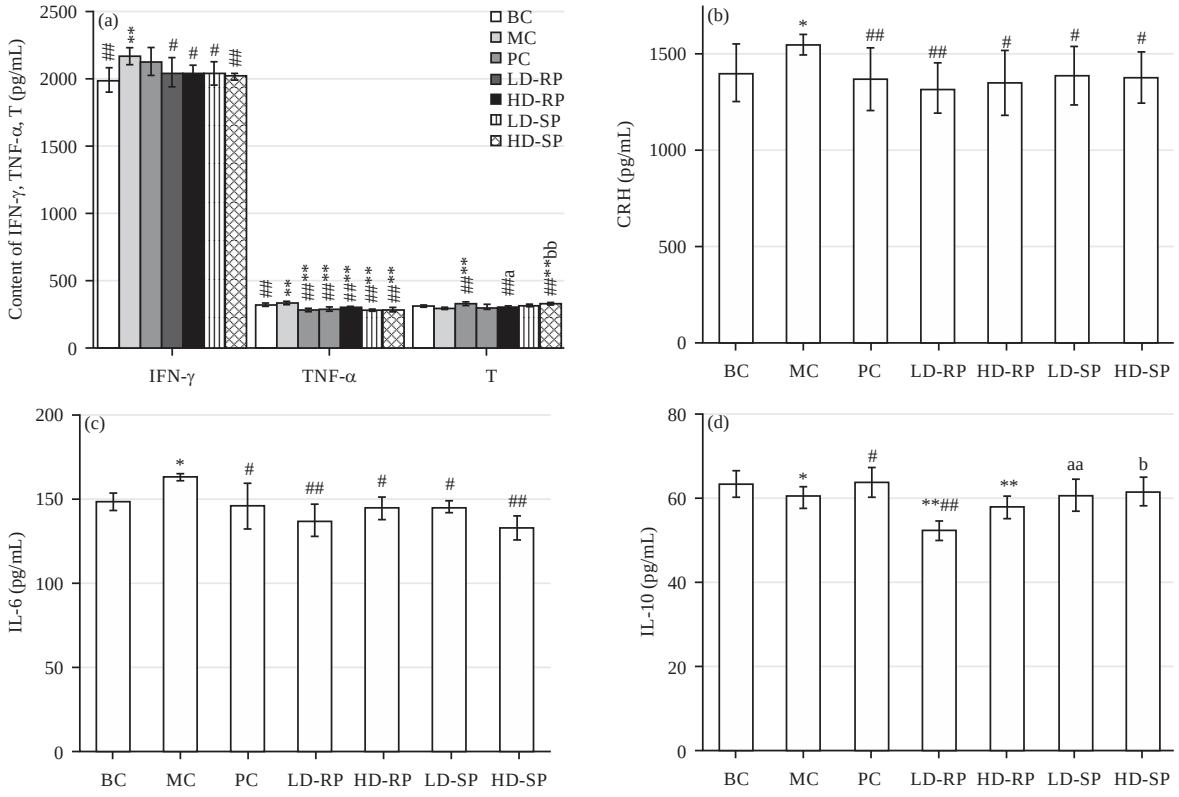

Fig. 5(a-d): Contents of TNF-α, IFN-γ, T, IL-2, IL-6 and IL-10 in rats in each experimental group, (a) Serum TNF-α, IFN-γ and T contents, (b) Serum IL-2 content, (c) Serum IL-6 content and (d) Serum IL-10 content

BC: Blank control group MC: Model control group PC: Positive control group LD-RP: Low dose group of RPC HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

the same dose condition. The above results indicated that all drug administration groups could regulate the relevant indexes in the serum of Kidney-Yin deficiency model rats and then improve thyroid function. The contents of TNF-α, IFN-γ, T, IL-2, IL-6 and IL-10 in rats were shown in Fig. 5. The contents of TNF-α, IFN-γ and T in the serum of rats in each experimental group is shown in the picture A, the contents of IL-2 in the serum of rats in each experimental group is shown in the picture B; the contents of IL-6 in the serum of rats in each experimental group is shown in the picture C and the contents of IL-10 in the serum of rats in each experimental group is shown in the picture D.

Pathological results of adrenal and pituitary tissues: In the blank control group, the adrenal cortex exhibited clear structures of globular zone, fascicular zone and reticular zone, with smooth nuclear membrane, visible nucleoli and lipid-like vacuoles. In contrast, the adrenal cortex of the model control group was thinned, with narrowed globular and fascicular zones, unclear structure, irregular cell arrangement, reduced volume, vacuolated cytoplasm, widened intercellular space and local necrosis and inflammatory infiltration. The positive control group showed a clear structure of globular zone, fascicular zone and reticular zone, with orderly arranged cells and occasional inflammatory infiltration, which was closest to the blank control group. Under the same dosage condition, the morphology of SPC was better than that of RPC. Figure 6(a-g) shows the H&E staining of rat adrenal tissue.

