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Intervention effects of Hellgrammites（Megaloptera：Corydalidae）on 'Kidney-Yang Deficiency syndrome' rats

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Abstract: The present study was conducted to explore the effects of hellgrammites protein hydrocortisone administration induced Kidney-Yang Deficiency syndrome (KYDS) rats. The proteins were extracted from hellgrammites. Protein content was determined by Kjeldahl method. 51 rats were divided randomly into 5 groups. (group 1: blank control; group 2: KYDS model; group 3: KYDS rats treated with desmopressin(DDAVP); group 4: KYDS rats treated with hellgrammites protein low dose group 100 mg/kg; group 5: KYDS rats treated with hellgrammites protein high dose group 200 mg/kg). The rats were injected intraperitoneally hydrocortisone once daily for 18 days to simulate 'Kidney-Yang Deficiency syndrome', then the ninth day administered orally hellgrammites protein extract once daily for the following 12 days. The ALD, AVP, CAMP, CAMP/CGMP and renal tissue expression in different groups were compared. Compared with blank control diet group, CAMP/CGMP in hyperlipemia model group decreased ($P < 0.05$) and increased ALD($P > 0.05$). After treatment with hellgrammites protein high dose group and DDAVP group, CAMP/CGMP of the Kidney yang deficiency rats increased ($P < 0.05$) and increased ALD($P < 0.05$). The hellgrammites protein extract have protective effects on the rat model of kidney yang deficiency and polyuria caused by hydrocortisone.

1. Introduction

'Kidney-Yang Deficiency syndrome' is one of the elementary syndrome patterns in traditional Chinese medicine and closely related to multiple disordered metabolic pathways（F. Gao et al, 2006). After intraperitoneal injection of a high dose of hydrocortisone，which shows the same symptoms as the essentials of'Kidney-Yang Deficiency syndrome'（Q. Chen et al, 1993). This model has been widely employed in the evaluation of therapeutic effect and action mechanism of Kidney-Yang toxifying herbs and preparations (Huang et al. 2013). It has been one of classical methods of establishing KDS-Yang to animals. Modern studies have shown that the animal model mimicked

the pathological state of suppression of hypothalamic-pituitary- adrenal (HPA) axis to some extent in KDS-Yang human and contributed greatly to important advances in the current understanding of KDS-Yang as well as treatments（Zhao et al. 2013; Q. Chen.1993; J.Yang et al. 2008; C.M.Wang. 2012)

With a growing world population, in order to get enough protein, we will face a serious challenge in the future. In the 21st century, insects will become the third largest source of protein after microorganisms and cell organisms are reported. Insects are highly nutritious and especially rich in proteins and thus represent a potential food and protein source, making them a subject of great interest in scientific research(Rumpold and Schluter 2013). In worldwide, approximately 1,900 insect species are eaten, and edible insects protein may have some beneficial health effect (van Huis 2016, 2013). In addition, some insects also have potential medicinal value, such as the antimicrobial, antioxidant and antihypertensive(de Castro et al. 2018).

Hellgrammites, the larval form of the dobsonfly, are a completely abnormal insect, scientific name: Corydalus cornutus (Linnaeus), belonging to the family Megaloptera. The insects are distributed in the Americas, Asia, and South Africa. It is a widely distributed insect in China (Jepson and Heads 2016). In addition to this kind of larvae is not only a favorite food for tourists, but also a common traditional Chinese medicine in China utilized to strengthening with tonics, toxifying the lungs, relieving cough, and treat kidney diseases（Cao CQ, 2014). It is reported that the extract of hellgrammites can not only significantly increase the number of fruit flies and improve their fecundity, but significantly increase the growth and fecundity of rats（Yang DM, 2012 and 2014). It is reported that hellgrammite is rich of proteins, and the insect extract is very effective in the treatment of nocturnal urine （Cao CQ, 2014). However, the composition of insect extracts for the treatment of kidney yang deficiency is not clear. There has not research on the relationship between hellgrammite protein and kidney yang deficiency syndrome. In this study, we established a model of Kidney-Yang Deficiency syndrome in rats. Additionally, we examined the effect of serum AVP, ALD, cAMP, cGMP, cAMP/cGMP and histopathological changes of kidneys. Then, we explored the effect of hellgrammite proteins on kidney yang deficiency syndrome.

