The Effect of ANGII-NADPH oxidase-ROS Channels to Study the Effect of Gastrodin on AngII Induced Vascular Smooth Muscle Cells

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Introduction. To study how gastrodin proliferates Ang II intervention through ANG II-NADPH oxidase-ROS pathways, the proliferation, ROS production and key protein expression.

Methods. After the mice VSMCS is stimulated by ANG II, intervention with different doses of gastrodin is used to observe its impact on VSMCS proliferation and ROS with crystalline purple experiments, DCFH-DA fluorescent probe, and detect its PKC with Western-Blot to PKC, EGFR, C-SRC kinase and NADPH oxidase protein expression.

Results. gastrodin can improve the abnormal proliferation status of mice VSMCS under ANG II stimulation, inhibit the expression of ROS levels and the expression of PKC, EGFR, C-SRC kinase, NADPH oxidase protein.

Conclusion. gastrodin may reduce ROS production by inhibiting ANG II-NADPH oxidase-ROS pathway, thereby inhibiting oxidation stimulation damage and ameliorating blood vessel reconstruction.

Keywords. ANG II, ROS, gastrodin, VSMCS

INTRODUCTION

According to preliminary studies, "vascular reconstruction" is the pathological basis of hypertension [1-2]. How to reverse vascular reconstruction is of great significance to improving hypertension and its complications. According to the "oxidation theory" based on vascular reconstruction, excessive accumulation of ROS in the body will damage the blood vessels [3], which eventually leads to vascular reconstruction. ROS is generated by AngII by activating NADPH oxidase, that is, after combining ANG II and AT1 receptors, by activating PKC, EGFR, c-Src kinase these three pathways, NADPH oxidase is activated by multiple channels, which then

generates ROS, causing vascular reconstruc， tion [4-5]. Early studies have found that the effective ingredients of Chinese medicine Gastrodia can reduce the Ang II concentration and AT1 protein level in serum [6], regulate the content of endothelium and nitric oxide, protect the function of vascular endothelial, and inhibit the smooth muscle cells of the vascular muscle muscle cells by removing the radicals. Oxidation damage [7-8]. According to the preliminary research, hypothesis: gastrodin may reduce ROS production by inhibiting ANGII-NADPH oxidase-ROS pathways, inhibit oxidation stimulation damage, and ameliorate blood vessel reconstruction. In this study, through cell experiments, the effect of observing the influence of Tiansu on Ang II -induced mouse vascular smooth muscle cell proliferation, ROS generation, and related kinase and oxidase protein expression, and initially verify its mechanism to ameliorate vascular reconstruction.

2 EXPERIMENTAL STEPS

2.1 Experimental cells

Buy mouse smooth muscle cells from Wuhan Pinoccue Company and cultivate with specific medium. The cells are transparent as long as 90%, and the first four generations of cells are frozen, and four to ten generations of cells are used for subsequent experiments.

2.2 Experimental drugs, reagents, consumables and instruments

2.2.1 Experimental drugs

gastrodin (production batch number: B21243, 20mg/branch) and vascular tension II (Angotnsin II, ANG II) (goods number: S25704) purchased at Yuanye Pharmaceutical Co., Ltd.

