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Mechanism of Wenyang Zhenshuai Granules in protecting injured H9C2 cardiomyocytes based on the exosomes-mediated miR-155/p38MAPK signal pathway

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Abstract

Purpose: To discuss about the mechanism of Wenyang Zhenshuai Granules in protecting injured H9C2 cardiomyocytes based on the exosomes-mediated miR-155/p38MAPK signal pathway.

Methods: The experiments are divided into two steps. Adriamycin (ADR) induced cardiomyocyte injury model was used in experiment 1. Normal group (N), ADR group (A), ADR + control serum group (A + B), ADR + Wenyang Zhenshuai-containing serum group (A + W), ADR + enalapril-containing serum group (A + Y) were set up. Cell status was monitored by microscope and MTT method. After 45 hours, p38MAPK and its phosphorylation expression were detected by WB method, p38MAPK mRNA and miR-155 content were detected by PCR method. In experiment 1, exosomes in A + W and A + Y groups were isolated after culture. After identification, fluorescence labeling was completed by transmission electron microscope and WB method. Co-culture system was established with cardiomyocytes injury model. Normal group (N), ADR group (A), ADR + A + W exosomes group (A + A + W) and ADR + A + Y exosomes group (A + A + Y) were set up. The process of exosomes aggregating and entering cardiomyocytes was observed by laser confocal system. After 45 hours, the cell viability, protein and gene were detected by light microscope, MTT, WB and PCR methods. The detection indexes were the same as those in experiment 1.

Results: Experiment 1: Light microscope showed vacuolar degeneration of modelling group cells, and degeneration of A + W group and A + Y group was improved. MTT experiment showed that compared with N group, OD value in modelling group decreased significantly. Compared with A group, OD values in A + W group and A + Y group increased significantly. The difference was statistically significant ($p < 0.05$ or $p < 0.01$). The results of WB and PCR showed that the mRNA of p38MAPK, p-p38MAPK and p38MAPK was up-regulated and the expression of miR-155 was down-regulated in the modelling group compared with that in the N group; compared with A group, the mRNA of p38MAPK, p-p38MAPK and p38MAPK were down-regulated and the expression of miR-155 was up-regulated in A + W group and A + Y group. The difference was statistically significant ($p < 0.05$ or $p < 0.01$). Experiment 2: Laser confocal system observed that exosomes can aggregate and enter damaged cardiomyocytes. MTT experiment showed that compared with N group, OD value in modelling group decreased significantly. Compared with A group, OD values in A + A + W group and A + A + Y group increased significantly. The difference was statistically significant ($p < 0.05$ or $p < 0.01$). The results of WB and PCR showed that the mRNA of p-p38MAPK and p38MAPK was up-regulated and the expression of miR-155 was down-regulated in the modelling group compared with that in the N group. Compared with A group, A + W group and A + Y group could down-regulate the mRNA of p-p38MAPK and p38MAPK and up-regulate miR-155 expression. The

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difference was statistically significant ($p < 0.05$ or $p < 0.01$).
Conclusion: Wenyang Zhenshuai Granules can protect damaged cardiomyocytes by up-regulating miR-155 and down-regulating target cell p-p38MAPK mediated by exosomes.
Keywords: Exosomes; Wenyang Zhenshuai Granules; H9C2 cardiomyocytes

Instruction

Wenyang Zhenshuai Granules is a preparation of the First Affiliated Hospital of Hunan University of Chinese Medicine, which has been well received by the majority of cardiovascular patients for many years. The experimental study [1][2][3][4][5][6][7][8][9]confirmed that Wenyang Zhenshuai Granules can positively regulate the cardiac function structure, serum inflammatory factors and cardiomyocytes apoptosis of the animal model of myocardial damage. Clinical research [10] shows that this recipe can safely and effectively improve the TCM symptoms and heart failure scores of patients with chronic heart failure and improve their quality of life. Cai Huzhi's research [11] suggests that the mechanism of Wenyang Zhenshuai Granules in treating chronic heart failure may be to up-regulate miR-155 of damaged cardiomyocytes and thus inhibit phosphorylation expression of p38MAPK, but its intercellular mechanism is still unclear. Exosomes are a group of small vesicles that naturally exist in various body fluids. In recent years, they have become a research hotspot due to the discovery that they can wrap a large amount of genetic materials and proteins and directly participate in intercellular communication. Therefore, we put forward a scientific hypothesis: Wenyang Zhenshuai Granules can protect damaged cardiomyocytes through exosomes transport. Specific experimental reports will be carried out below.