2. Materials and methods

2.1 Instruments

Pharmaceutical Ultrasound Processor (Model: FS-150N, Shanghai Shengsen Ultrasonic Instrument Co.), Freeze Dryer (Model: BT-85, Millrock Technology Company), Microplate reader (Model: 1575, BIO-RAD Company), UV-visible spectrophotometer (Model: UV1000, Shanghai Tianmei Scientific Instrument Co), DigiPREP TKN Systems(Model: Kjeltec8400, Shanghai Rui Yin International Trade Co.), Hitachi L-8900 automatic amino acid analyzer. Fresh hellgrammites(HGM) were purchased from local farmers' markets in Panzhihua. India ink (batch number: 150301, Shanghai JinSui Bio-Technology Co), Hydrocortisone injection (batch number: 20180101, Harbin Sanma Veterinary Pharmaceutical Co), Trypsin and Alkaline protease(product lot number: 20180314, Beijing Aoboxing Biotechnology Co), argininevasopressin (AVP), aldosterone (ALD), Cyclic Adenosine monophosphate(CAMP), Cyclic guanosine monophosphate (CGMP) and Elisa kits(batch number: 20180319, Nanjing Jiancheng bioengineering institute), Desmopressin acetate(batch number: N16464C, Huiling (Switzerland) Pharmaceutical Co), Saline(batch number: W218022102, Sichuan Kelun Pharmaceutical Co) and the remaining reagents were of analytical grade. Fifty one SPF grade SD male rats were weighed (180±20) g, provided by Animal Laboratory of Sichuan Medical University.

2.2 Process of proteins extract

Fresh hellgrammites (HGM) were purchased from local farmers' markets in Sichuan Panzhihua, China. Remove head, tail and viscera, freeze dry insect, get dry ingredients, crush and reserve. Ultrasonic degreasing with petroleum ether (60-90℃), ultrasonic power 350W, extraction temperature 40℃, liquid to material ratio 12:1, extraction time 30 min for two times. The defatted powder was obtained by vacuum filtration, and the protein was extracted by trypsin: the enzyme amount was 1.5%, the temperature was 55℃, the ratio of material to liquid was 1:10, the pH value was 8.0, the extraction time was 5 h, and after the protein extraction was completed, the temperature was raised to 70℃. Incubate the enzyme for 0.5 h, and refrigerate overnight at 4℃. Vacuum the protein extract, filter the spoil, and lyophilize the supernatant into protein powder, which was applied to the following test in this study.

2.3 Total protein determination experiment

The content of total protein in the samples were determined by Kjeldahl method and a certain amount of the insect powder and insect protein powder were taken. All weighing was made with balances accurate to 0.0001 g. Copper sulfate 0.8g, potassium sulfate 7g, concentrated sulfuric acid 12ml, nitrification preheating 220 ° C for 20 min, 420 ° C for 1.5 h, after cooling, the protein content of the sample was measured by Kjeldahl.

2.4 Determination of amino acids

In this experiment, Hitachi automatic amino acid analyzer was used, and the amino acid type and content of the hellgrammites were determined by referring to the national standard GB5009.124-2016.

2.5 Animals and Experimental design

The mental and activity status of the experimental rats were observed daily: fur, tail color, stool, foraging, and presence or absence of abdomen arch back, recorded once a day. Through general behavioral observation to determine whether the modeling is successful.

Fifty one SD male rats of SPF grade were weighed (180±20) g, provided by Animal Laboratory of Sichuan Medical University. The animals were housed under fixed conditions of temperature (23 ± 2℃) and humidity with a 12-hour light/dark schedule, and fed standard rats diet and filtered tap water ad libitum. All the animal studies were conducted in accordance with the Regulations of Experimental Animal. After one-week habituation, in addition to the blank group, all rats were injected intraperitoneally hydrocortisone at a dose of 10 mg/kg once daily for 18 days and then the ninth day administered orally DDAVP(0.06mg/kg), hellgrammite proteins(low dose: 100 mg/kg; high dose: 200 mg/kg) once daily for the following 12 days. After the final treatment, the rats were weighed and then sacrificed. Blood was collected from rats through the abdominal aorta, then take the nephridial tissue. All liver specimens in each group were fixed with 10% buffered formalin and embedded in paraffin for slicing after weighing. Five sections in each group were used for histopathology examination. Sections were stained with HE for histopathology examination. The blood sample was taken for AVP, ALD, cAMP, cGMP and kidney function test.

