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Der Pharmacia Lettre, 2016, 8 (3):53-61
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The effect of Syringin on the expression of TNF- α , iNOS, ICAM-1 and its' mRNA in the heart, brain and kidneys of spontaneously hypertensive rats

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ABSTRACT

To investigate the effect of syringin (SYN) on TNF- α , iNOS, ICAM-1 and its' mRNA expression in the heart, brain and kidneys of spontaneously hypertensive rats (SHR), and reveal the mechanism of its anti-inflammatory injury in hypertension target organs. SHRs were randomly divided into 5 groups including the model group (Group-2), the high-dose (G-4), medium-dose (G-5), low-dose (G-6) SYN group and the benazepril group (G-3), with 5 rats in each group. Wistar Kyoto (WKY) rats were used for the normal control group (G-1). Non-invasive blood pressure (BP) instruments were used to measure systolic blood pressure in the rats tail artery; used western blot to analyze the expression of TNF- α , iNOS, ICAM-1 and used reverse transcription-polymerase chain reaction (RT-PCR) to analyze the expression of TNF- α , iNOS, ICAM-1 mRNA. Compared with the normal control group, the model group's BP level was significantly increased ($P < 0.01$), but the SYN had no significant lowering effect ($P > 0.05$); compared with the normal control group, the expression of TNF- α , iNOS, ICAM-1 and its' mRNA in the model group was significantly increased ($P < 0.05$ or $P < 0.01$), and SYN could reduce the level of expression of these inflammatory cytokines ($P < 0.05$ or $P < 0.01$); between the benazepril group and each dose SYN group, most of the indicators had no significant difference ($P > 0.05$). SYN had no significant BP lowering effect; SHR showed inflammatory injury in the heart, brain and kidneys; improvement on the inflammatory injury, and in turn, the anti-inflammation mechanism may be associated with lowering TNF- α , iNOS and ICAM-1 and its' mRNA expression.

Keywords: Syringin, Spontaneously Hypertensive Rat, inflammatory factor, RT-PCR, Western Blot.

INTRODUCTION

Syringin, a phenyl propanoid glycoside belongs to eleutheroside derivative. This bioactive compound was identified in *Musa paradisiaca*. The pharmacological properties of syringin includes scavenging the free radicals [1,2], protection against neuronal cell damage [3], inhibition of apoptosis [3], antidiabetic effect [4], antiulcer [5], anti-inflammatory potential [6], etc. Hypertension is a low-grade inflammation state of the disease [7-8], and easily complicated by heart, brain, kidneys and other organs' inflammatory response [9], however studies on SYN anti-hypertensive inflammatory injury is still poorly reported. In previous research, we found that SYN had a protective effect on the GIT [10]. This paper will reveal the mechanism of SYN protection on heart, brain and kidneys' inflammatory injury in SHRs by observing the effect of SYN on inflammatory factors including Tumor necrosis factor alpha (TNF- α), Inducible nitric oxide synthase (iNOS), Intercellular Adhesion Molecule 1 (ICAM-1) and its' mRNA expression in heart, brain and kidneys. This will provide an experimental basis for finding a new drug to reduce hypertensive target organs' inflammatory injury in the same time.

MATERIALS AND METHODS

Experimental Animals

The ten-week-old male SHRs and ten-week-old male normotensive Wistar-Kyoto (WKY) rats were used. The

weights ranged between 280g to 320g, and were obtained from Universiti Sains Malaysia, Penang. These rats were randomly divided into the model group (G-2), the high-dose (G-3), medium-dose (G-4), low-dose (G-5) SYN group and the benazepril group (G-3), with 5 rats in each group. The WKY rats (5 numbers) were used for the normal control group (G-1). The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the Institutional ethical guidelines (UNISZA/AEC/14/007). The animals were kept under controlled conditions for temperature, humidity and light, with unrestricted access to food and water available throughout all experimental stages.

Drugs and Main Reagents

SYN (CAS No: 118-34-3, molecular formula: $C_{17}H_{24}O_9$) is an extract of tepals of *Musa paradisiaca*, was provided by Sigma-Aldrich (M) Sdn Bhd, Selangor, Malaysia.

Primary antibodies of TNF, iNOS, ICAM-1 rabbit polyclonal antibodies and goat anti-rabbit horse radish peroxidase labeled secondary antibodies were from Santa Cruz, SDS-PAGE gel preparation kit were from Biyuntian, RIPA (R0095), protein extracts were from Axon Scientific Sdn Bhd, RNA enzyme A (RNase A) were from Sigma; Trizol(15596-026)reagent extracts were from Invitrogen, reverse transcription kit (MBI 00032062) were from the Revert Aid RTTMFISRT, TNF- α , iNOS and ICAM-1 mRNA primers were from Axon Scientific Sdn Bhd.