2.2.2 Main reagent

Table 1 main reagent

Reagent name	manufacturer (goods number)
Vascular tension II	source leaves, S25704
Gastrodia	source leaves, B21243
Mouse aorta smooth muscle cells completely cultivat e the base	Pino, CM-M076
BL539A 4%polymerization formaldehyde/universal tis sue fixing solution	Biosharp, BL539A
PBS drift buffer (liquid)	Doctoral, AR0192
RPMI1640 liquid medium (including HEPES/Double Anti -)	Doctoral, pyg0126
PKC Alpha Rabbit PolyClonal Antibody	Proteintech, 21991-1-AP
EGFR (C-Terminal) Rabbit Polyclonal Antibody	PROTEINTECH, 51071-2-AP
C-SRC MOUSE MONOCLONAL ANTIBODY	PROTEINTECH, 60315-1-Ig
Nox1 rabbit polyClonal antibody	PROTEINTECH, 17772-1-AP
Crystal purple staining solution	Biyuntian, C0121-500ml
Active oxygen (ROS) detection kit	ServiceBio, G1706-100T
Enhanced RIPA cracking solution	Doctoral, AR0102
Proteinase inhibitor PMSF	Doctoral, AR1192
BCA protein concentration measurement kit	Doctoral, AR0146
Snaponic buffer on SDS-PAGE protein (5 ×)	Biyuntian, P0015L
Protein conventional molecular weight marking (10-1 80kda	Wuhan Three Eagle, PL00001
SDS-PAGE gel preparation kit	Doctoral, AR0138
Tris-Glycine-SDS Electric Polytes Caps (1 × Powde r)	Doctoral, AR0139
Western dedicated to one anti -two anti -diluted solu tion	Dr. De, AR1017
Western one anti -two anti -removal liquid (strong a lkali)	Biyuntian, P0025-1L
10 × fast rotor fluid	
TBS-T (containing 0.05%TWEEN-20) drift buffer	Doctoral, AR0042
Special super -sensitivity chemical light emitting testing kit	Doctoral, AR-0195

2.2.3 Main instruments and materials

Table 2 Main instruments and materials

Instrument and equipment name (model)	Manufacturer
6 -hole cell culture plate, flat bottom, TC treatment	Sorfa, 220100-block
Vortex hybrid [vortex-6]	Fuzhou Kanghe Er Biotechnology Co., Ltd.
Optical-1300	Like Biomedical Company
Gradient PCR instrument [MV-C155-OV71]	Hangzhou Mio Instrument Co., Ltd.
QPCR c [Archimed X6]	Kunpeng Gene (Beijing) Technology Co., Ltd.
SPECTRAMAX absorption reader CMAX Plus	Meigu Molecular Instrument (Shanghai) Co., Ltd.
Frozen centrifuge [JID-17R]	Guangzhou Jidi Instrument Co., Ltd.
Dycp-31DN	Beijing Liuyi Biotechnology Co., Ltd.
Hiscript Q RT Supermix for QPCR (+GDNA Wiper) [R123-01]	Nanjing Nuoweizan Biotechnology Co., Ltd.
Chamq universal sybr QPCR MASTER MIX [q711-02]	Nanjing Nuoweizan Biotechnology Co., Ltd.
Laboratory ultra-pure water machine [wp-up-yj-10]	Sichuan Waterwater Treatment Equipment Co., Ltd.
Ultra-micro ultraviolet visible spectrophotia [ND-100C]	Hangzhou Mio Instrument Co., Ltd.

2.3 Experimental medicinal liquid preparation

According to the requirements, the AngI (1 μmol/L) group, Gastrodin (5, 10, 20 μmol/L), are used for the configuration of the three dose groups for subsequent cell experiments.

2.4 Experimental method

2.4.1 Cell culture

2.4.1.1 Frozen deposit

(1) When the degree of convergence of cells reaches 80-90%, abandoned the medium, 2 ml PBS cleaning cells, 2.5 ml ispelinase digesting 30 s, plus 5 ml complete medium to terminate digestion;

(2) Gently blow the cells and collect it to the 15 mL centrifugal tube, 1000 RPM

centrifugal 3 min;

(3) Discard the clearing, add 3 ml of freezing cell frozen liquid, evenly lift the cells to 3 freezing bottle, seal the freezing deposit at -80 ° C.

2.4.1.2 recovery

(1) Take out the cells, quickly transfer to 37 ° C water bath pot, shake to all dissolve;

(2) Transfer the cell suspension to the 7 ml centrifugal tube, add 1: 5 to add a complete medium, mix well, 1000 RPM room temperature centrifugal 3 min;

(3) Abandon the upper clearing, add a 1 mL culture base suspension, transfer to the T25 cell culture bottle, and then add 4 ml complete medium, and cultivate in a 37 ° C culture box.

2.4.1.3 Cell Biography

(1) When the degree of convergence of cells reaches 80-90%, it is passed on; the same method of digestion and centrifugation is the same

(2) Use a complete culture base for suspension cells, pack it to 5 T75 culture bottles, and add 15ml to complete the cultivation group to continue cultivation.