2.6 The rat serum AVP, ALD change

At the end of the experiment, the rats were anesthetized with 4% chloral hydrate, and 2 ml of

blood was taken from the abdominal aorta. The ethylenediaminetetraacetic acid was anticoagulated, centrifuged, and the supernatant was taken. Then, according to the operation requirements of the kit instructions, the serum AVP and ALD content was determined by ELISA method.

2.7 The rat serum cAMP, cGMP and cAMP/cGMP change

The above method was used to collect 2 ml of blood, centrifuged, and serum was taken. According to the operation requirements of the kit instructions, serum CAMP and CGMP content was determined by ELISA, and the ratio was calculated.

2.8 Histopathological examination of the kidney

At the end of the experiment, the rat was killed when the blood was completed. The abdominal cavity was opened quickly, the left kidney was removed and the left kidney was removed. Kidney was taken and fixedin10 % formaldehyde. After several treatments of dehydration in alcohol, sections having 4μm thickness were cut and used conventional HE staining and histopathological analysis was performed by using a microscope.

2.9 Statistical analysis

Results were expressed as means ± SD. The significance of difference among groups was determined by using one-way ANOVA. P values <0.05 were considered significant.

3. Results

3.1 Determination Protein content results

According to Kjeldahl method, Total protein ratio= Measured protein content (g)/ Sample (g) ×100%. The results showed that the protein content of the hellgrammites powder and the hellgrammites protein powder were 39.18% and 59.9%, respectively.

3.2 Amino acid analysis

The amino acid profile of the hellgrammites powder was determined by Hitachi automatic amino acid analyzer. The results are shown in Figure 1. The amino acid species and content results are shown in Table 1. Hellgrammites contain 17 amino acids, of which 7 are essential amino acids and 10 are non-essential amino acids. The ratio of amino acids to total amino acids is about 40%.

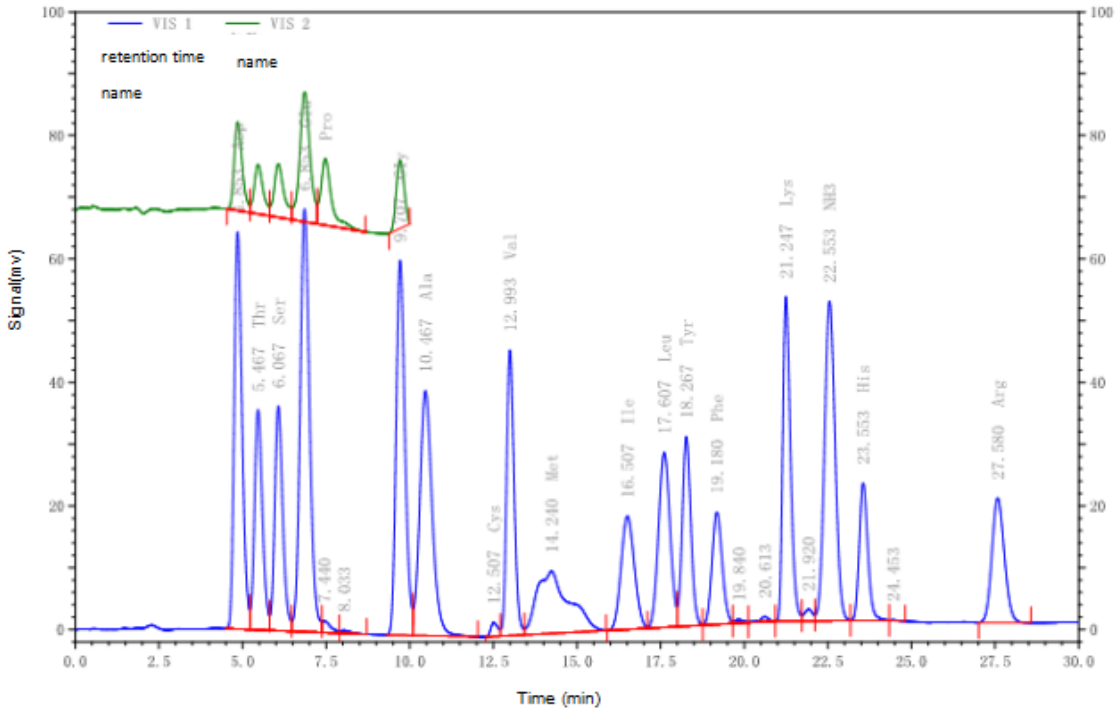

Figure 1 Amino acid profile

Table1 Kinds and contents of amino acids.