In the blank control group, the pituitary gland was divided into anterior lobe and posterior lobe under the microscope. The anterior lobe was composed of many glands and the glandular epithelium had eosinophils, basophils and chromophobe cells. The posterior lobe was composed of nerve fibers and a few pituitary cells, with uniformly arranged eosinophils, basophils and neutrophils and well-defined boundaries without any abnormalities. Compared with the blank control group, the model control group showed increased eosinophils and decreased basophils with vacuolar degeneration in pituitary tissue. The boundary of pituitary tissue cells in the positive control group was not very clear but it was close to the normal group, with increased eosinophils and decreased basophils. There was a certain degree of

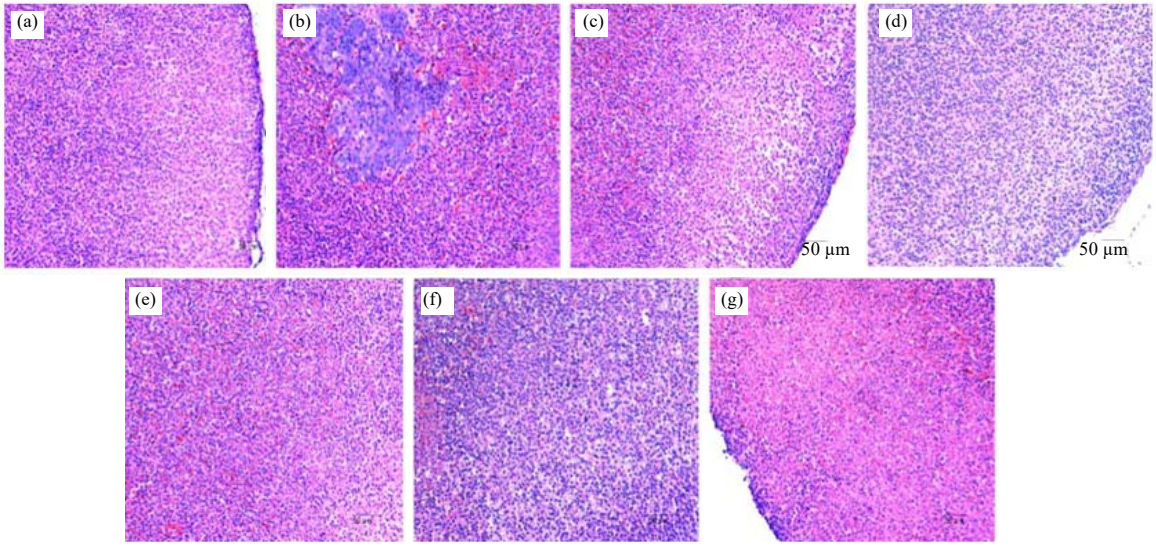

Fig. 6(a-g): H&E staining of adrenal tissue of rats in each experimental group (×100), (a) Blank control group, (b) Model control group, (c) Positive control group, (d) Low dose group of RPC, (e) High dose group of RPC, (f) Low dose group of SPC and (g) High dose group of SPC

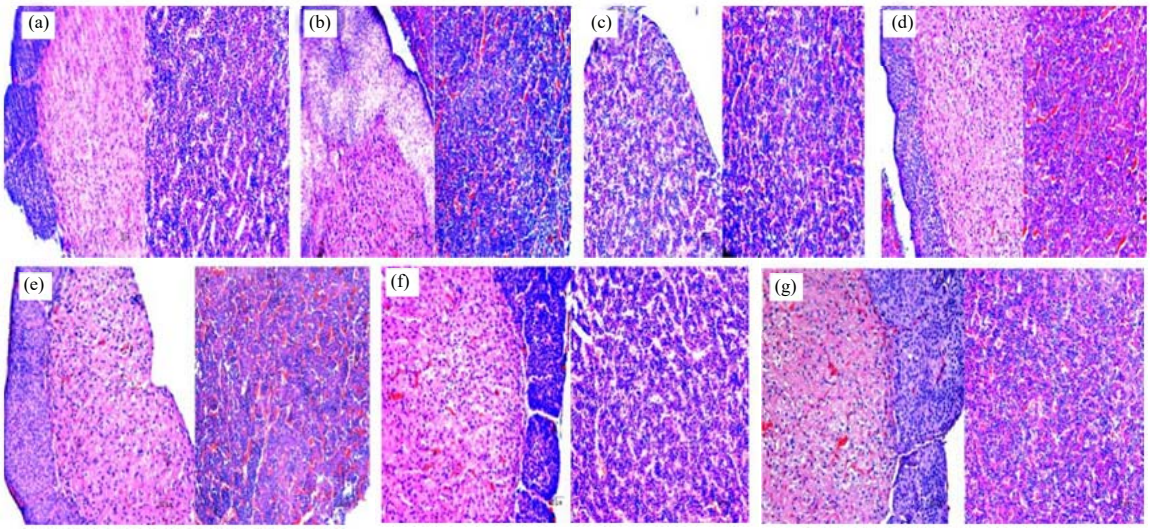

Fig. 7(a-g): H&E staining of pituitary tissue of rats in each experimental group (×100), (a) Blank control group, (b) Model control group, (c) Positive control group, (d) Low dose group of RPC, (e) High dose group of RPC, (f) Low dose group of SPC and (g) High dose group of SPC

improvement in each PC group and at the same dosage, the SPC group showed better effects than the RPC group. Figure 7(a-g) shows the H&E staining of rat pituitary tissue.