Drug Treatment

SYN was dissolved in heated distilled water, dissolved into desired concentration, respectively 4, 2, 1 g/ L. The rats in the SYN groups were treated with a high, medium and low dose of SYN [40, 20, 10 mg/ (kg·d), respectively, intragastrically (ig)]; benazepril [10 mg/(kg·d),ig] was administered in the benazepril group; distilled water was applied to the rats in the normal control group and the model group, all rats were treated for 8 weeks consecutively [10 mL/(kg·d), ig].

Blood Pressure Measurement

Non-invasive blood pressure instruments used to measure systolic blood pressure in the rats' tail artery. Before taking measurements the rats were placed in a 35°C incubator to warm up for 10 minutes to dilate the vessels, then pressure was measured; took 4 to 6 measurements; and then took the average.

Tissue collection

Fasting 12 hours after the last drugs' administration, the rats were anesthetized intra-abdominally with chloral hydrate (0.3 mg/kg). Firstly an incision was made in the abdomen to expose the kidneys which were removed and washed in saline. The heart was removed, washed in saline. Lastly the skull was opened and the brain was removed.

Tissue preparation for Western Blot

The tissues were rapidly frozen in tissue wells, then placed directly on dry ice, and stored at -80°C. In each 100mg tissue added 1mL RIPA and 10 μ L 100mM PMSF and 10 μ L of phosphatase inhibitors (not previously added to RIPA, otherwise lose performance PMSF). Homogenized, avoid foaming and heating, the samples were transferred to 1.5mL tubes, ice placed 30min. 1.5mL tubes containing samples, 4° 12000g / min high speed centrifugation 30min. Carefully transfer the supernatant to a clean sterile centrifuge tube, -20° preservation.

Tissue preparation for RT-PCR

The tissues were cut into pieces (maximum surface can not be more than 0.5 cm), and 1 mL Trizol was added to the sample, then placed into a 4°C refrigerator overnight and then moved to a -80°C refrigerator.

Western Blot procedure

20 μ L of tissue lysate and add 5 μ L 5 × electrophoresis sample buffer (Prestained Maker fetch 5 μ L). The boiling water bath for 5min, 12000g / min centrifugal 3 min, remove insoluble proteins, samples were loaded and separated by electrophoresis (5% stacking gel 90V, 12% separating gel 110V, not constant current) about 1.5h. Take separated glue transfer film (the electric meter wiped film, 90V 30min). Open the electroporation instrument, remove the nitrocellulose membrane washed with deionized water, lotion equilibrated with 5% skim milk (diluted with PBS or TBS) blocking at room temperature 30min, then 4°C overnight, the next day at room temperature equilibrium. Add diluted primary antibodies of TNF, iNOS, ICAM-1 (1: 2000, diluted solution containing 2% skim milk), 4 °C overnight. PBS washing the membrane, the first 15min, after three each 5min. Add 5mL 1: 5000 dilution (diluted solution containing 2% skim milk powder) of goat anti-rabbit secondary antibody, 37°C reaction for 1h. And after washing thoroughly, dried, apposed to Kodak XAR5 film (Sigma), and exposed at -70°C. Use WD-9413B gel imaging system comes with software to analyze the film Gelpro32 of protein bands of gray value.

RT-PCR procedure

Total RNA extraction, identification, reverse transcriptase in the PCR instrument, PCR reaction, results observation in gel electrophoresis imaging analyzer. All gel electrophoresis results were analyzed with quantity one 4.6.2 software, the average A of each group was calculated, use the ratio of gene A and the corresponding β -actin as the mRNA relative expression level.

Relative expression level = A target gene / A β -actin gene

The primers and their sequences are shown in Table 1 and the primers' PCR cycles parameters are shown in Table 2.