Name	Amino acid content (%)
Threonine	1.8339
Valine	2.2209
Methionine	2.7432
Isoleucine	1.8556
Leucine	2.6899
Phenylalanine	1.7989
Lysine	2.9694
Aspartic acid	3.8597
Histidine	1.6586
Arginine	2.6804
Proline	2.1982
Serine	1.6351
Glutamate	5.1307
Glycine	2.3573
Alanine	2.4983
Cysteine	0.1055
Tyrosine	2.7978

The rats in the blank control group were active, the diet was normal, and the fur was shiny. The model group showed obvious polyuria, dry hair, sparse flaking, chills and atrophy, arched back, slow response, pale tail and other symptoms; DDAVP, HGM200 mg/kg and HGM100 mg/kg groups had no obvious symptoms such as polyuria, dry body hair, sparse shedding, chills, depression, bowing, slow reaction and so on, indicating the success of the hydrocortisone-induced Kidney-Yang Deficiency syndrome model.

3.3 Effect of Hellgrammite proteins on serum AVP and ALD levels

The results are shown in Figure 2. The serum of ALD level in the KYDS rats are significantly higher than blank control group ($P < 0.05$), indicating the success of the KYDS model. The total

proteins from hellgrammites extract increased arginine vasopressin (AVP) and aldosterone (ALD). AVP level of HGM100 mg/kg and ALD level of HGM200 mg/kg showed statistically significance ($P < 0.05$). AVP level of HGM200 mg/kg and ALD level of HGM100 mg/kg showed no statistically significance. The serum AVP and ALD in DDAVP group decreased ($P < 0.05$), showed statistically significant differences.