(3) When the degree of convergence of cells is 70 to 80%, the cell suspension is made according to the previous steps. After the ratio of 0.2%is mixed with 0.2%, the injecting cell counting plate is taken.

(4) Cell counting meter count, take the average value;

(5) Dilute the cells into the required concentration according to follow -up experimental needs.

2.4.2 Cell experiment grouping

Divide VSMCS into 5 groups: blank control group, ANGII (1 μmol/L) group, ANGII + gastrine (1 μmol/L + 5, 10, 20 μmol/L) group.

2.4.3 The impact of gastrodicin on ANGII -induced VSMCS proliferation

Crystal Purple Experiment: VSMCS is inoculated on 24 -hole board according to 8 × 104/holes and cultivated 24h. Add the corresponding drug treatment 24h respectively, abandon the medium, add 250 μL of crystal purple chromatin per hole, set up for 30 minutes, dry naturally after washing, add 500 μL 20%acetic acid solution to dissolve the crystals, read each value of each hole under 540nm wavelength, calculate the calculation, calculate Survival rate.

2.4.4 DCFH-DA fluorescent probe Dyeing detection ROS

(1) Clean cells with 2 × 104/ml, cultivate in a 37 ° C cell culture box;

(2) When cells convergence reaches 50-60%, 0.5mlpbs washing cells;

(3) ANGII and gastrodin intervention of specific doses of each group are added to each group; 6h;

(4) Prepare the DCFH-DA reagent to add 1ml probe, incubate for 20min, and wash 3 times PBS.

(5) Dilute Hoechst33342, add 0.5ml staining solution for dyeing, avoid light incubation for 10 minutes, and wash 3 times PBS. Add 1ml blank medium for observation.

2.4.5WESTRNBLOT detection ANGII-NADPH oxidase-ROS channel-related protein expression

2.4.5.1 QPCR Step

(1) Add an appropriate amount of chloroform, isopropanol, 75%alcohol, etc. to extract and dissolve RNA after the cell cracking;

(2) Preparation of agarose gel;

(3) Design primers are synthesized by Shanghai Boshang Biological Engineering Technology Co., Ltd..

(4) The concentration measurement and quantification of the RNA obtained by the appeal step. Reverse transcription system (30 μL).

Table 3 reversing transcription system

reaction system	dose	Reaction conditions
Step 1 reaction--		
4 × GDNA Wiper Mix	6 μL	42 ℃ 2min
Rnase-free ddh2o	Up to 18 μL	
RNA	2 μg	
Step 2 reaction--		
5 × qrt supermix II	6 μL	50 ℃ 15min, 85 ℃ 2min, 4 ℃

(5) The QPCR amplification is performed in accordance with the response system and reaction conditions of Table 4, and the reaction system (20 μL) is performed.

Table 4 SYBR's fluorescent quantitative PCR reaction system

reaction system	dose	Reaction conditions
2×ChamQ SYBR qPCR Master Mix	10μL	95℃ 30s 95℃ 10s (40 Cycles)
F	0.5 μL	60℃ 30s
R	0.5 μL	72℃ 30s
cDNA	1μL	72℃ 10min
Nuelease-Free Water	Up to 20 μL	4℃ ∞

2.4.5.2 Western blot steps

(1) The centrifugal tube containing a cell suspension is 12000rpm centrifugal 15min.

(2) Discard the liquid, add 100 μl of cracking solution per tube, mix and mix it, and stop.

(4) 4 ° C, 12000 RPM, centrifuged 15 min, absorb the upper clearing, and save it at -80 ° C.

(5) Add the volume of the standard (2mg/ml) to 100, 75, 50, 75, 50, 50, 25, 5, 0 μL to 1.5ml of EP tubes, and correspond to 0, 25, 50, 125, respectively. , 150, 350, 375, 395, 400 μL of the physiological saline are diluted, 25 μL per hole, repeated 3 times.

(6) Mix physiological saline and samples to prepare samples to be tested.

(7) Configure the BCA working liquid, place 30 min at 37 ° C;

(8) 562 NM wavelength measure OD value and draw standard curves.