Table 1. The Primers and their Sequences

TNF- α	Sense	Primer: 5'-TTGACCTCAGCGCTGAGTTG-3'
	Antisense	Primer: 5'-CCTGTAGCCCACGTCGTAGC -3'
iNOS	Sense	Primer: 5'- AGCTCCTCCCAGGACCACAC -3'
	Antisense	Primer: 5'-ACGCTGAGTACCTCATTGGC -3'
ICAM-1	Sense	Primer: 5'-AAACGACGCTTCTTTTGCTC-3'
	Antisense	Primer: 5'-GGTGTTCCTTTCTTCTCTTGCT-3'
β -actin	Sense	Primer: 5'-TGGAATCCTGTGGCATCCATGAAAC -3'
	Antisense	Primer: 5'-TAAAACGCAGCTCAGTAACAGTCCG -3'

Table 2. The Primers' PCR Cycles Parameters

Number of cycles	Temperature(°C)	Time	Process
1	95	3min	preparatory denaturation
35	94	30sec	Denaturation
35	56	30sec	annealing
35	72	1.5min	Extending
1	72	8min	heat preservation
	4		preservation

Data Management and Statistical Analysis

The data was analyzed using SPSS11.0. The data was presented as mean $\pm$ standard deviation. The significance of the difference between multiple means was calculated by a single-factor analysis of variance. Probability values of $P < 0.05$ was considered to represent significant differences.

RESULTS**Effect of SYN on Blood Pressure levels of the SHRs**

Before intragastric administration, compared with the normal control group, the model group's blood pressure level was significantly increased ($P < 0.01$). After intragastric administration for 4 weeks and 8 weeks, compared with the normal control group, the model group's BP level was significantly increased ($P < 0.01$) compared with the model group. The benazepril groups' BP level was significantly decreased ($P < 0.01$). SYN group's BP level was slightly decreased, but were not statistically different ($P > 0.05$). Among the intervention groups, SYN groups' BP level was significantly higher than that of the benazepril group ($P < 0.01$) (Table 3).

Table 3 Comparison of Blood Pressure levels among different groups before and after 4-week, 8-week intervention

($\bar{X} \pm s$, n=5, mmHg)

Group	BP before intervention	BP after 4-week intervention	BP after 8-week intervention
Normal control	113.05 $\pm$ 2.34	112.36 $\pm$ 3.45	114.33 $\pm$ 6.58
Model	162.43 $\pm$ 30.55**	174.39 $\pm$ 31.33**	187.59 $\pm$ 37.77**
Benazepril	164.37 $\pm$ 32.34	147.36 $\pm$ 23.35**	155.25 $\pm$ 34.54**
High-dose SYN	163.38 $\pm$ 31.28	170.21 $\pm$ 29.50 ^{AA}	181.21 $\pm$ 34.40 ^{AA}
Medium-dose SYN	162.56 $\pm$ 30.36	171.26 $\pm$ 30.40 ^{AA}	183.26 $\pm$ 32.60 ^{AA}
Low-dose SYN	165.49 $\pm$ 29.38	172.45 $\pm$ 32.78 ^{AA}	184.45 $\pm$ 33.58 ^{AA}

Note: ** $P < 0.01$, compared with the normal control group; ** $P < 0.01$, compared with the model group; ^A $P < 0.01$, compared with the benazepril group.

Effect of SYN on the expression of TNF- α , iNOS and ICAM-1 in the heart of SHR

Compared with the normal control group, the expression of TNF- α , iNOS and ICAM-1 in the model group was significantly increased ($P < 0.01$); compared with the model group, the expression of TNF- α , iNOS and ICAM-1 in the Benazepril group was significantly decreased ($P < 0.05$ or $P < 0.01$), that of each dose SYN group was significantly decreased ($P < 0.05$ or $P < 0.01$) (Figure 2).