1. Experimental Materials

1.1 Cells and animals

H9C2 cardiomyocytes were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.; SPF male SD rats were purchased from Hunan STJ Laboratory Animal Co., Ltd., with license number being SCXK (Hunan) 2016-0002.

1.2 Main instruments

Super clean workbench (Beijing Yntnl Kelong YT-CJ-2NB); inverted biomicroscope (Beijing Cnmicro DSZ2000X); multifunctional enzyme-labeled analyzer (Huisong MB-530); Desktop high-speed freezing centrifuge (Xiangyi H1650R); transmission electron microscope (JEOL JCM6000, Japan); electrophoresis apparatus (Bio-rad 164-5050, USA); Membrane transfer instrument (Beijing Liuyi DYCZ-40A); Fluorescence quantitative PCR apparatus (Thermo PIKOREAL96, USA).

1.3 Drugs and main reagents

Wenyang Zhenshuai Granules (The First Affiliated Hospital of Hunan University of Chinese Medicine); Enalapril Tablets (Cisen Pharmaceutical Co., Ltd.); MTT (Sigma); Exosomes extraction reagent (SBI); Exosomes quantitative kit (Bio Vision); CD9 antibody (Proteintech 60232-1-Ig); CD63 antibody (Proteintech 25682-1-AP); P38 antibody (Proteintech 14064-1-AP); GAPDH antibody (Proteintech 10494-1-AP); p-p38 antibody (CST 9211S); mRNA reverse transcription kit (Beijing Cowin Biotech CW2569); miRNA reverse transcription kit (Beijing Cowin Biotech CW2141); UltraSYBR Mixture (Beijing Cowin Biotech CW2601).

2. Experimental Methods

2.1 Preparation of serum

2.1. 1 Wenyang Zhenshuai Granules and Enalapril Serum [12]

The rats were treated with Wenyang Zhenshuai Granules (144 mg · mL-1) and enalapril (1 mg · mL-1) at a dose of 10 mL · kg-1 for 7 days, once a day. Anesthetized rats (7% chloral hydrate, 5 mL · kg-1) were subjected to abdominal aorta blood extraction, with centrifugation (2500r/min, 15min) and supernatant inactivation (56 ℃, 30min), filtration and sterilization were conducted with 0.45 μm microporous filter membrane, and then they were stored in -20 ℃ refrigerator for later use.

2.1.2 Blank rat serum

Pure water was used for rats' lavage at a dose of 10 mL · kg-1 for 7 consecutive days, once a day. The next steps are the same as before.

2.2 Cell culture, modeling and grouping

H9C2 cardiomyocytes were cultured in 90% DMEM + 10% FBS (37 ℃, 5% CO_2, saturated humidity). Adriamycin (ADR) 2.67 μmol/L was added to induce cardiomyocytes injury model. Experiment 1: 10% blank rat serum / Wenyang Zhenshuai-containing serum / enalapril-containing serum were added to the culture medium in the intervention group for a total culture time of 45h. It was divided into 5 groups: normal group (N); ADR group (A); ADR + control serum group (A + B); ADR + Wenyang Zhenshuai-containing serum group (A +

W); ADR + enalapril-containing serum group (A + Y). Experiment 2: The total culture time of exosomes obtained from 15% A + W group / A + Y group added to the culture medium of intervention group is 45h. It was divided into 4 groups: normal group (N), ADR group (A), ADR + A + W exosomes group (A + A + W) and ADR + A + Y exosomes group (A + A + Y).

2.3 Morphological observation of cells

Morphological observation was observed by inverted microscope at 45h of culture.

2.4 MTT

MTT assay was used to detect cell proliferation activity. The cells in logarithmic growth phase were digested and counted and inoculated into 96-well plate at a density of 4 * 10^3 cells/well, with 200 μL per well. 5 multiple wells were set up in each group. After the cells adhered to the wall, 5mg/ml MTT 20 μl/well was added to each well. The cells were incubated at 37 ℃ and 5% CO_2 for 4 hours. The supernatant was carefully sucked and discarded, and 150 μl/well dimethyl sulfoxide was added. The absorbance (OD) value at 490nm was analyzed by enzyme-labeled analyzer, and the average value was taken and the growth curve was drawn.