Table 5 The preparation of separation glue

Separation glue (5ml)	6%	8%	10%	12%	15%
The best separation range	>140KD	>100 KD	30-100 KD	10-30 KD	<10 KD
Pure water (ml)	2.0	1.7	1.3	1.0	0.5
30%Acr-Bis(29:1) (mL）	1.0	1.3	1.7	2.0	2.5
1.5M Tris，pH8.8（mL）	1.9	1.9	1.9	1.9	1.9
10%SDS(mL）	0.05	0.05	0.05	0.05	0.05
10%ammonium sulfate (ml)	0.05	0.05	0.05	0.05	0.05
TEMED（mL）	0.004	0.003	0.002	0.002	0.002

Note: For ammonium sulfate, the concentration is 10%(0.1g/1 ml).

Table 6 Configuration of concentrated glue

Concentrated glue	pure water	30%Acr-Bis	1M Tris，pH6.8	10%SDS	10%super sulfate	TEMED
2（mL）	1.4（mL）	0.33（mL）	0.25（mL）	0.02	0.02（mL）	0.002（mL）

(12) Sample: 10 μL per hole, Protein Marker 5μL;

(13) 30V 10 minutes, 80V 30min, 120V 40-50min for electrophoresis until the dye reaches the bottom;

(14) Cut the glue and put it in a methanol to activate 30s.

(15) Soaking filter paper and making the diaphragm sandwich.

(16) Closure: TBST drove 3 times, 10min/time, put in 5%skimmed milk powder /BSA (0.1g/1ml), and incubate at room temperature for 2h or 4 ° C for overnight.

(17) Dilute one resistance to the working concentration. The membranes that have been performed (such as turning the film, closed, etc.) have been performed in an anti -solution, and the room temperature of the shake is incubated for 2 h or 4 ° C for overnight.

(18) washing: Place the membrane in TBST, rinse 3 times, 10min/time.

(19) Incubation Two Resistance: Select the right two -resistance according to the source of the first resistance, dilute the two -resistance of the HRP mark to the working concentration, and the room temperature is incubated by 1.5h.
(20) Washing: Step of the same (20), exposure development.

3 STATISTICAL RESULTS

Use SPSS23.0 version statistical software processing. As a result, ± s is represented by ± s. Multi -sets of data are compared with single factor variance analysis. The two sets of data are compared with T test, and the count data is tested with the card party. P <0.05 is a significant difference and is statistically significant.

4 –CELL EXPERIMENTAL RESULTS

4.1 The effect of gastrodin on ANGII -induced VSMCS proliferation

After each group of processing, the survival rate of each group of cells is calculated using crystal purple experiments; the experimental results show that ANG II causes VSMCS abnormal proliferation, while different doses of gastrodin can inhibit this proliferation, and the high -dose group effect is the best.