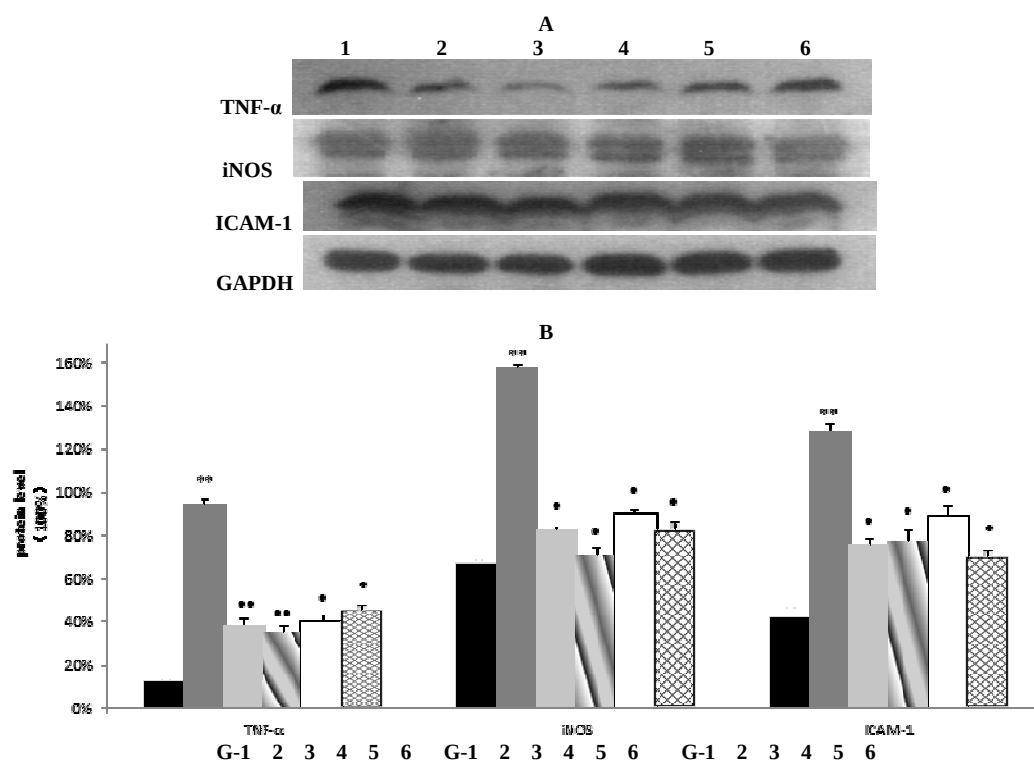


Figure 2 Comparison of TNF- α , iNOS and ICAM-1 expression in the heart of SHR among different groups

Notes: A: Electrophoresis. 1: Normal control group; 2: Model group; 3: Benazepril group; 4: High-dose SYN group; 5: Medium-dose SYN group; 6: Low-dose SYN group.

B: ** $P < 0.01$, compared with the normal control group; * $P < 0.05$, ** $P < 0.01$, compared with the model group.

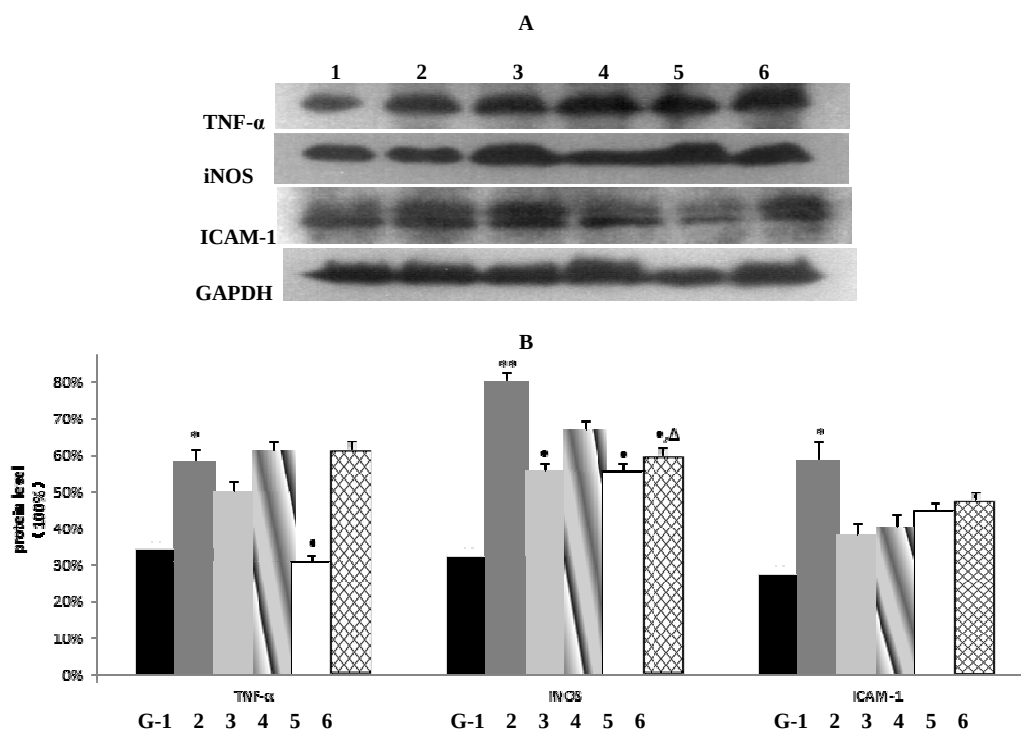


Figure 3 Comparison of TNF- α , iNOS and ICAM-1 expression in the brain of SHR among different groups

Notes: A: Electrophoresis. 1: Normal control group; 2: Model group; 3: Benazepril group; 4: High-dose SYN group; 5: Medium-dose SYN group; 6: Low-dose SYN group.

