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Coicis Semen Inhibits Inflammatory Factors and Neutrophils in Anti-Inflammation Pathway

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Abstract. Ischemic stroke can be treated through anti-inflammation pathway.Coicis Semen is a kind of common traditional herbal medicine with long history. It is a Chinese medicine for both treatment and food. It contains fatty acids and esters, polysaccharides, flavonoids, triterpenes, alkaloids, sterols, lactam, starch, and other compounds, which play different effects and achieve the purpose of treatment. It has been proven to protect against ischemic stroke in TGFβ/ALK1 signaling pathway, which stimulates angiogenesis. This project aims to verify if Coicis Semen can treat ischemic stroke in anti-inflammation pathway. Both inflammatory factors and neutrophils were tested. Inflammatory factors were collected from bEnd.3 cells. The expression level of P-NF-kB, P-IkB and HIF-1α was evaluated by Western Blot. The production of NO was analyzed through Griess reaction. Zebrafish was applied to mimic the neutrophil production in ischemic stroke. The neutrophils in zebrafish embryos were stained by Sudan black and could be observed under optical microscope. Coicis Semen had positive effect on inhibiting expression of inflammatory factors and production of neutrophils. It can be proven that Coicis Semen treating ischemic stroke in anti-inflammation pathway is feasible.

Keywords: schemic stroke, Coicis Semen, Anti-inflammation, NF-κB

1. Introduction

1.1. Ischemic stroke

Stroke is one of the most common diseases that cause death worldwide. It is mainly found in the elderly group and causes approximately 10% of death, which is only after ischemic heart disease [1]. There are two types of strokes. One is hemorrhagic stroke and the other one is ischemic stroke [2]. Ischemic stroke is critical for its high incidence and limited effective therapies. It can cause a series of inflammation reactions and oxidative stress, which is associated with the level of factors such as NF-kB, IkB and HIF-1α. The inflammatory cells release inflammatory factors, reactive oxygen species (ROS) and nitric oxide (NO) [3]. Leukocytes are activated and infiltrate into brain causing disease, and induce the synthesis of NO, cytokines and ROS in return [3]. Neutrophils are found to be the first type of leukocyte infiltrate brain of ischemic stroke patients [4]. The level of neutrophils can be an crucial index of ischemic stroke.

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1.2. Coicis Semen

Coicis Semen is the dried and mature seed of Coix lacryma-jobi of the grass family. It is a common Chinese medicine for both medicine and food. Coicis Semen has a long planting history in China. The main active ingredients of Coicis Semen are esters, unsaturated fatty acids, sugars, lactam and so on. In traditional Chinese medicine, it is conducive to removing dampness, relaxing the muscles, blood circulation, clearing heat, anti-inflammatory effect, and detoxification [5]. Modern pharmacological studies have shown that triglycerides in Coicis Semen have anti-tumor effects [6]. Coicis Semen has been proven to have a positive therapeutic effect on ischemic stroke [7]. In this report, the western blot result displayed that the contain of tight junction protein ZO-1 and Occludin increased with the treatment of Coicis Semen.

2. Material and methods

2.1. Coicis Semen (CS) powder preparation

CS seeds were pulverised by pulverizer first. Then accurately weighed CS powder were soaked in 70% ethanol in a ratio of 1:10 (Weight: volume) overnight. After that, the extract was ultrasonic for 2 hours. The extracted solution was then vacuum filtered. The extraction step was repeated for 3 times and all filtrate was combined. Ethanol was evaporated at 55-60℃, in vacuum environment. The rest mixture solution was then frozen at -80℃ in aliquots. To get the pure CS powder, the solution was freezing dried. These powders were weighed and the extraction rate was calculated according to the ratio of raw material to end products. The powder was stored at -20℃ for further using.

2.2. bEnd.3 inflammation model

Medium for bEnd.3 cells contained DMEM (HG), heat inactivated FBS, PS and L-glutamine. Medium for RAW cells contain DMEM (HG), PS and 10%FBS. RAW were kept in the environment of 1μg/ml LPS. To stimulate the synthesis of inflammatory factors, RAW cells were incubated at 37℃ for 1h. The medium of LPS treated RAW were added into the bEnd.3 6-well plate. The 6-well plate with treated medium and bEnd.3 cells were kept in 37℃ for 24h for further use.