2.5 Extraction, identification and fluorescent labeling of exosomes

Cell culture solution was collected and centrifuged (3000g, 15min). Cells and debris were removed and transferred to a new centrifuge tube. Exosomes extraction reagent was added according to the proportion (cell supernatant: ExoQuick TC=5: 1), mixed well, and allowed to stand at 4 °C overnight. The next day, centrifuge again (1500g, 30min), discard the supernatant, centrifuge again (1500g, 5min), carefully suck and discard the supernatant, and the precipitate obtained is exosomes. Exosomes were identified combined with transmission electron microscope observation and specific protein WB qualitative. In the end, exosomes were quantified by kit. The marker PKH26 was diluted with serum-free DMEM high glucose medium to 5 μM working solution. 10 μL of extracted exosomes were added, mixed and incubated at 37 ℃ for 20min to complete exosomes labeling. 100 μL of 10% BSA termination solution was added to terminate that reaction. The labeled exosomes were added to the cell climbing slice, the cells were covered, and the cells were incubated at 37 ℃ to a specified time point. After PBS rinsing, 1 mg/mL DAPI was added, incubated at 37 ℃ for 10 min, rinsed again and then sent to fluorescence microscope for observation.

2.6 Western-Blot

Samples of logarithmic growth cells and exosomes were taken, added with RIPA lysate, lysed on ice for 10 min, and centrifuged (4 ℃, 12000 rpm, 15 min). The supernatant was quantified by BCA method and then subjected to standard SDS-PAGE gel electrophoresis. Then the separated protein was transferred to nitrocellulose membrane by wet transfer method (300 mA, CD9 about 50 min, CD63 about 55 min, p38, p-p38, GAPDH about 1h), blocked and added with primary antibody, the dilution concentration was p38 (1: 1000), p-p38 (1: 1000), CD9 (1: 2000); CD63 (1: 500); GAPDH (1: 3000), respectively, staying overnight at 4 ℃. The next day, diluted secondary antibodies (rabbit antibodies 1: 6000, mouse antibodies 1: 5000) were added and incubated for 90 min. In the end, ECL was used for color observation.

2.7 RT-PCR

The total RNA was extracted by standard Trizol method, incubated with reverse transcription primers, cooled and diluted, and then added with reverse transcription system for specific reverse transcription of mRNA and miRNA to obtain the target cDNA product. After diluting the cDNA stock solution, searching the sequence of the target gene on NCBI, designing and synthesizing primers, SYBR real-time fluorescence quantitative PCR detection can be carried out. Amplification procedure: pre-denaturation at 95 ℃ for 10 min; denaturation at 95 ℃ for 15 s, annealing at 60 ℃ for 30 s and 40 cycles. The expression level of the target gene was expressed by 2-△△CT value. The primer design is shown in Table 1.

Table 1. **Primer for Genes to be Tested and Internal Reference Genes**

Primer	Sequence
GAPDH	F：5’-ACAGCAACAGGGTGGTGGAC-3’
	R：5’-TTTGAGGGTGCAGCGAACTT-3’
5S	F：5’-GCCTACAGCCATACCACCCGGAA-3’
	R：5’-CCTACAGCACCCGGTATCCCA-3’
P38	F：5’-AGCTTACCGATGACCACGTT-3’
	R：5’-CACGTAGCCGGTCATTTCGTC-3’
rno-miR-155-3p	5’-GGCACCTCCTACCTGTTAGCATT-3’