B: * $P < 0.05$, ** $P < 0.01$, compared with the normal control group; * $P < 0.05$, compared with the model group; ^Δ $P < 0.05$, compared with the benazepril group.

Effect of SYN on the expression of TNF- α , iNOS and ICAM-1 in the brain of SHR

Compared with the normal control group, the expression of TNF- α , iNOS and ICAM-1 in the model group was increased ($P<0.05$ or $P<0.01$); compared with the model group, the expression of iNOS in the Benazepril group was decreased ($P<0.05$), the expression of TNF- α , iNOS in medium and low dose SYN group was decreased ($P<0.05$), compared with the Benazepril group, the expression of iNOS in low-dose SYN group was decreased ($P<0.05$) (Figure 3).

Effect of SYN on the expression of TNF- α , iNOS and ICAM-1 in the kidneys of SHR

Compared with the normal control group, the expression of TNF- α in the model group was increased ($P<0.05$); compared with the model group, the expression of TNF- α in the medium-dose SYN group was decreased ($P<0.05$), the expression of iNOS in the low-dose SYN group was decreased ($P<0.05$) (Figure 4).

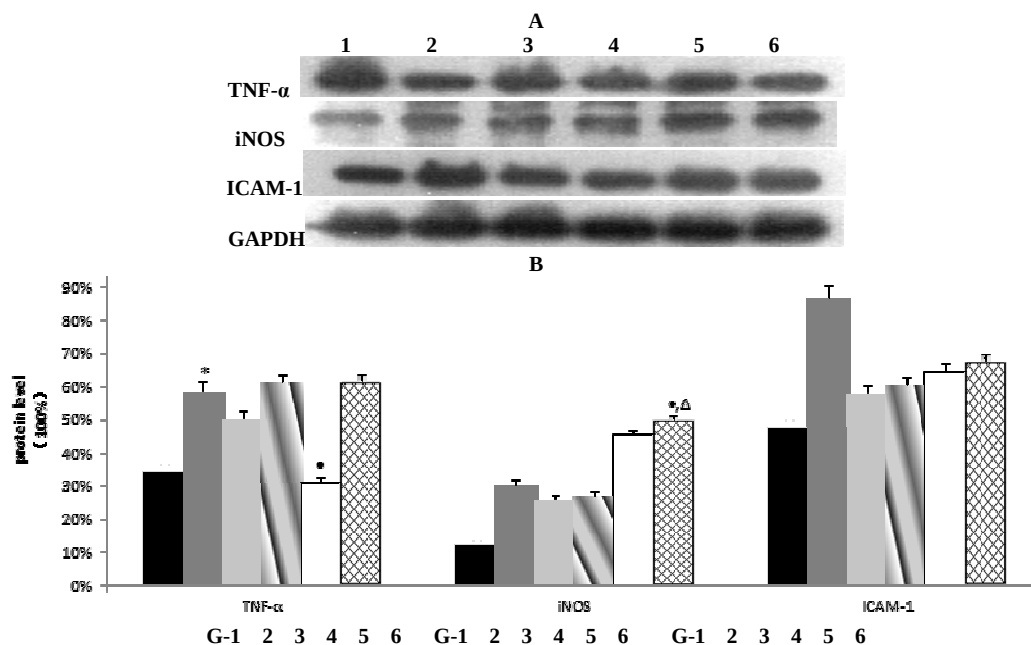


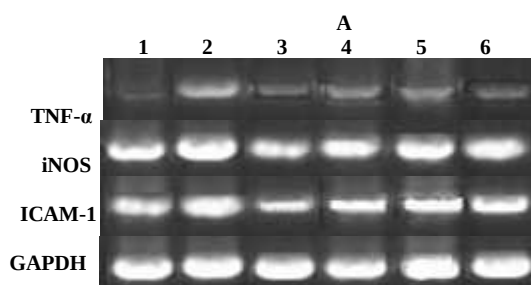
Figure 4 Comparison of TNF- α , iNOS and ICAM-1 expression in the kidneys of SHR among different groups

Notes: A: Electrophoresis. 1: Normal control group; 2: Model group; 3: Benazepril group; 4: High-dose SYN group; 5: Medium-dose SYN group; 6: Low-dose SYN group.

B: * $P<0.05$, compared with the normal control group; * $P<0.05$, compared with the model group.