2.3. CS treatment

The medium of LPS treated bEnd.3 was removed, with 1ml PBS washing each well twice. 2ml medium was added into each well. CS powder was dissolved in the medium to make 5mg/ml CS stoke solution. Then, medium with 1.25μg/ml, 2.5μg/ml and 5μg/ml CS was prepared and added into the appropriate well. Then the 6-well plate with CS treated bEnd.3 was kept at 37℃ for 24h.

2.4. NO test

Griess reagent was applied to test the concentration of NO. Stock solution A (2% sulfanilamide in 10% phosphoric acid) and stock solution B (0.2% naphtylethylenediamine dihydrochoride in Milli-Q water) were mixed by 1:1 to make working Griess reagent. 100μl Griess reagent were added into the well of 96-well plate with 100μlmedium collected in 2.4 in the order of control, model, 1.25, 2.5, 5. The process was duplicated 3 times. Then, the plate was incubated at room temperature for 10 min, followed by absorbance test through spectrometer.

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2.5. Western blot

7.5% resolving gel or lower gel was made and 4% stacking gel or upper gel was prepared later. Two batches of samples were tested in one running of Western Blot in two gels. The samples and biomarkers were run in SDS polyacrylamide gel (SDS-PAGE) until the loading dye and biomarker reach the bottom of the gel. PVDF membrane was soaked in methanol for 30 seconds to activate it. To transfer protein to the activated membrane, the transfer was run at 100V for 2 hours. After transfer, these two membranes were blocked in 5% BSA for 2 hours. The membrane was cut according to the molecular size for the incubation of β-Actin (43kDa), HIF-1α (120kDa), P-NF-kB (65kDa) and P-IkBα (40kDa). These strips were shaken in the primary antibody at 4oC for two nights. Then the stripes were shaken in goat anti-rabbit secondary antibody (Invitrogen) at 4 ℃ overnight. These stripes were soaked in ECL (Thremo Scientific) and the signal was measured using digital imager.

2.6. Neutrophil analysis through zebrafish

Three days old embryos of zebrafish were used in this experiment. The embryos were grown in 26 ± 1℃ water (pH 7.2-7.6, salinity 0.03%-0.04%). 1μM CuSO4 solution (1mM) was for model control. 1μM CuSO4 stock solution with 1μM Indomethacin was added for positive control. 1μM CuSO4 with 5μg/ml and CS 2.5μg/ml CS were added for CS treatment group. 20 zebrafish embryos were added into each well with water moving away. Normal water was added into the one labeled with -ve. The rest wells were added with corresponding solution. The plate was incubated at 28 ± 1℃ for 60 min. Each group of 20 embryos were then transferred to a 2ml tube. The solution in it was removed and 4% PFA was added. The embryos were then fixed by PFA at 4℃ overnight.

In the next day, PFA was removed, and the embryos were rinsed by 1ml PBST for 5 min 3 times. After PBST was removed, the embryos were washed with 50% ethanol for 3 min. Working Sudan black staining solution was added into the embryos. The stained embryos were washed by 1ml 70% ethanol for 5 min 4 times on a mini orbital shaker. The embryos were then washed with PBST 5 min at room temperature twice on a mini orbital shaker after the 70% ethanol was removed. 1ml bleach solution was added into each tube and incubated at room temperature for 10 min on the shaker with cap of the tube open. Then the bleach solution was removed. The embryos were then washed with 1ml ethanol for 5 min, followed by 1ml PBST for 1 min on the shaker. After removing PBST of the previous step, 1ml clearing solution 1 was added into the tube, followed by shaking for 15 min at room temperature. Then, the clearing solution 1 was removed and 1ml clearing solution 2 was added. The tubes were then shaken for 10 min at room temperature. Clear solution 2 was then removed and the embryos were washed by 1ml PBST for 3 min on the shaker at room temperature. After that, 1ml 50% glycerol (in PBS) was added and the embryos were incubated at 4℃ overnight. last, the image of each embryo was taken.