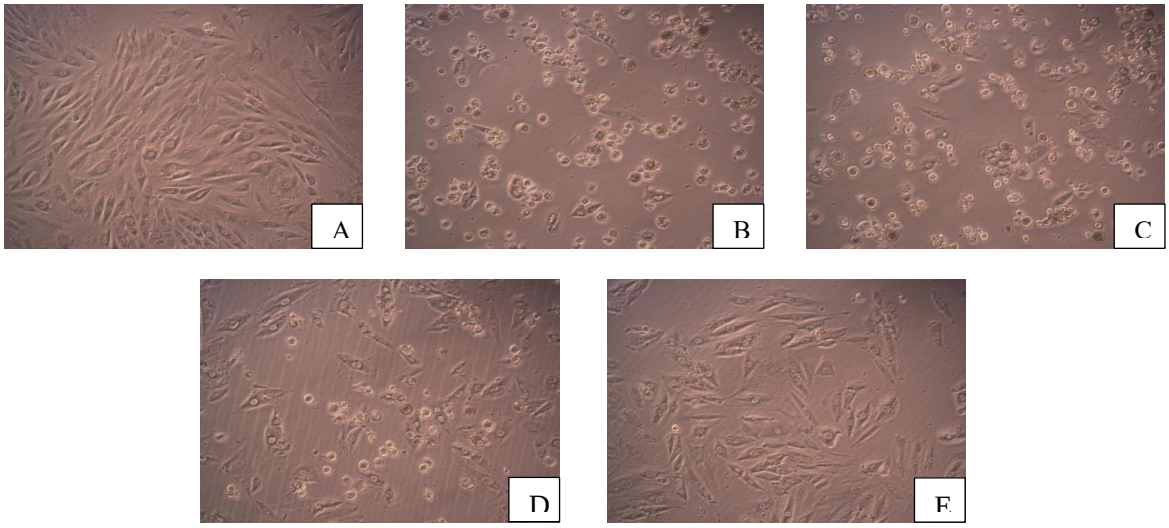
3. Statistical analysis

SPSS 21.0 statistical software was used to process the data. The measurement data were all expressed by mean ± standard deviation ($\bar{x} \pm s$). One-way ANOVA was used to compare the diversity among the groups. First, Levene method was used to test the homogeneity of variance. LSD method was used when the variance was homogeneous, and Tamhane'T2 method was used when the variance was heterogeneous. The significance level was *P*=0.05.

4. Result

4.1 Morphological observation of H9C2 cardiomyocytes in each group

The inverted microscope showed that the cells in N group grew well. Morphology, cytoplasm, stroma and transverse striation are normal. Most of the cells in A group and A + B group were round and floating on petri dishes, with myofibrolysis and cytoplasmic vacuolar degeneration, accompanied by different degrees of inflammatory cell infiltration and interstitial edema. The cells in A + W group and A + Y group were better than those in A group. After 45h of culture, spindle-shaped cells adhered to the wall and grew. See Figure 1 for details.



Note: A: N group, B: A group, C: A + B group, D: A + W group, E: A + Y group.

Figure 1. **Cell Morphology after 45h Culture (100 ×)**

4.2 Detection of proliferative activity of H9C2 cardiomyocytes in each group

After 45 hours of culture, compared with N group, A group decreased significantly. Compared with A group, A + B group, A + W group and A + Y group all increased significantly or showed an upward trend. The difference was statistically significant ($p < 0.05$ or $p < 0.01$). See Table 2 for details.

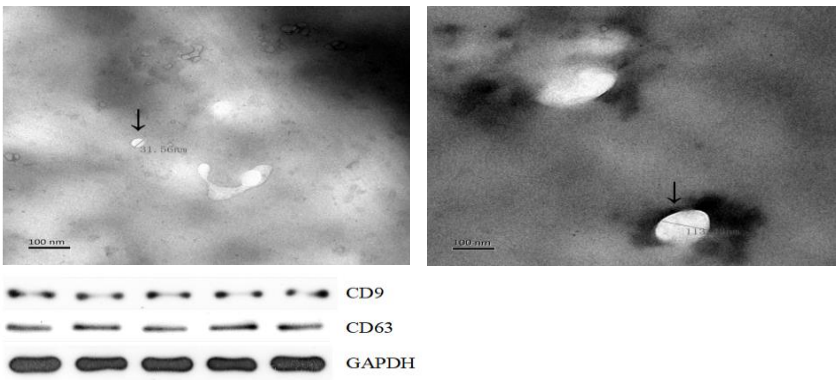
Table 2. **Comparison of 45h Proliferation Activity of H9C2 Cardiomyocytes ($\bar{x} \pm s$, n=6)**