Effect of SYN on the expression of TNF- α , iNOS and ICAM-1 mRNA in the heart of SHR

Compared with the normal control group, the expression of TNF- α , iNOS and ICAM-1 mRNA in the model group was significantly increased ($P<0.01$); compared with the model group, the expression of TNF- α , iNOS and ICAM-1 mRNA in the Benazepril group was significantly decreased ($P<0.05$ or $P<0.01$), that of each dose SYN group was significantly decreased ($P<0.01$); Among the intervention groups, the expression of TNF- α mRNA in the medium-dose and low-dose SYN group was higher than that of Benazepril group ($P<0.05$ or $P<0.01$), the expression of iNOS mRNA in the high-dose and medium-dose SYN group was lower than that of the Benazepril group ($P<0.05$ or $P<0.01$) (Figure 5).



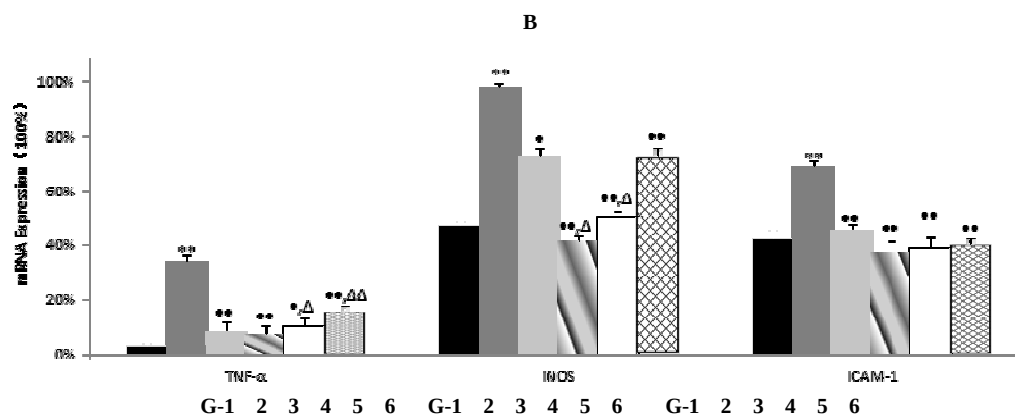


Figure 5. Comparison of TNF- α , iNOS and ICAM-1 mRNA expression in the heart of SHR among different groups

Notes: A: Electrophoresis. 1: Normal control group; 2: Model group; 3: Benazepril group; 4: High-dose SYN group; 5: Medium-dose SYN group; 6: Low-dose SYN group.

B: ** $P < 0.01$, compared with the normal control group; * $P < 0.05$, ** $P < 0.01$, compared with the model group; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, compared with the benazepril group.

Effect of SYN on the expression of TNF- α , iNOS and ICAM-1 mRNA in the brain of SHR

Compared with the control group, the expression of TNF- α and ICAM-1 mRNA in the model group was significantly increased ($P < 0.05$ or $P < 0.01$); compared with the model group, the expression of TNF- α and ICAM-1 mRNA in benazepril group was significantly decreased ($P < 0.05$ or $P < 0.01$), that of each dose SYN group was significantly decreased ($P < 0.01$), the expression of iNOS mRNA in the high-dose and medium-dose SYN group was significantly decreased ($P < 0.01$); Among the intervention groups, the expression of iNOS mRNA in the high-dose SYN group was lower than that of the Benazepril group ($P < 0.05$) (Figure 6).