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3. Result

3.1. Effect of CS on NO production

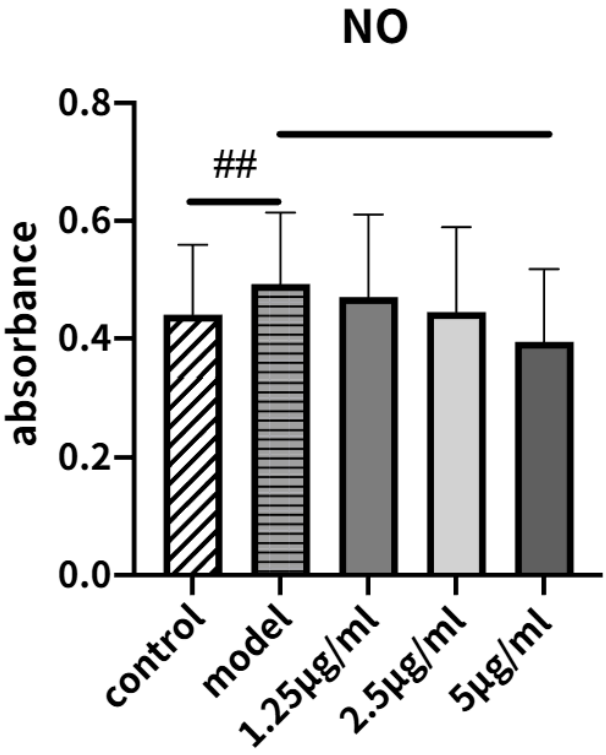
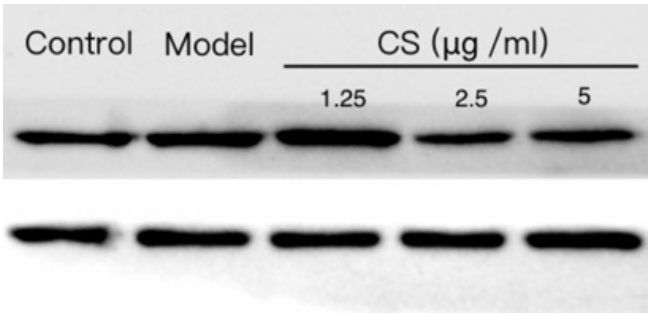


Figure 1. Evaluation of effect of CS on NO production in bEnd.3 cells

In figure 1, absorbance is positively correlated with the concentration of NO products. Model had the highest absorbance. Absorbance decreased steadily with the adding concentration of CS. NO concentration of the medium of model is higher than that of control with ## $p < 0.01$. NO concentration decreases with the increase of concentration of CS. However, ** $p < 0.01$ only appears for 5μg/ml versus model.

3.2. Evaluation of P-NF-kB expression by Western bloting



(A) P-NF-kB β-Actin

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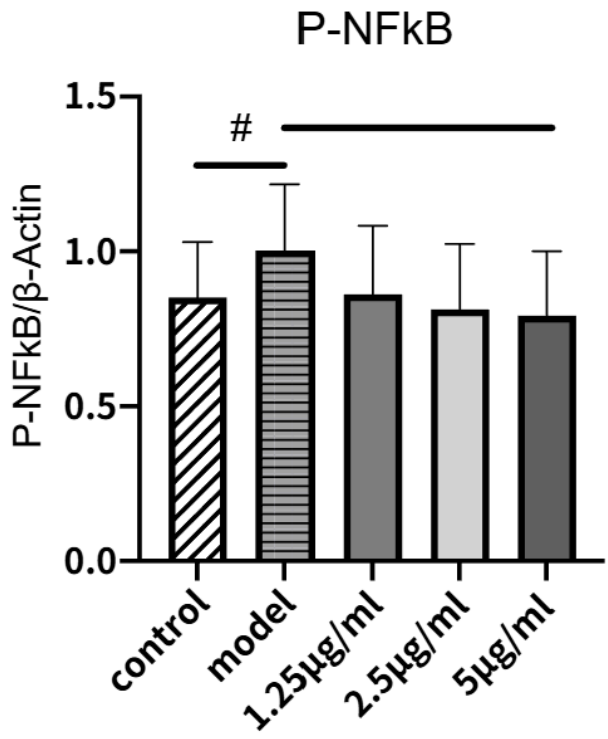
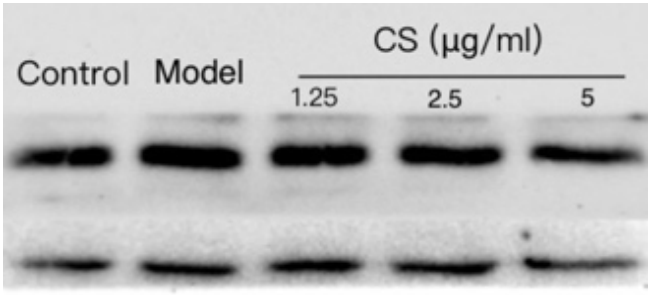