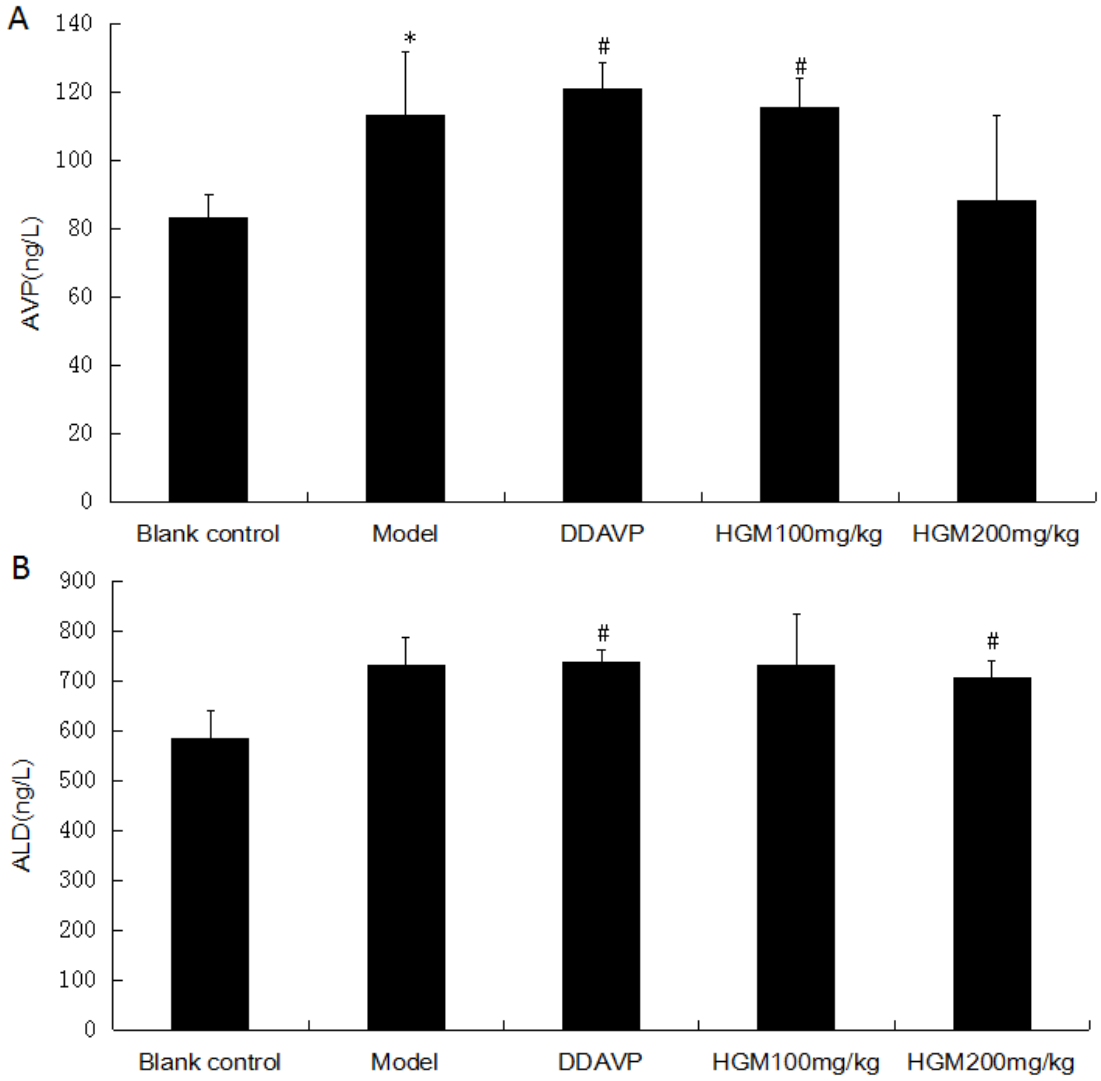

Figure 2 A: The serum AVP level ; B: The serum ALD level

Compared with the blank control group: $*P<0.05$, Compared with the model group: $^{\#}P<0.05$.

3.4 Effects of Hellgrammites proteins on serum cAMP, cGMP and cAMP/cGMP

The results are shown in Figure 3. The serum cAMP level and cAMP/cGMP in the KYDS rats are significantly lower than blank control group ($P < 0.05$), but cGMP level in the KYDS rats are significantly higher than blank control group ($P < 0.05$). The total proteins from hellgrammites extract increased cAMP level, cAMP/cGMP and decreased cGMP level. The serum cGMP level of HGM100 mg/kg and cAMP/cGMP of HGM200 mg/kg showed statistically significance ($P < 0.05$). The serum cAMP level of HGM100 mg/kg, HGM200 mg/kg and DDAVP showed no statistically significance.