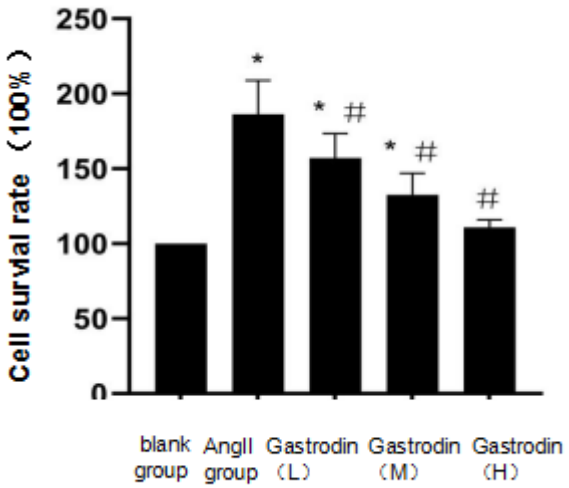
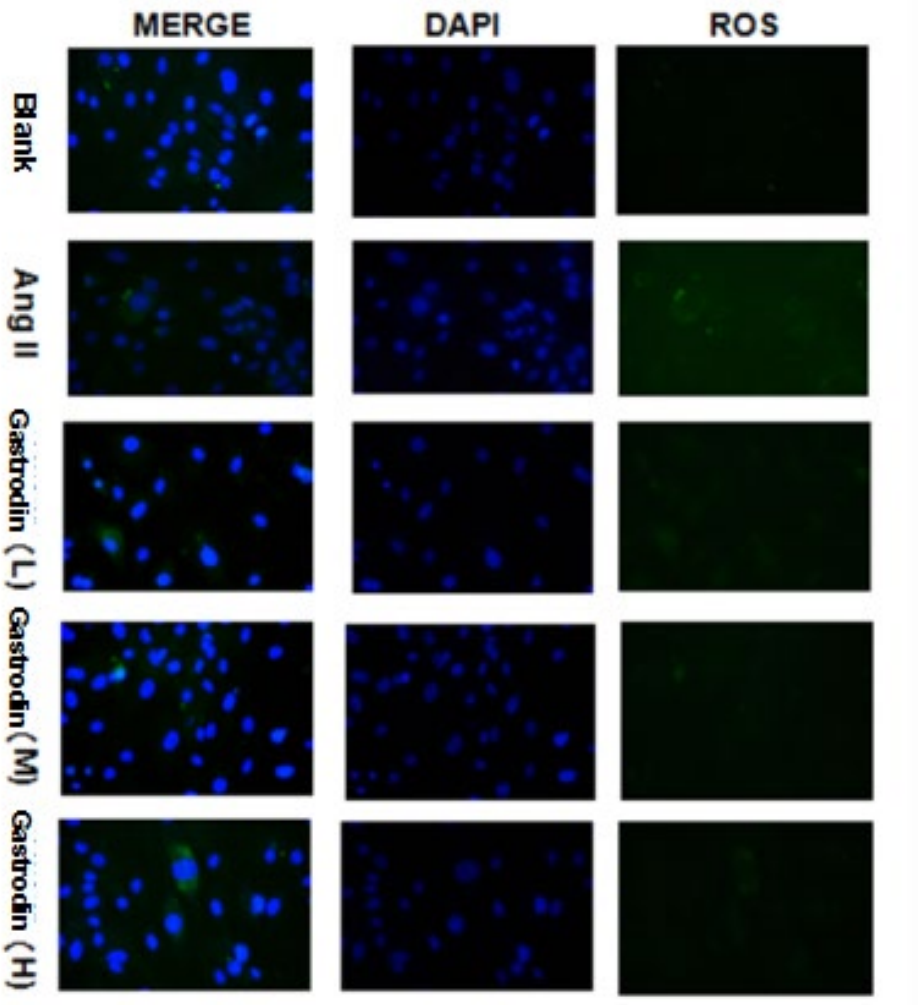


Figure 4-1 The impact of different concentrations of gastrodin on VSMCS proliferation

4.2 The effect of gastrodin on the ROS content in VSMCS induced by ANGII

After the experiental intervention of each group of ANGII and the corresponding concentration, the DAPI dyeing cell nucleus is used as a reference, and the DCFH-DA

fluorescent probe is used to detect the level of ROS levels of each group of cells. The experimental results show that the green fluorescence (characterized ROS level) of the ANG II group is enhanced, indicating that ANG II can cause ROS levels in VSMCS to rise. The green fluorescence of the Gastrodin Group shows that gastrodin can inhibit the vsmcs caused by ANGII Increased ROS level. (As shown in Figure 4-2)