(B) CS concentration

Figure 2. Evaluation of sythesis of P-NF-kB in bEnd.3

In Figure 2, the area of the band of CS treated cells in P-NF-kB is significantly smaller than that of model. Model has the highest amount of P-NF-kB. The ratio of P-NFkB reached the lowest value when CS concentration is 5μg/ml. A sharp decrease can be observed between model and 1.25μg/ml, followed with a smooth decline from the group treated with 1.25μg/ml to 2.5μg/ml. # $p < 0.05$ for control versus model. Cells treated with 5μg/ml CS has the lowest amount of P-NF-kB and * $p < 0.05$ versus model.

3.3. Evaluation of P-IkB expression by Western bloting



(A) P-IkB β-Actin

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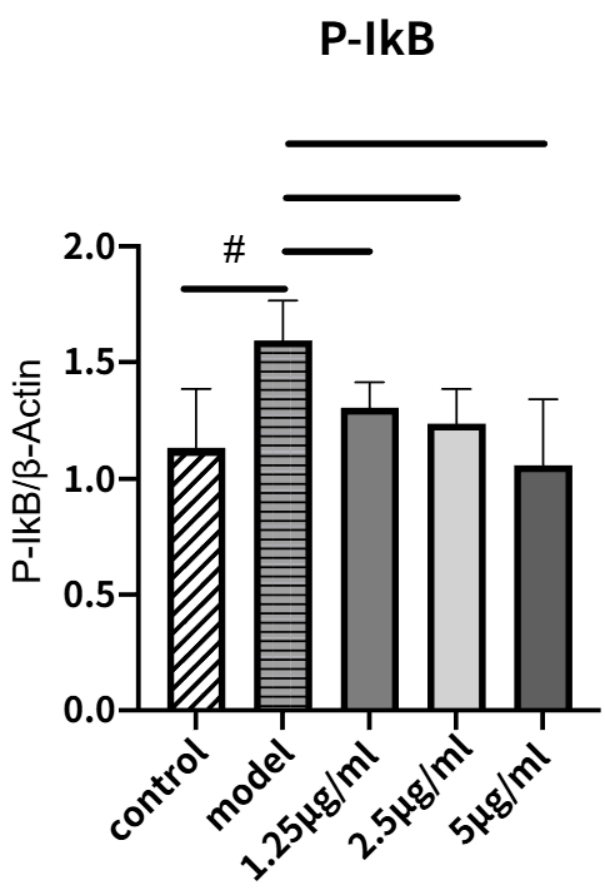
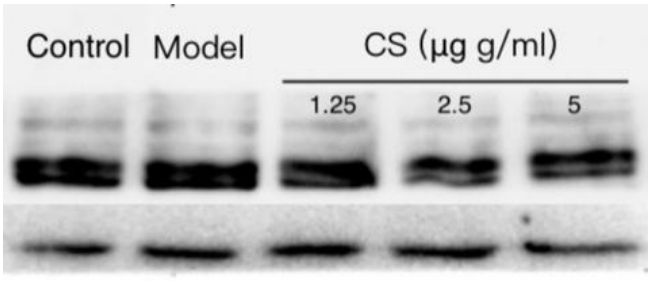