Group	Absorbance (OD value)
N	2.25±0.09
A	0.38±0.01**★★■■
A+B	1.51±0.05**★★■■
A+W	0.38±0.01$^{**\#\#}$▼▼■■
A+Y	1.88±0.02$^{**\#\#}$★★▼▼

Note: Compared with N group, * P < 0.05, * * P < 0.01; compared with A group, # P < 0.05, ## P < 0.01; compared with A + B group, ▼ P < 0.05, ▼▼ P < 0.01; compared with A + W group, ★ P < 0.05, ★★ P < 0.01; compared with A + Y group, ■ P < 0.05, ■■ P < 0.01;

4.3 Identification of exosomes of H9C2 cardiomyocytes in each group

Under transmission electron microscope, it was observed that the extract was evenly distributed in the field of vision, roughly showing a microcapsule structure, with circular or oval shapes, different sizes and diameters ranging from 30 nm to 120 nm. Western-Blot results showed that the extracts were positive in exosomes marker proteins CD9 and CD63. Combining the microscopic results with the qualitative analysis of specific protein WB, the extract can be identified as exosomes. See Figure 2 for details.



Note: The arrow in the figure refers to exosomes.
They are N group, A group, A + B group, A + W group and A + Y group at gray strips from left to right.
Figure 2. **Identification Results of Exosomes**

4.4 Western-blot

P38MAPK in A group was significantly higher than that in N group. Compared with A group, A + B group, A + W group and A + Y group decreased significantly. The above differences were statistically significant (P < 0.05). p-p38MAPK in A group was significantly higher than that in N group. Compared with A group, A + W group and A + Y group decreased significantly. The above differences were statistically significant (P < 0.05). See Table 3 and Figure 3 for details.

Table 3. **Expression of p38MAPK and p-p38MAPK in H9C2 Cardiomyocytes ($\bar{x}$ ± s, n=6)**

Group	p38MAPK	p-p38MAPK
N	0.38±0.01	0.10±0.01
A	0.53±0.05$^{*▼★■}$	0.33±0.01$^{*★■}$
A+B	0.46±0.02$^{*#■}$	0.34±0.03$^{*★■}$
A+W	0.43±0.03$^{#}$	0.22±0.02$^{*#▼}$
A+Y	0.40±0.03$^{#▼}$	0.18±0.03$^{*#▼}$

Note: Compared with N group in the same group, * P < 0.05; compared with A group in the same group, # P < 0.05; compared with A + B group in the same group, ▼ P < 0.05; compared with A + W group in the same group, ★ P < 0.05; compared with A + Y group in the same group, ■ P < 0.05;

They are N group, A group, A + B group, A + W group and A + Y group at gray strips from left to right.
Figure 3. **Expression of p38MAPK and p-p38MAPK in Cardiomyocytes of Each Group**

4.5 RT-PCR

Compared with N group, miR-155 in A group decreased significantly. Compared with A group, A + W group and A + Y group increased significantly. The above differences were statistically significant (P < 0.05). p38MAPK mRNA in A group was significantly higher than that in N group. Compared with A group, A + W group and A + Y group decreased significantly. The above differences were statistically significant (P < 0.05). See Table 4 for details.