Immunohistochemical results of Bax, Ccl-2, caspase-3 and Fas proteins: Bax and Bcl-2 proteins were expressed in the adrenal cytoplasm and the staining appeared as yellow granules. The average optical density of Bax protein in the model control group was significantly increased ($p<0.01$) compared to the blank control group. However, the average optical density of all the drug administration groups, except for the high dose group of RPC and SPC, was decreased compared to the model control group. There was no significant difference between the same dose group and the group of SPC. This may be due to the high concentration of PC

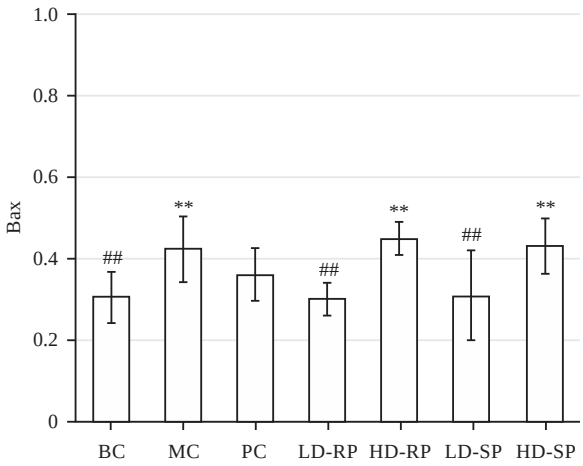

Fig. 8: The mean optical density of Bax protein in the adrenal tissue of rats in each experimental group

Compared with the blank control group, *p<0.05, **p<0.01, compared with the model control group, #p<0.05, ##p<0.01, BC: Blank Control group MC: Model control group PC: Positive control group LD-RP: Low dose group of RPC HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC HD-SP: High dose group of SPC

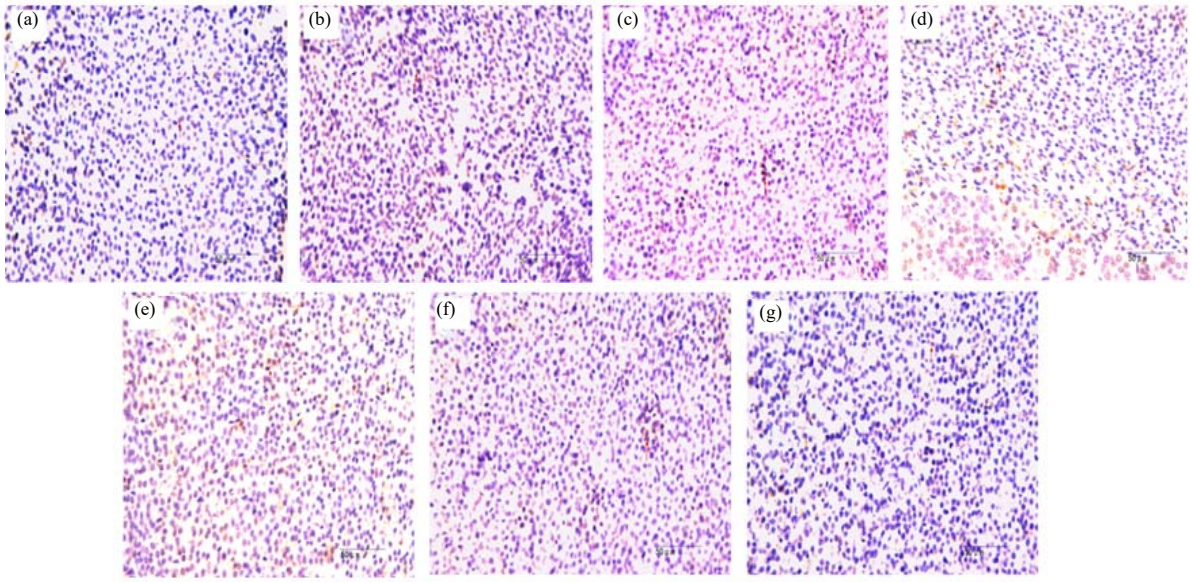

Fig. 9(a-g): Immunohistochemical section of adrenal Bax protein (×100), (a) Blank control group, (b) Model control group, (c) Positive control group, (d) Low dose group of RPC, (e) High dose group of RPC, (f) Low dose group of SPC and (g) High dose group of SPC

solution, which could have interfered with the rats' bodies. The average optical density of Bax in adrenal tissue was shown in Fig. 8 and the immunohistochemical section diagram was shown in Fig. 9(a-g).

The average optical density of Bcl-2 protein in the model control group was significantly decreased ($p<0.05$) compared to the blank control group. However, the average optical density of the low dose group of SPC was significantly increased ($p<0.01$), while the average optical density of the high dose group of RPC was not significantly increased. The mean optical density of Bcl-2 in adrenal tissue was shown in Table 2 and the immunohistochemical section diagram was shown in Fig. 10(a-g).

The caspase 3 protein primarily exists in its original form in the cytoplasm of normal tissues and its expression is low. In comparison to the blank control group, the model control group showed a significant increase in caspase 3 expression, while the low dose group of RPC and the high