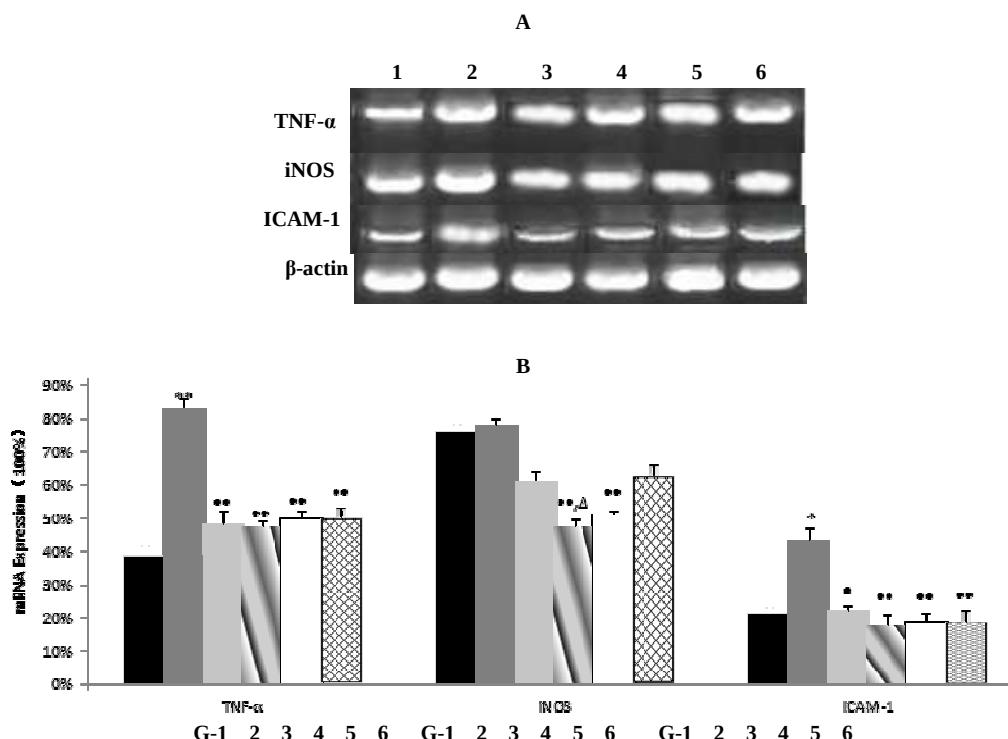


Figure 6 Comparison of TNF- α , iNOS and ICAM-1 mRNA expression in the brain of SHR among different groups.

Notes: A: Electrophoresis. 1: Normal control group; 2: Model group; 3: Benazepril group; 4: High-dose SYN group; 5: Medium-dose SYN group; 6: Low-dose SYN group.

B: * $P < 0.05$, ** $P < 0.01$, compared with the normal control group; * $P < 0.05$, ** $P < 0.01$, compared with the model group; $^{\Delta}P < 0.05$, compared with the benazepril group.

Effect of SYN on the expression of TNF- α , iNOS and ICAM-1 mRNA in the kidneys of SHR

Compared with the control group, the expression of TNF- α , iNOS and ICAM-1 mRNA in the model group was significantly increased ($P < 0.01$); compared with the model group, the expression of TNF- α , iNOS and ICAM-1

mRNA in the Benazepril group was significantly decreased ($P < 0.01$), that of each dose SYN group was significantly decreased ($P < 0.01$); Among the intervention groups, the expression of iNOS mRNA in the high-dose SYN group was lower than that of the Benazepril group ($P < 0.05$) (Figure 7).

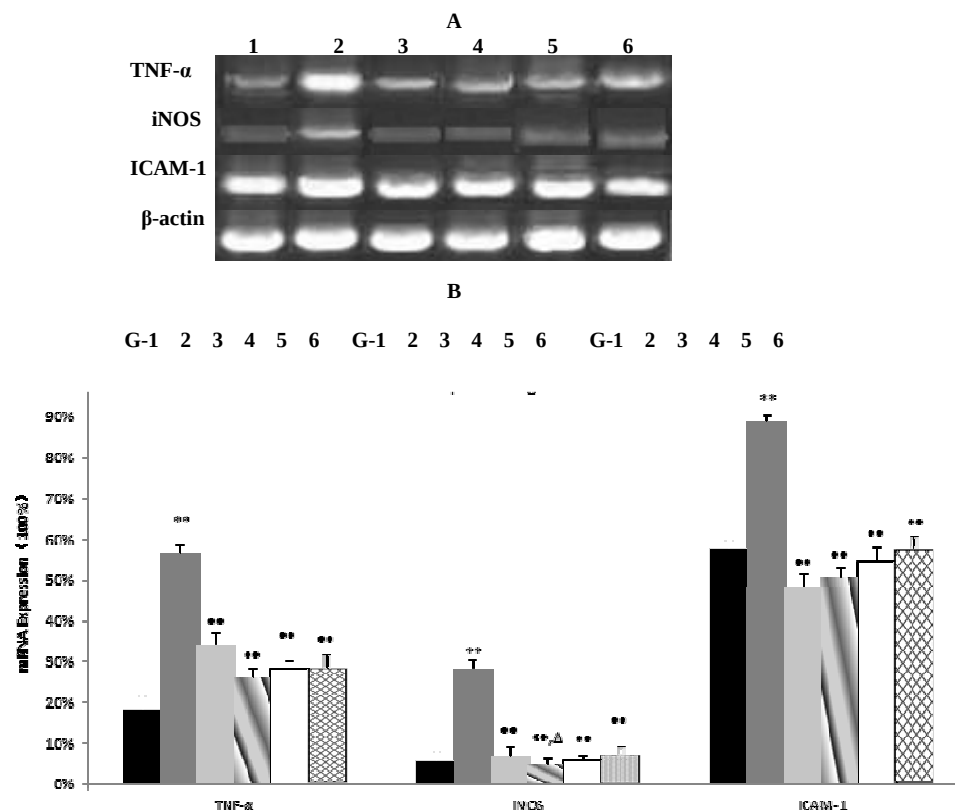


Figure 7. Comparison of TNF-α, iNOS and ICAM-1 mRNA expression in the kidneys of SHR among different groups

Notes: A: Electrophoresis. 1: Normal control group; 2: Model group; 3: Benazepril group; 4: High-dose SYN group; 5: Medium-dose SYN group; 6: Low-dose SYN group.