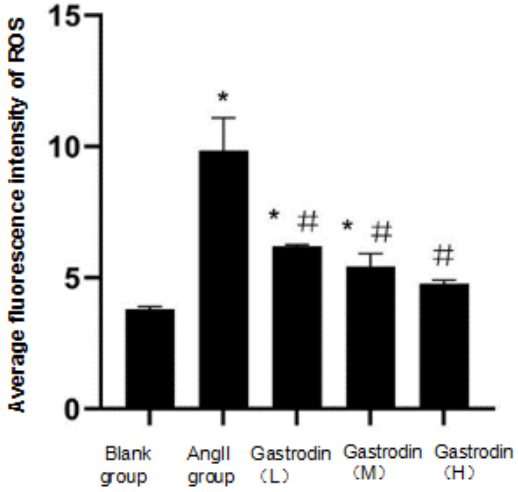
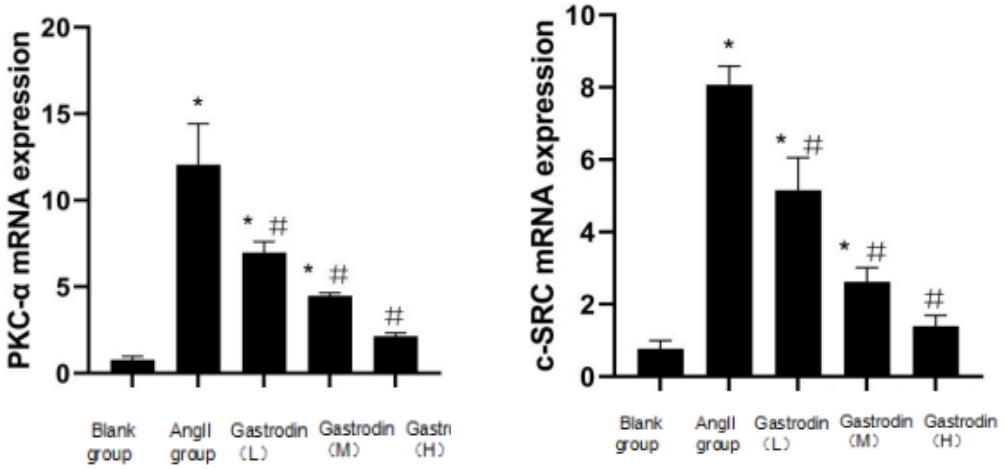


Figure 4-2 The impact of ROS content in VSMCS induced by Gastrodin (400 ×; Label ruler: 20 μm)

4.3 QPCR was used to detect the effect of Gastrodin on PKC, EGFR, c-Src kinase and NADPH oxidase mRNA expression

After the intervention of ANGII and Gastrodin in each group, the QPCR results showed that ANG II caused high expression of PKC, EGFR, C-SRC kinase , and NADPH oxidase mRNA. Gastrodin can inhibit this high expression state, and high-dose group suppression effect is the best. (As shown in Figure 4-3)



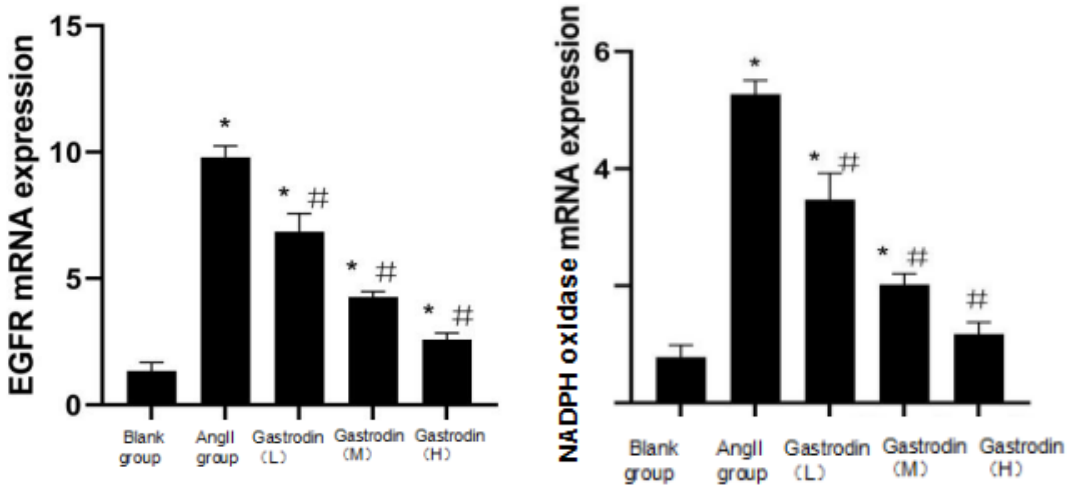


Figure 4-3 QPCR was used to detect the effect of Gastrodin on PKC, EGFR, c-Src kinase and NADPH oxidase mRNA expression