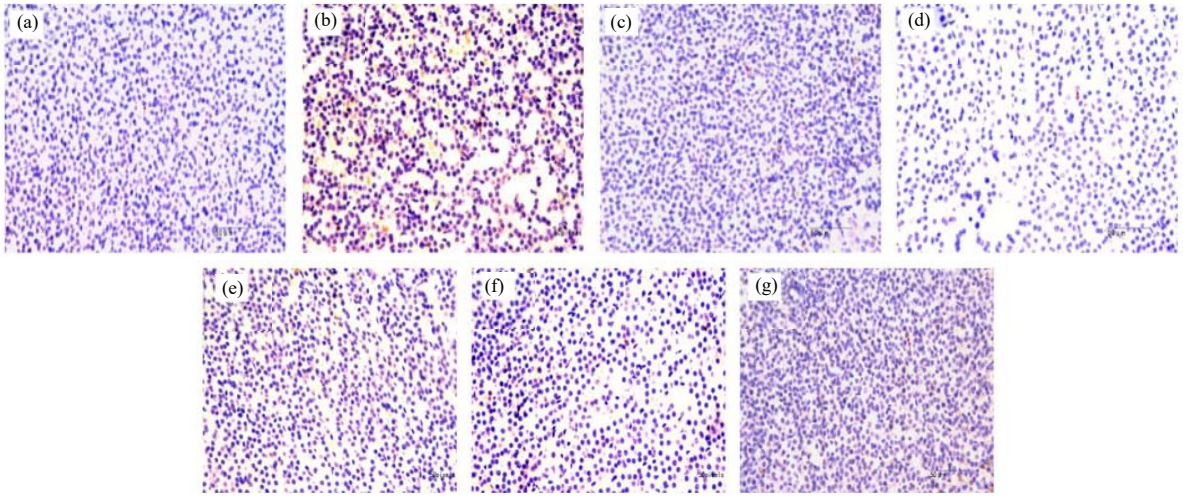

Fig. 10(a-g): Immunohistochemical sections of adrenal Bcl-2 protein in rats of each experimental group (×100), (a) Blank control group, (b) Model control group, (c) Positive control group, (d) Low dose group of RPC, (e) High dose group of RPC, (f) Low dose group of SPC and (g) High dose group of SPC

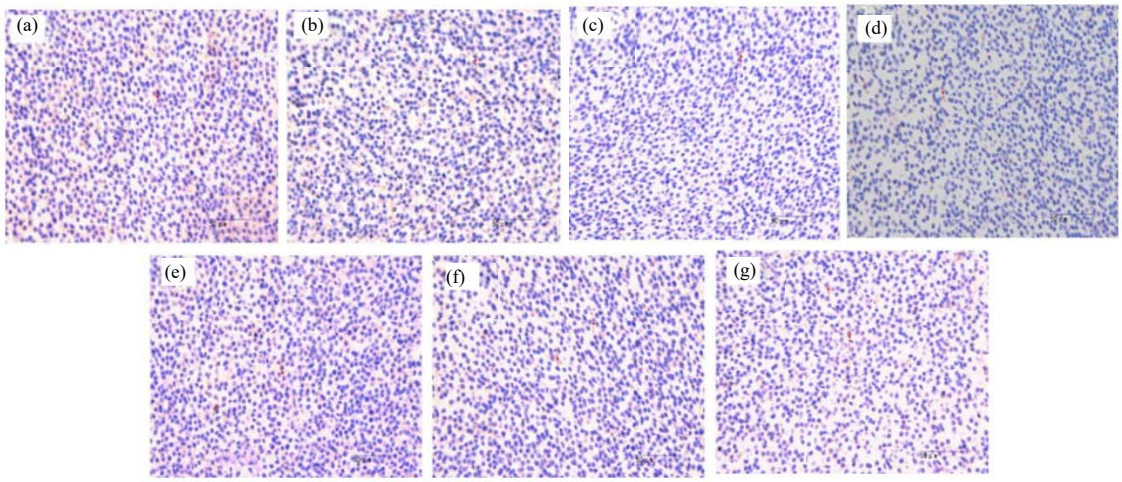

Fig. 11(a-g): Immunohistochemical sections of adrenal caspase 3 protein in rats of each experimental group (×100), (a) Blank control group, (b) Model control group, (c) Positive control group, (d) Low dose group of RPC, (e) High dose group of RPC, (f) Low dose group of SPC and (g) High dose group of SPC

Table 2: Mean optical density of adrenal Bcl-2 protein in rats of each experimental group (x±s n = 8)

Group	Bcl-2
BC	0.6347±0.0815$^{\#}$
MC	0.4896±0.0767*
PC	0.5436±0.1433
LD-RP	0.4403±0.0715**
HD-RP	0.5958±0.1298
LD-SP	0.6809±0.1491$^{\#\#aa}$
HD-SP	0.4894±0.1997*

Compared with the blank control group, *p<0.05, **p<0.01, compared with the model control group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, compared with the low dose group of SPC, ^{a}p<0.05, ^{aa}p<0.01, BC: Blank control group, MC: Model control group, PC: Positive control group, LD-RP: Low dose group of RPC, HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

dose and low dose groups of SPC exhibited a significant decrease (p<0.05). Furthermore, compared to the model control group, the low dose group of RPC and the high dose and low dose groups of SPC showed a significant increase in caspase 3 expression (p<0.01). At the same dose, the high dose group of SPC had a lower expression of caspase 3 than the RPC group (p<0.01). Table 3 presents the average optical density of caspase 3 in adrenal tissue and Fig. 11(a-g) shows the immunohistochemical section diagram.

The Fas gene expression was less in normal tissues and the positive signal was brownish yellow. Compared with the blank control group, the mean optical density of Fas protein