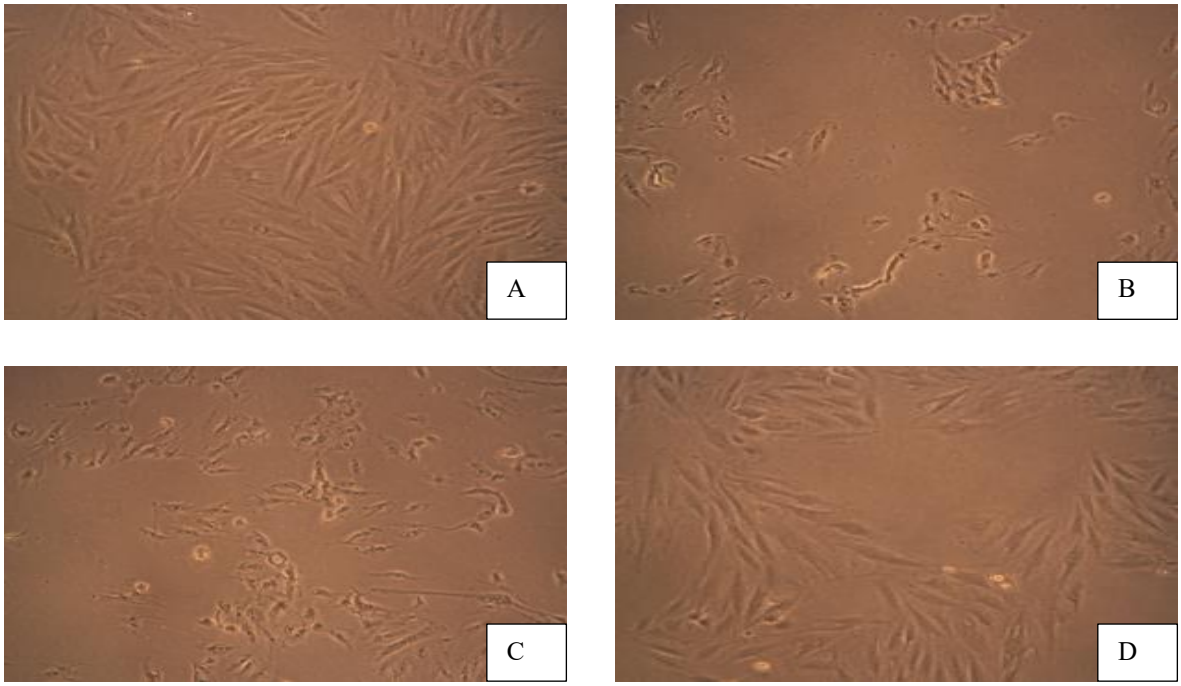
Table 4. **Expression of miR-155 and p38MAPK mRNA in H9C2 Cardiomyocytes ($\bar{x}$ ± s, n=6)**

Group	miR-155	p38MAPK mRNA
N	1.18±0.04	0.99±0.03
A	0.56±0.04$^{*★■}$	3.05±0.02$^{*★■}$
A+B	0.51±0.02$^{*★■}$	3.08±0.01$^{*★■}$
A+W	0.70±0.03$^{*#▼■}$	1.90±0.02$^{*#▼■}$
A+Y	0.78±0.02$^{*#▼★}$	1.43±0.03$^{*#▼★}$

Note: Compared with N group in the same group, * P < 0.05; compared with A group in the same group, # P < 0.05; compared with A + B group in the same group, ▼ P < 0.05; compared with A + W group in the same group, ★ P < 0.05; compared with A + Y group in the same group, ■ P < 0.05;

4.6 Morphological observation of H9C2 cardiomyocytes in each group

Inverted microscope showed that the cells in N group grew well and their morphology, cytoplasm, stroma and transverse striations were normal. The cells in A group showed myofibrolysis and cytoplasmic vacuolar degeneration. After exosomes were added, spindle cells grew adherently in A + A + W group and A + A + Y group, with a small number of round cells floating in petri dishes. See Figure 4 for details.



Note: A: N group, B: A group, C: A + A + W group, D: A + A + Y group.

Figure 4. **Cell Morphology after 45h Culture (100 ×)**

4.7 Real-time fluorescence labeling imaging

H9C2 cardiomyocytes showed blue fluorescence under microscope after adding DAPI cell membrane dye, while exosomes showed red fluorescence under microscope after adding PKH26 dye. After cross-incubation, exosomes aggregate and enter H9C2 cardiomyocytes, as shown in Figure 5.