Note:*P <0.05 Compared with the blank group,#P <0.05 Compared with the model (ANGI) group

4.4 The effects of gastrodin on ANGII-NADPH oxidase-ROS pathway-related protein expression

After the intervention of ANGII and gastrodin in each group, the Western Blot results showed that ANG II caused high expression of PKC, EGFR, C-SRC kinase, and NADPH oxidase. Gastrodin can inhibit this high expression state, and high-dose group suppression effect is the best. (As shown in Figure 4-4)

	Blank	Ang II	L	M	H
c-SRC					
Density value	38674	218796	133749	103324	78297
Grayscale value	0.153403119	0.886653753	0.546175112	0.421875255	0.304050669
PKC-α					
Density value	69528	175746	141494	116369	97273
Grayscale value	0.270401238	0.702731017	0.549728232	0.457046239	0.379149192
NOX1					

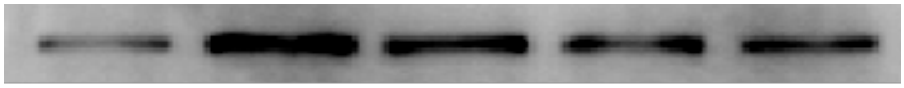
Density value	124302	232801	201512	187490	167864
Grayscale value	0.488247679	0.960693446	0.844899876	0.74907609	0.658765541
EGFR					
Density value	63202	241692	174451	145387	121997
Grayscale value	0.250695141	0.97943801	0.712385098	0.593619853	0.47375084
GAPDH					
Density value	252107	246766	244883	244916	257513

Figure 4-4 The effects of different concentrations of gastrodin on PKC, EGFR, C-SRC kinase, NADPH oxidase protein expression

Note:*P <0.05 Compared with the blank group,#P <0.05 Compared with the model (ANGI) group

5 DISCUSSION

Hypertension is mostly in the middle -aged and elderly people. It is a clinical syndrome that is characterized by the increase in circulating arteries. It is one of the most common clinical chronic diseases. According to a large number of preliminary research observations, the pathological basis of hypertension is "vascular reconstruction". The reversal process is essential for improving hypertension and its complications. Hypertensive vascular reconstruction involves a variety of cell changes and changes in matrix. ANG II, as a key peptide substance, participates in blood pressure regulation, sodium preservation, water preservation, potassium exhaustion and other processes [9], which is closely related to the diagnosis and treatment of hypertension [10]. ANG II can combine with its specific receptor vascular tension 1 (AT1) to activate a variety of pathways [11-13], which in turn promotes the generation of ROS, and ROS is an important damage factors for vascular reconstruction.

ROS accumulation leads to oxidation stress, which is a key factor in vascular reconstruction. A large amount of ROS damage the vascular endothelial cells, damage and decrease its function, and then change the function and structure of blood vessels. ROS can also lead to abnormal proliferation, skeleton reorganization, and fibrosis of vascular smooth muscle cells, thereby promoting the occurrence and development of hypertension [14]. At present, Western medicine treats hypertension mainly to control blood pressure, but has limited effect on prevention, delaying or reverse blood vessel

reconstruction and protection of target organs. Therefore, simple antihypertensive drugs have been improved on the overall condition of some patients [15].

Although the classics of Chinese medicine have no "hypertension" name, they can be attributed to "headache" or "dizziness" according to symptoms [16]. Chinese medicine masters and modern physicians in -depth analysis of their causes and pathogenesis, taking "flat liver and submerging" as the rule, using classic prescriptions to add subtraction and treatment to achieve good results [17]. In recent years, the results of Chinese medicine complex and effective ingredients have achieved results in improving vascular reconstruction and blood pressure. Gastrodin in Chinese medicine Gastrodia has a antihypertensive effect, and combined with commonly used antihypertensive drugs to synergistic antihypertensive [20]. Cell experiments have initially showed that Gastrodin may reduce the possibility of active oxygen generating, inhibiting oxidation stress damage, and the possibility of improving vascular reconstruction by inhibiting ANGII-NADPH oxidase-ROS pathways [6]. However, mechanism research also requires more animal experiments and clinical research support.

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