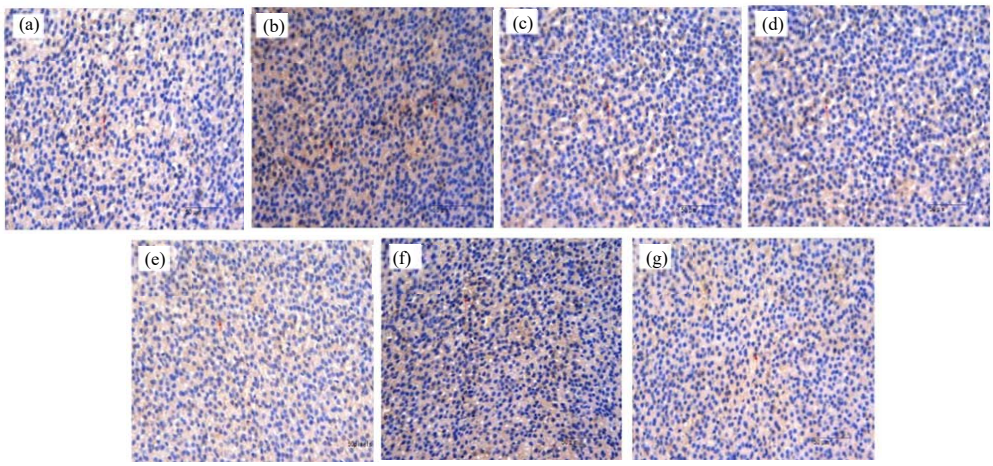

Fig. 12(a-g): Immunohistochemical sections of Fas protein in adrenal glands of rats in each experimental group (×100), (a) Blank control group, (b) Model control group, (c) Positive control group, (d) Low dose group of RPC, (e) High dose group of RPC, (f) Low dose group of SPC and (g) High dose group of SPC

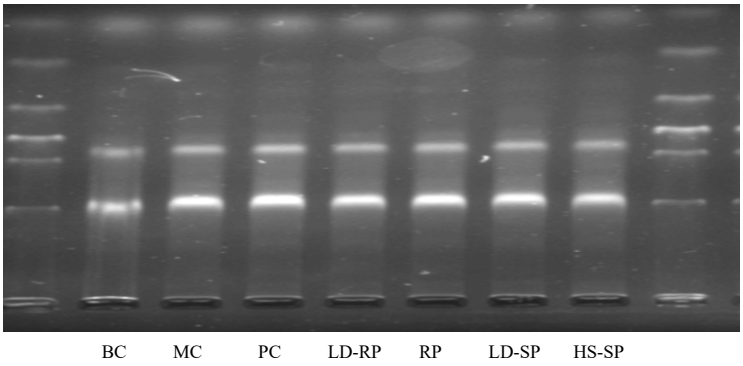

Fig. 13: Electrophoresis of CRH mRNA in hypothalamus of rats in each experimental group,
BC: Blank control group MC: Model control group PC: Positive control group, LD-RP: Low dose group of RPC HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

Table 3: Mean optical density of adrenal caspase 3 protein in rats in each experimental group (x±s n = 8)

Group	Caspase 3
BC	0.3853±0.0786
MC	0.3961±0.0420
PC	0.3948±0.0232
LD-RP	0.3439±0.0182*##
HD-RP	0.4032±0.0311
LD-SP	0.3411±0.0208*##
HD-SP	0.3427±0.0276*##bb

Compared with the blank control group, *p<0.05, **p<0.01, compared with the model control group, #p<0.05, ##p<0.01, compared with the high dose group of SPC, bp<0.05, bbp<0.01, BC: Blank control group, MC: Model control group, PC: Positive control group, LD-RP: Low dose group of RPC, HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

was significantly higher (p<0.01) in the model control group and significantly lower (p<0.01) in the high dose group of RPC (p<0.05), compared with the model control group, the mean optical density was significantly lower (p<0.01) in the positive control group, the high dose and low dose group of RPC and the high dose group of SPC. Under the same dose conditions, the mean optical density in the groups of SPC were both significantly higher than that in the groups of RPC (p<0.01). The mean optical density of Fas in the adrenal tissue was shown in Table 4 and the immunohistochemical sections were shown in Fig. 12(a-g).

Results of CRH mRNA expression in hypothalamus: Compared to the blank control group, the model control group showed a significant decrease in the expression of CRH mRNA in the hypothalamus. However, the other groups showed an increase in the expression of CRH mRNA to varying degrees when compared to the model control group. The positive control group had the highest expression of CRH

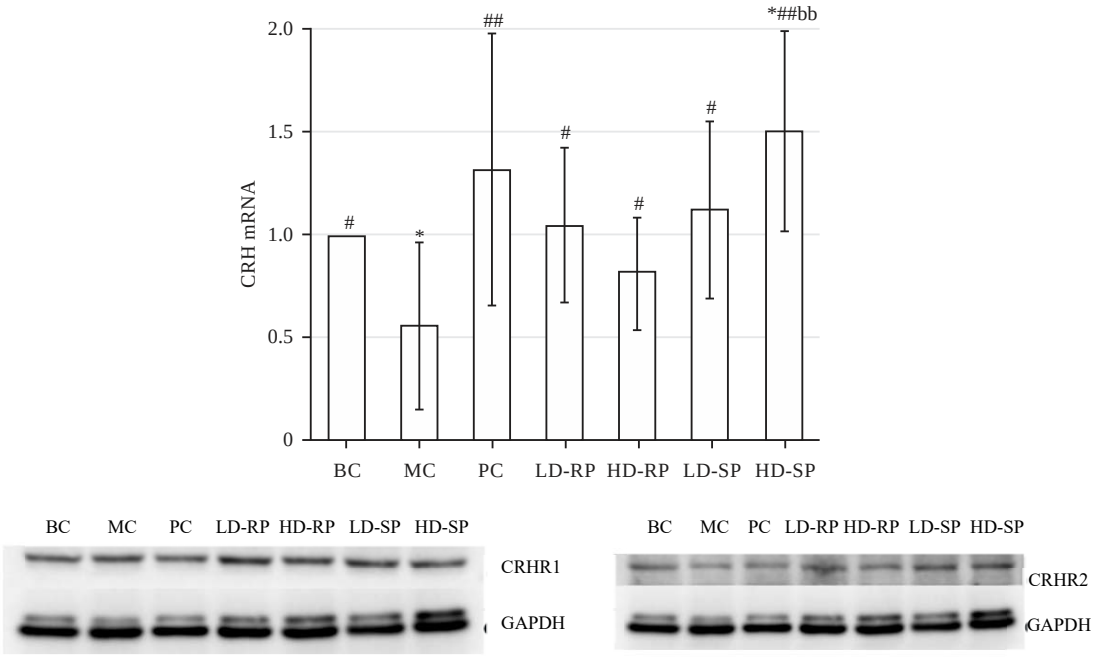

Fig. 14: mRNA expression of CRH in hypothalamus of rats in each experimental group