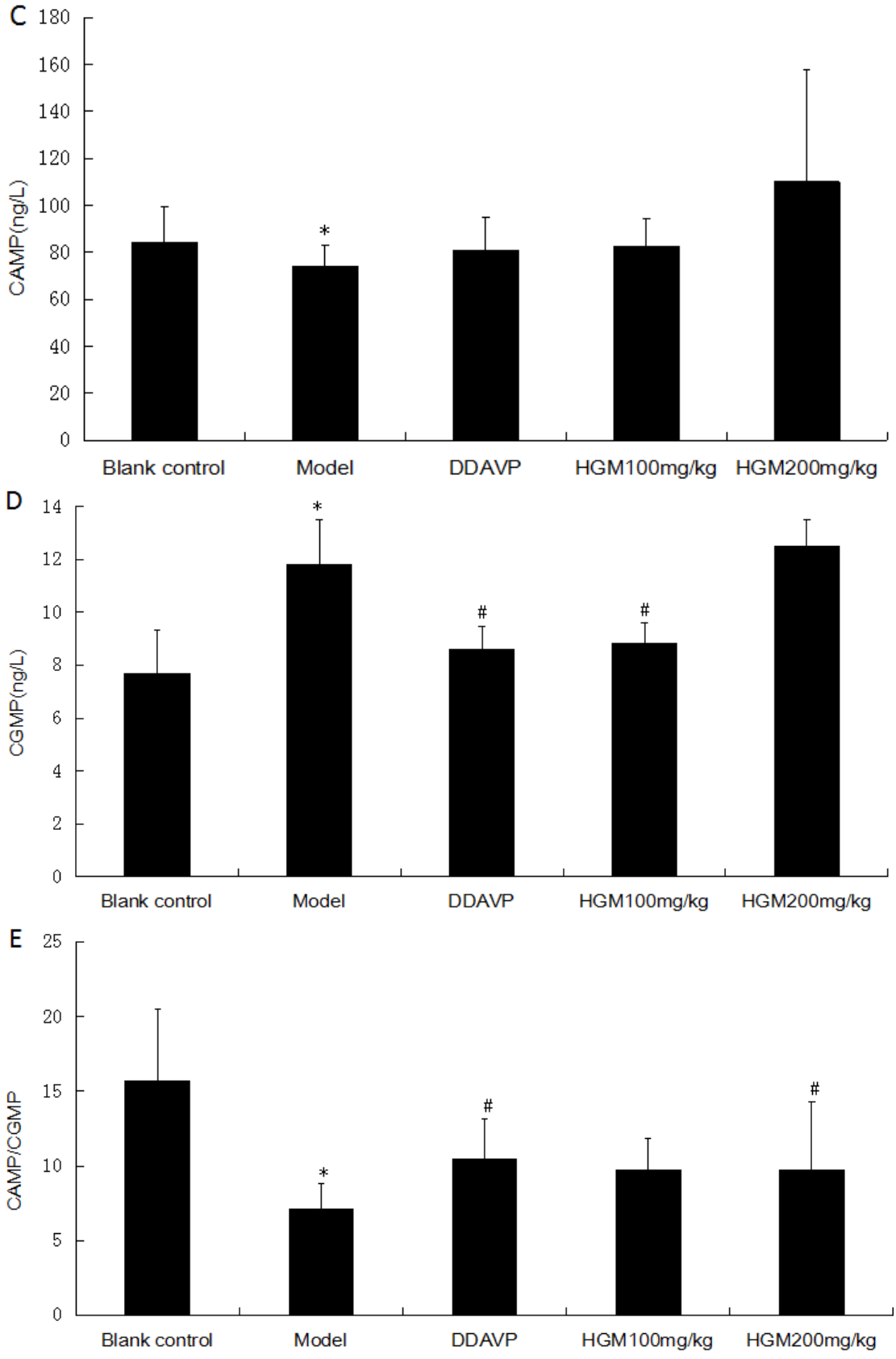

Figure 3 C: The serum cAMP level ; D: The serum cGMP level; E: cAMP/cGMP

Compared with the blank control group: *$P<0.05$, Compared with the model group: $^{\#}P<0.05$.

3.5 Effects of Hellgrammites proteins on histomorphology changes of kidneys

Photomicrographs of kidney specimens stained with H&E were shown in Figure 4. In the blank control group, the kidneys showed normal glomerular and tubular forms, no glomerular atrophy, no obvious tissue swelling. Compared with the blank group, the glomerulus of the model group was sparsely distributed, atrophied, and the cystic cavity expanded. Compared with the model group, the glomerulus distribution of the DDAVP and HGM200 mg/kg groups were concentrated, and the volume and the number of glomeruli per unit of view were larger than that of the model group.

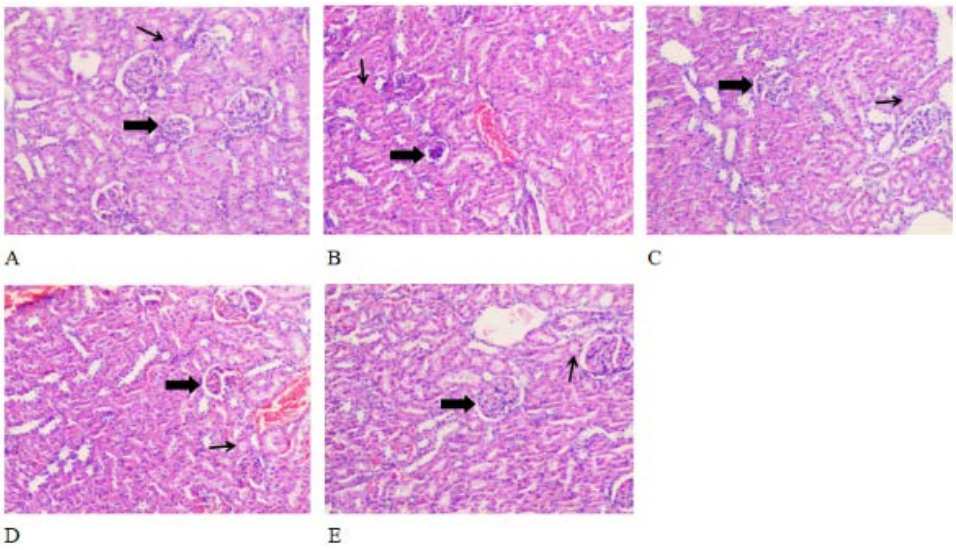

Figure 4 Histopathological changes of rat kidney in different groups (HE stained 100X lower).
⬆ Glomeruli, ↑ Renal tubule
A: Blank control group; B: Model group; C: DDAVP group; D: HGM100 mg/kg group; E: HGM200 mg/kg group