(B) CS concentration

Figure 3. Evaluation of synthesis of P-IkB in bEnd.3

In Figure 3, the area of the band of CS treated cells in P-IkB is significantly smaller than that of model. Model has the highest amount of P-IkB. The content of P-IkB decreased with the adding of concentration of CS and reached the lowest level in 5μg/ml CS. A sharp increase of protein ratio can be viewed from control group to model. The ratio of protein did not change much from 1.25μg/ml CS group to 2.5μg/ml group. However, an obvious decrease happened from 2.5μg/ml CS group to 5μg/ml CS group. # $p < 0.05$ for control versus model. Cells treated with 5μg/ml CS has the lowest amount of P-IkB and * $p < 0.05$ versus model. The amount of P-IkB decreased progressively from model to 5μg/ml. * $p < 0.05$ for 1.25μg/ml treated versus model. ** $p < 0.01$ for 2.5μg/ml.

3.4. Evaluation of HIF-1α Expression by Western bloting



(A)HIF-1α β-Actin

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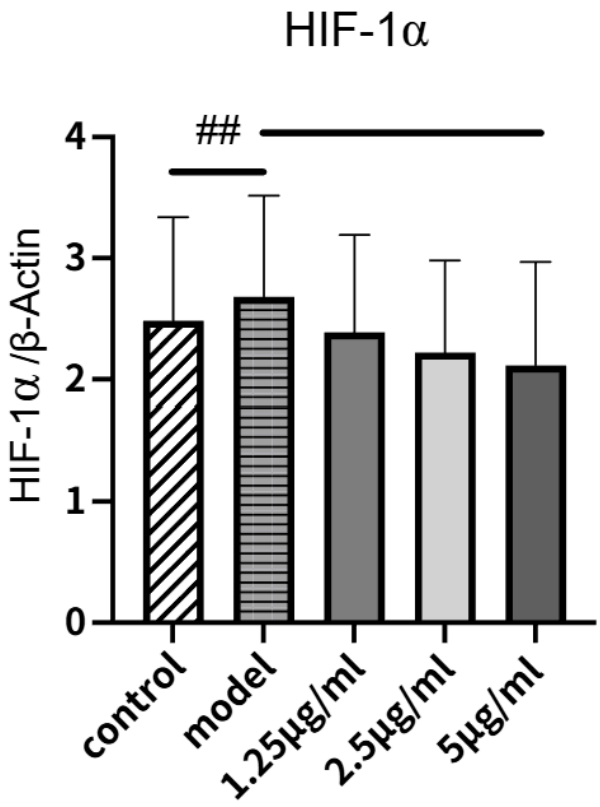

(B) CS concentration

Figure 4. Evaluation of synthesis of HIF-1α in bEnd.3

In Figure 4, the area of the band of CS treated cells in HIF-1α is significantly smaller than that of model. Model has the highest amount of P-NF-kB. The ratio of HIF-1α decreases with the adding of CS concentration, which shared similar trend with that of P-NF-kB. ## $p < 0.01$ for control versus model. Cells treated with 5μg/ml CS has the lowest amount of P-NF-kB and * $p < 0.05$ versus model.

3.5. Evaluation of neutrophil in zebrafish

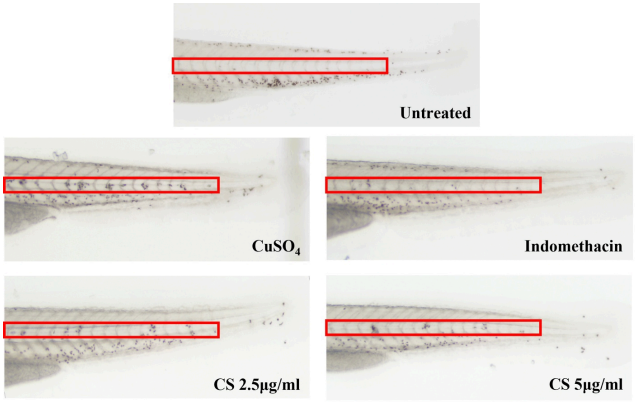

Figure 5. image of neutropils in zebrafish embryos

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The neutrophils in the red square were counted for further analysis. The untreated group had the lowest number of neutrophils and very few purple dots, which are neutrophils, could be found in the red square. Obvious purple dots can be viewed in model ($CuSO_4$ treated group). The number of purple dots decreases significantly in positive control (Indomethacin treated group) and CS treated group.