BC: Blank control group, MC: Model control group, PC: Positive control group, LD-RP: Low dose group of RPC, HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

Table 4: Mean optical density of adrenal Fas protein in rats in each experimental group (x±s n = 8)

Group	Fas
BC	0.4598±0.0604$^{\#\#}$
MC	0.5602±0.0128**
PC	0.4558±0.0821$^{\#\#}$
LD-RP	0.4764±0.0129$^{\#\#}$
HD-RP	0.4012±0.0079$^{*\#\#}$
LD-SP	0.6210±0.0295$^{**\#\#bb}$
HD-SP	0.4830±0.0756$^{\#\#aa}$

Compared with the blank control group, *p<0.05, **p<0.01, compared with the model control group, $^{\#}$p<0.05, $^{\#\#}$p<0.01, compared with the low dose group of SPC, ^{a}p<0.05, ^{aa}p<0.01, compared with the high dose group of SPC, ^{b}p<0.05, ^{bb}p<0.01, BC: Blank control group, MC: Model control group, PC: Positive control group, LD-RP: Low dose group of RPC, HD-RP: High dose group of RPC, LD-SP: Low dose group of SPC and HD-SP: High dose group of SPC

mRNA, followed by the high dose group of SPC. On the other hand, the RPC groups and the low dose group of SPC had the lowest expression of CRH mRNA. Figure 13 and 14 depicted the expression of CRH Mrna.

Results of CRHR1 and CRHR2 protein expression in the hypothalamus: Compared to the blank control group, the model control group exhibited a significant decrease in the expression levels of CRHR1 and CRHR2 proteins in the hypothalamus. Conversely, the other groups showed varying degrees of increased expression levels of CRHR1 and CRHR2 proteins compared to the model control group. The highest expression levels were observed in the high dose of RPC and high dose of SPC groups, with no significant difference between the two. Figure 15(a-b) illustrated the protein expression of CRHR1 and CRHR2.

DISCUSSION

Recent research on traditional Chinese medicine has revealed a close association between the clinical manifestations of Kidney-Yin deficiency and the Hypothalamic-Pituitary-Adrenal (HPA) axis dysfunction. The HPA axis is a vital neuroendocrine function axis comprising the hypothalamus, pituitary and adrenal gland, which regulates the fundamental life activities of the human body[10]. The paraventricular nucleus of the hypothalamus secretes adrenocorticotropic hormone-releasing hormone, which travels through the pituitary portal system to the adenohypophysis and binds to CRHR-1 on the membrane of ACTH cells[11]. This promotes the secretion of ACTH by the adenohypophysis, which acts on adrenocortical cells, stimulating the proliferation of the adrenal cortex and promoting the synthesis and secretion of cortisol in the zona fasciculata. Cortisol is involved in the metabolism, immunity, growth and development of the body and plays a crucial role in maintaining the body's homeostasis.

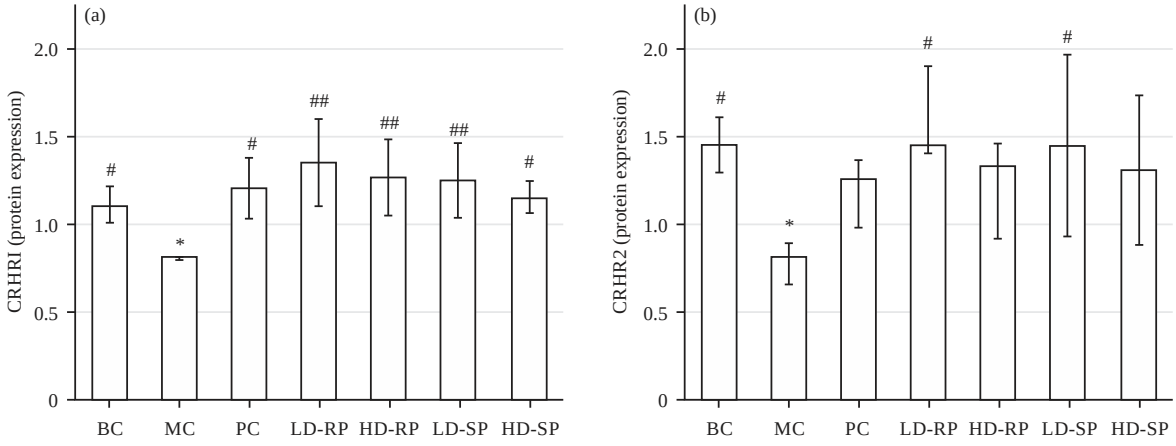

Fig. 15(a-b): Protein expression of CRHR1 and CRHR2 in hypothalamus of rats in each experimental group, (a) CRHR1 and (b) CRHR2

Compared with the blank control group, *p<0.05, **p<0.01, compared with the model control group, #p<0.05 and ##p<0.01