4. Discussion

In this study we investigate the active ingredient identification and biomedical function of hellgrammite on kidney yang deficiency. Kjeldahl determination proved that proteins are functional ingredients abundant in hellgrammite. The species and content of amino acids were analyzed and determined. The ratio of amino acids to total amino acids is about 40%, indicating that hellgrammite is a good source of protein.

Hellgrammite have been used traditionally in China to strengthening with tonics, tonifying the lungs, relieving cough, and treat kidney diseases（Cao CQ, 2014). Yang DM et al（Yang DM, 2012 and 2014) found that the bioactivity in hellgrammite extracts can not only significantly increase the number of fruit flies and improve their fecundity, but significantly increase the growth and fecundity of mice. Here we investigate its effect on Kidney-Yang Deficiency syndrome. 'Kidney-Yang Deficiency syndrome' is one of the elementary syndrome patterns in traditional Chinese medicine（F. Gao et al, 2006). Besides, intraperitoneal injection of a high dose of hydrocortisone has been widely employed in the evaluation of therapeutic effect and action mechanism of Kidney-Yang tonifying herbs and preparations (Huang et al. 2013). The behavioral observation of rats and the determination of serum ALD, AVP, and CAMP/CGMP ratio all proved

that the model of kidney yang deficiency polyuria was successful. As the first choice for the treatment of kidney yang deficiency, minirin has significant anti diuretic effect. However, after stopping the drug, the recurrence rate is high, and it may increase the risk of side effects such as hypertension（Deng HY et al, 2006). It is particularly important to find better resources for the treatment of kidney yang deficiency polyuria.

The decline in CAMP/CGMP is a recognized indicator of kidney yang deficiency. cAMP can activate the protein kinase PKA and release the enzymatic substance. After a certain process, the aquaporin AQP-2 on the vesicle membrane is inserted into the luminal membrane of the main cell of the collecting duct to increase the permeation permeability of the luminal membrane（Chen X and Huang ZX. 2010). CAMP plays an important regulatory role in the regulation of AQP-2 and the water permeability of cell membranes (Xie et al. 2010) . AQP-2 is present in the main cells of the renal collecting duct and is the molecular basis for regulating the water balance of the body. The kidneys reabsorb the water to regulate the urine. The results of this experiment show that the high dose group of the hellgrammite protein extract can increase the concentration in the serum of rats, while the elevated cAMP helps phosphorylate AQP-2, enhance its ability to run water and achieve the inhibition of urine output（Maurice DH et al, 2003).

ALD can regulate the reabsorption of renal sodium ions, maintain sodium and potassium, maintain the balance of body fluid volume and osmotic pressure, and is closely related to the production of polyuria（Mo, JM et al, 2003). When serum ALD levels increase, sodium reabsorption increases, discharge decreases, and urine decreases. After modeling, ALD levels were significantly increased in the DDAVP group and the high-dose protein extracts intervention group. It is indicated that the hellgrammite dose group has significant effects on regulating the reabsorption of renal sodium ions, maintaining sodium and potassium, and maintaining body fluid volume and osmotic pressure balance. Experimental observation, AVP and ALD change trend is consistent, but there is no research to prove the role of serum AVP in the regulation of urine volume, AVP can be used as an indicator of kidney yang deficiency polyuria model, it is worth further exploration.

5. Conclusions

In summary, the experimental results showed that hellgrammite is a rich protein resource and proteins from hellgrammite extract can decrease AVP, ALD, cGMP, and increasing cAMP, cAMP/cGMP. In addition, renal tissue sections showed that the high and low dose groups of hellgrammite had protective effects on damaged kidney tissue. Therefore, the protein of hellgrammites may have a corrective effect on rats with kidney yang deficiency induced by hydrocortisone. This experiment provides a certain experimental basis for the development and utilization of hellgrammites. However, whether the hellgrammite can be developed into a drug for treating kidney yang deficiency and polyuria, whether it has side effects still needs further investigation.

Acknowledgements

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