3.5.1. Number of neutrophils

Table 1. Counting result of neutrophils

No.	Untreated	Model	1μM indomethacin	2.5μg/ml CS	5μg/ml CS
1	2	28	6	16	8
2	2	11	12	10	7
3	2	22	12	6	8
4	1	28	9	9	10
5	9	15	7	10	8
6	1	27	3	14	10
7	1	25	3	12	8
8	7	12	10	15	13
9	10	14	11	9	11
10	1	20	8	6	7
11	4	22	6	10	7
12	2	21	5	12	13
13	3	15	10	6	15
14	2	18	10	13	12
15	0	19	7	13	12
16	1	17	3	9	9
17	7	21	4	10	14
18	4	33	8	9	12
19	3	18	10	19	6
20	1	46	6	6	7
Average	3.15	21.6	7.5	10.7	9.85

The number of neutrophils of each embryo was counted in red square shown in the photo above.

Anti-inflammatory efficiency = (neutrophil No. in model control-neutrophil No. in testing sample)/(neutrophil No. in model control) ×100%

The anti-inflammatory efficiency of indomethacin is 65.3%. The anti-inflammatory efficiency of 2.5μg/ml CS is 50.4%. The anti-inflammatory efficiency of 5μg/ml CS is 54.4%. The positive control had the highest efficiency. The efficiency of 5μg/ml CS was higher than that of 2.5μg/ml.

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3.5.2. Evaluation of the number of neutrophils

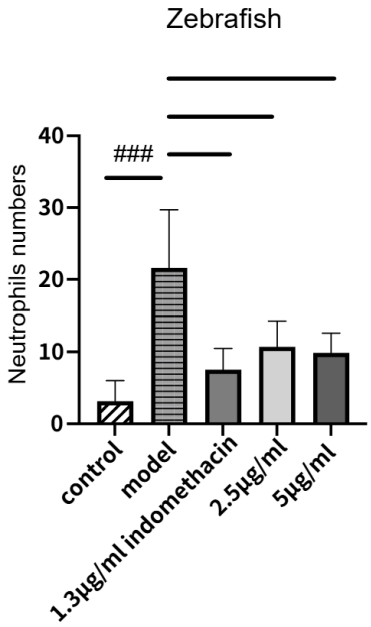

Figure 6. Evaluation of the number of neutrophils

All other groups of neutrophil number have ### or *** $p < 0.001$. A sharp decline of number of neutrophils can be viewed in both +ve group (indomethacin) and CS treated group. The number of 5μg/ml treated is a little smaller than that of 2.5μg/ml. Untreated embryos had the lowest amount of neutrophils. Although CS treated embryos had much less neutrophils, the number of neutrophils is still higher than +ve.

4. Conclusion

CS can be applied in anti-inflammation pathway. LSP induced bEnd.3 monoculture inflammatory model failed to show an obvious inflammatory reaction. LPS treated co-culture of RAW and bEnd.3 was applied to make the inflammatory model. 5μg/ml CS can be proven to inhibit the expression of P-NF-kB, P-IkB and HIF-1α, and the production of NO. More repetitions are needed to increase the significance. Both 2.5μg/ml and 5μg/ml CS have high anti-inflammatory efficiency in zebrafish experiment. The production of neutrophils showed a sharp decrease when treated with CS. The experiment on bEnd.3 is expected to be repeated more times. Test of iNOS can be optimized by using new anti-iNOS antibody. CS has a good efficiency in anti-inflammation. It is an essential Chinese medicine with a wide range of application value in the field of anti-inflammation.

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