Compared to the model control group and the drug administration groups, the rats in the blank control group exhibited obvious hypertrophy, dry and smooth fur and good mental state. The rats in the model control group gradually displayed symptoms of Kidney-Yin deficiency, such as weight loss, increased anal temperature, hair loss, irritability, piling up, arching back, dry stool and significantly reduced water intake and activity. These symptoms were consistent with those of human Kidney-Yin deficiency, indicating that the rat Kidney-Yin deficiency model was successful. To minimize the stimulation of high dose hydrocortisone gavage on the body, a preventive administration method was selected and the corresponding solution was given on the 11th day at 3 PM when hormone secretion in the body was low. This approach aimed to improve the phenomenon of excessive inhibition of adrenal cortex by high dose exogenous glucocorticoids, which could lead to a large number of deaths. The symptoms of rats in other treatment groups were relieved to varying degrees. The treatment effect of rats in the positive control group was the best and the treatment effect of rats in the SPC group was closest to that in the positive control group and better than that in the RPC group.

The rats in the blank control group exhibited complete and well-defined thymic edges after dissection. Conversely, the rats in the model control group displayed atrophic and shapeless thymus, while the other drug administration groups showed varying degrees of improvement in thymus shape. Among the groups administered the same dose, the low dose group of SPC exhibited the highest degree of recovery in thymus index and its ability to repair the thymus was higher than that of the positive control group. No significant difference was observed in the recovery degree of thymus index in the other groups. Additionally, we observed that the high dose group of SPC had the lowest renal index and a better therapeutic effect than the positive control group. The thymus and spleen are two crucial immune organs in the body and their indexes can be used to reflect the body's immune function. Hydrocortisone is a glucocorticoid and high doses of hydrocortisone can cause organ damage and inhibit the body's immune function. The significant decrease in thymus and spleen indexes in the model control group suggests that the HPA axis and immune system were rapidly damaged.

Patients with Kidney-Yin deficiency and immunodeficiency exhibit hyperfunction of the Hypothalamic-Pituitary-Adrenal (HPA) axis, resulting in increased levels of glucocorticoids in the blood and endocrine dysfunction[12]. This is manifested as increased hypothalamic CRH and ACTH, which stimulate the adrenal cortex to release glucocorticoids, significantly increasing the level of plasma glucocorticoids. The massive release of CRH and glucocorticoids can also inhibit the gonadal axis at the hypothalamus, pituitary and gonad levels, leading to reproductive endocrine disorders. In the model control group, rats exhibited increased serum cortisol content due to the administration of large doses of exogenous mineralocorticoid hormone. This increase in serum cortisol content promoted the hypothalamus to secrete

adrenocorticotropic hormone releasing hormone, which increased the level of CRH in serum. This, in turn, caused the pituitary gland to release more adrenocorticotropic hormone, increasing the level of ACTH in serum. The results of this study showed that the blood levels of CRH, ACTH and cortisol in the model control group were decreased to varying degrees and the body weight of the model control group was significantly decreased. Additionally, the body temperature was increased, the frequency of activity was increased, the hair was dry and colorless and the amount of water was increased. After drug treatment, the serum level of CRH in the model control group was decreased and the pathological changes of adrenal tissue were alleviated. These results indicated that the intervention of PC has a certain protective effect on the HPA axis in rats. Compared with the model control group, the levels of CRH, cortisol and ACTH in the serum of rats in the other drug administration groups were decreased to varying degrees. The level of CRH in the positive control group decreased most significantly ($p<0.01$), followed by the high dose group of SPC and the level of CRH in the RPC groups increased the least. The content of cortisol in the group of RPC was the lowest and it was lower than that in the blank control group, positive control group and SPC group. There was no significant difference in content. The content of ACTH in the positive control group was the lowest. At the same dose, the SPC group was lower than the RPC group and the therapeutic effect of the SPC group was the closest to the positive control group, which was better than the other groups.

The IL-2 is a growth factor secreted by T cells and macrophages that participates in the regulation of inflammation, regulates the body's immunity and increases the body's ability to resist infection. The IL-10 is an anti-inflammatory factor mainly derived from Th2 and some regulatory T cells that can inhibit Th1 cell response and cytokine synthesis, as well as inhibit the antigen presentation function and cytokine synthesis of macrophages[13]. The results showed that the expression of IL-2 and IL-6 in the model control group increased, while the expression of IL-10 decreased. After drug treatment, the levels of IL-2 and IL-6 in each drug administration group decreased and IL-10 increased to varying degrees. This suggests that PC has a certain effect on regulating the body's immunity and a certain concentration of SPC has a better effect than RPC.

The IFN-γ is mainly produced by macrophages and enhances the anti-tumor activity of macrophages[14]. The TNF-α is a pro-inflammatory cytokine that activates macrophages, induces vascular endothelial cells to express adhesion molecules, activates inflammatory cell migration and aggravates inflammation[15,16]. The results showed that the levels of IFN-γ and TNF-α in the serum of rats in the model control group significantly increased, while the levels of IFN-γ and TNF-α in the serum of rats in the other drug administration groups decreased to varying degrees. The high dose group of SPC had the lowest level of IFN-γ and the content of TNF-α in the other drug administration groups significantly reduced. Compared with normal rats, the content of TNF-α in each treatment group significantly decreased. The serum T content of the other positive control groups and the high dose group of SPC significantly increased compared to the blank control group. Compared with the model control group, the serum T content of the positive control group and the high and low dose group of SPC significantly increased. The serum T content of the SPC group significantly increased under the same dose. It is concluded that PC not only has a certain effect on regulating the body's immunity of normal rats but also has an effect on Kidney-Yin deficiency model rats and SPC has a better effect than RPC.