B: ** $P < 0.01$, compared with the normal control group; *** $P < 0.01$, compared with the model group; ^a $P < 0.05$, compared with the benazepril group.

DISCUSSION

Musa paradisiaca grows in humid lowland to upland tropical areas comprising banana and plantain; it is among the world's leading fruit crops, which are large perennial herbs growing from a sympodial rhizome. It has traditionally been used for antihypertensive since it is rich in syringin. [11-12]. Apart from syringin, the flowers of *Musa paradisiaca* was reported to contain various biologically active phytochemicals such as pectin, leucocyanidin, quercetin, syringin, β sitosterol and terpenoid glucosides [13-14]. Syringin, a phenylpropanoid glucoside is a found to be distributed in the tepals of *Musa pardisiaca*. Various pharmaceutical actions of syringin have been reported. Syringin is effective for the treatment of hypertension.

Hypertension is one of the most common cardiovascular diseases [15]. Hypertension is has a high incidence of important risk factors for cardiovascular diseases, it can cause heart, brain and kidney damage, the treatment for hypertension must attach great importance to the protection of the heart, brain, kidney [16-18]. In recent years, the effect of inflammation in the occurrence and development of hypertension has attracted the attention of more and more scholars [19].

SHR model is the model closest to the genetic background of human essential hypertension, can increase blood pressure level spontaneously, so it is widely used in the study of medical research [20]. The heart, brain and kidneys of SHR have significant inflammatory injury [21].

TNF-α plays an important role in the regulation of neuroendocrine, vascular endothelial injury, vascular wall inflammation, smooth muscle cell proliferation and vascular remodeling, it may be involved in the pathological process of essential hypertension's occurrence and development [22]. ICAM-1 belongs to a member of the immunoglobulin superfamily, it is a transmembrane single-chain glycoprotein, and it was reported that ICAM-1

expression level was significantly increased in hypertensive rats [23]. Under normal physiological conditions, iNOS has no expression generally, but in the pathological state, endotoxin and various cytokines can induce expression of iNOS gene in macrophage and leukocyte, lead to a lot of NO production to inhibit the effect of endotoxin and various cytokines, in the same time, cause strong blood pressure lowering effect [24].

In this study, compared with the normal control group (G-1), the model group's (G-2) BP level was significantly increased; before and after 4 weeks, the blood pressure was continuously increasing. The trend of changes in blood pressure was consistent with the results in the past report [25]. However SYN had no significant lowering BP effect which is in contrast to the previous research by Mansoor A and Khalid Aftab (1995) [26]. Additionally, compared with the normal control group, the expression of TNF- α , iNOS, ICAM-1 mRNA in model group was significantly increased, this showed that hypertension rats had inflammatory injury in target organs, this was consistent with the results reported by Sun L et al (2006) [27]. SYN could reduce the level of expression of these inflammatory cytokines. It showed that SYN could reduce inflammation response, thereby protects the target organs. This was consistent with the results that SYN can lower the inflammatory factors' expression in the heart, brain and kidney reported by Jung et al (2007) and Niu et al (2008) [29-30]. Benazepril is an antihypertensive drug commonly used in clinic [31], in the same time, it can inhibit the inflammatory response of the whole body or some organs [32, 33]. In brief, between the benazepril group (G-3) and SYN groups (G-3, 4, 5), most of the indicators had no significant difference.

These results suggest that: SYN had no significant lowering BP effect, SHRs showed inflammatory injury in the heart, brain and kidneys, SYN showed improvement on the injury of the organs, the anti-inflammation mechanism may be associated with lowering TNF- α , iNOS, ICAM-1 and its' mRNA expression, this also explained that lowering BP and anti-hypertensive inflammation are two independent pharmacological effects, they have no positive correlation.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' contributions

Hu XQ, Pan WG and Deng JG participated in the design of the study; Zhou B carried out animal experiments, Luo P carried out western blot and RT-PCR experiments, Sun J drafted the manuscript, Zeng XW analyzed the data, made the tables and drew the figures, all authors read and approved the final manuscript.

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