The H&E staining method has many advantages such as good cell transparency, strong permeability of the staining solution, sharp contrast between the nucleus and plasma, excellent staining effect, etc. The most widely used in the study of the internal structure of animal tissues is the conventional paraffin section and staining technique, which has formed a fixed operation procedure over the years and has been commonly adopted by people[17,18]. Immunohistochemistry, with its high specificity and sensitivity, can be used to observe the products of antigen-antibody reactions occurring in the cell under the microscope and to determine the distribution and content of certain chemical components in cells or tissues *in situ*, using the average optical density value as a reference value[19].

The Bcl-2 family proteins are a class of key proteins that mediate the mitochondrial pathway to maintain mitochondrial integrity by regulating the balance between pro- and anti-apoptosis[20] and Bcl-2 proteins can play an anti-apoptotic role by inhibiting the activation of a series of signals triggered by apoptotic proteins. Bax can contribute to the release of cytochrome C into the cytoplasm, causing caspase-3 to be activated and triggering cell death, by enhancing the permeability of mitochondrial membranes and altering transmembrane potential. Bax can enhance mitochondrial membrane permeability and alter the transmembrane potential, thereby prompting the release of cytochrome C into the cytoplasm to cause a cascade reaction of cysteine aspartase, which ultimately leads to the activation of

caspase-3 and triggers apoptosis. Caspase-3 is a widely and deeply studied indicator of apoptosis and is involved in apoptosis induced by a variety of factors[21], the Fas system belongs to the tumor necrosis factor receptor family and Fasl is the only natural ligand and it has been considered that the apoptosis signaling pathway mediated by the Fas/Fasl system is one of the important pathways of apoptosis[22]. The Bc1-2, Fas, Fasl and Bax genes have the closest relationship with apoptosis, in which Bc1-2 as an apoptosis inhibitory gene and the pro-apoptotic genes, Bax, Fas and Fasl, whose expression level determines whether a cell survives or death[23], meanwhile, it was found that the kidney tonic Chinese medicine-Liuwei Dihuang free decoction granules down-regulated the expression of key genes related to apoptosis (Fas) and the granules significantly reduced the expression of key proteins in the Fas-mediated apoptotic signaling pathway of granular cells, Fas and caspase-3, inhibited the Fas-mediated apoptotic signaling pathway of granular cells and reduced the apoptosis of granular cells[24,25]. Therefore, the changes of apoptosis indexes have important significance for Kidney-Yin deficiency syndrome.

The immunohistochemical experiment revealed that the adrenal tissue of the model control group exhibited significant lesions. High-dose exogenous hydrocortisone reduced the expression of anti-apoptotic Bcl-2 protein and increased the expression of pro-apoptotic Bax, caspase-3 and Fas protein, leading to cell apoptosis. The results indicated that, at the same dose, the low dose group of SPC significantly reduced the expression of Bax and caspase-3 protein, while significantly increasing the expression of Bcl-2 protein[26]. Additionally, a certain concentration of RPC and SPC improved the expression of the four proteins in normal rats. The decoction of RPC and SPC can alleviate the low immunity condition in patients with Kidney-Yin deficiency by enhancing the stability of the expression of anti-apoptotic and pro-apoptotic proteins. In conclusion, a certain concentration of RPC and SPC can improve the immune function of rats, which may be attributed to the up-regulation of Bcl-2 protein expression and the down-regulation of Bax, caspase-3 and Fas protein expression. This may be one of the mechanisms of PC's pharmacological effect[27].

Short-term and high-dose administration of exogenous hydrocortisone can inhibit the expression of CRH mRNA, CRHR1 and CRHR2 proteins in the hypothalamus of rats. The RPC has the best effect on up-regulating the expression of CRHR1 and CRHR2 protein and SPC has the best effect on up-regulating the expression of CRH mRNA. By up-regulating the expression of CRH mRNA, CRHR1 and CRHR2 proteins in the hypothalamus of rats, SPC can antagonize the HPA axis dysfunction in Kidney-Yin deficiency model rats. These findings suggested that PC can improve the Hypothalamic-Pituitary-Adrenal (HPA) axis disorder, with SPC having a better effect, which may be one of the mechanisms of its intervention on Kidney-Yin deficiency.

CONCLUSION

The experiment indicates that among different treatment groups of processed product of PC, the SPC and the positive control group has the best treatment effect to the KYDS. Both the RPC and the SPC can increase the body weight, decrease the anal temperature, increase the organ indices of the kidney, spleen and thymus. The SPC can improve the inhibition of HPA axis function in rats with exogenous high-dose of hydrocortisone. And the content of CRH in blood of rats was down-regulated, the values of IL-10, T, lymphocyte subpopulation $CD4^+$ and $CD4^+/CD8^+$, CRH mRNA in hypothalamus and Bc1-2, Fas, caspase-3 protein expression in adrenal gland tissues were reduced and caspase-3 protein expressions were upregulated in the hypothalamus and adrenal tissue. The therapeutic effect of PC on KYDS can be enhanced by salt-water processing.

SIGNIFICANCE STATEMENT

This study aims to better understand the efficacy mechanism of PC processed by salt-water in improving Kidney-Yin deficiency syndrome rats. The experiment indicates that both the raw product and the salt-water processing product of PC improved the inhibition of HPA axis function in rats with exogenous high dose of hydrocortisone and the anti-inflammatory, immunomodulatory and regulatory effects on hypothalamus and adrenal tissue were enhanced after salt-processing of PC. The therapeutic effect of PC on KYDS can be enhanced by salt-water processing.

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