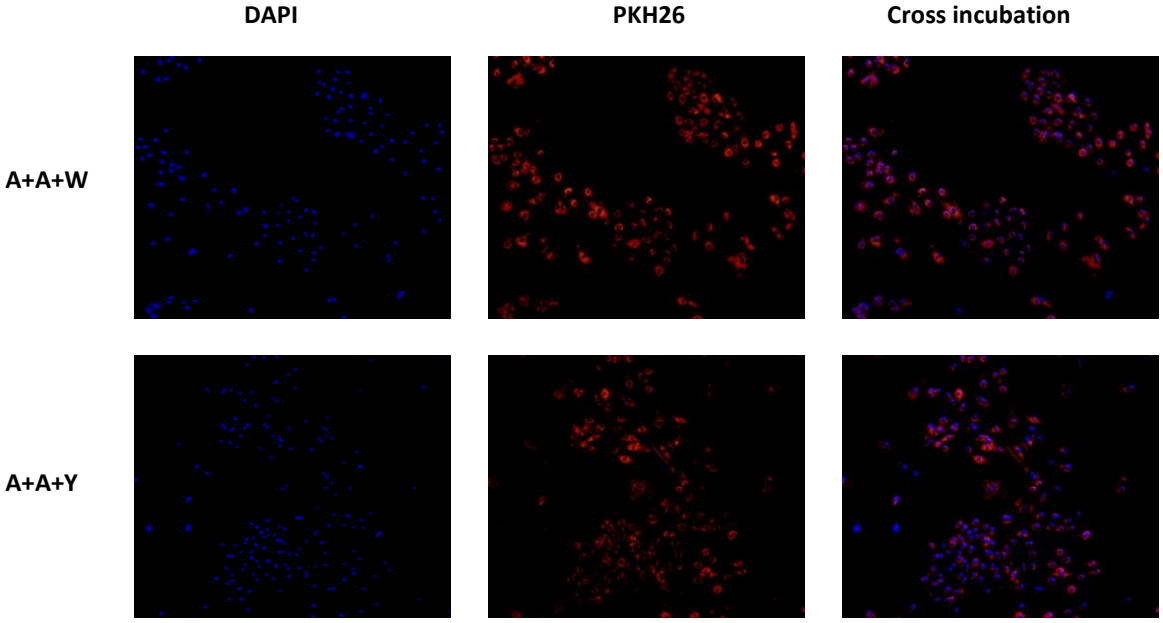


Figure 5. **Real-time Fluorescence Labeling Imaging of Cells after 45h Culture (100 ×)**

4.8 Detection of proliferative activity of H9C2 cardiomyocytes in each group

After 45 hours of culture, compared with group N, group A decreased significantly. Compared with A group, A + A + W group and A + A + Y group increased significantly. The difference was statistically significant ($p < 0.05$ or $p < 0.01$). See Table 5 for details.

Table 5. **Comparison of 45h Proliferation Activity of H9C2 Cardiomyocytes ($\bar{x} \pm s$, n=6)**

Group	Absorbance (OD value)
N	1.916±0.487
A	0.363±0.015*■■☆☆
A+A+W	1.382±0.025▲▲☆☆
A+A+Y	1.631±0.015▲▲■■

Note: Compared with N group, * p < 0.05, ** p < 0.01; compared with A group, ▲ p < 0.05, ▲▲ p < 0.01;
Compared with A + A + W group, ■ p < 0.05, ■■ pp < 0.01; compared with A + A + Y group, ☆ p < 0.05, ☆☆ p < 0.01;

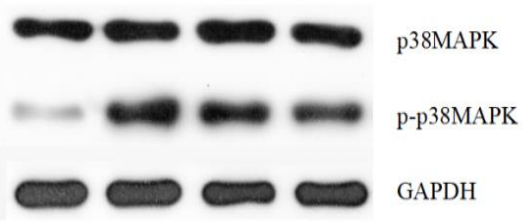
4.9 Western-blot

Comparison of p38MAPK: There was no significant difference in the total protein value of p38MAPK in each group, and there was no significant difference after statistical analysis. Comparison of p-p38MAPK: Compared with N group, A group increased significantly. Compared with A group, A + A + W group and A + A + Y group decreased significantly. The above differences were statistically significant (P < 0.05). See Table 6 and Figure 6 for details.

Table 6. **Expression of p38MAPK and p-p38MAPK in H9C2 Cardiomyocytes ($\bar{x} \pm s$, n=6)**

Group	p38MAPK	p-p38MAPK
N	0.44±0.02	0.07±0.02
A	0.40±0.02	0.36±0.04*■☆
A+A+W	0.43±0.05	0.28±0.04*▲☆
A+A+Y	0.38±0.03	0.12±0.02▲■

Compared with A + A + W group, ■ p < 0.05; compared with A + A + Y group, ☆ p < 0.05; Note: Compared with N group, * p < 0.05; compared with group A group, ▲ p < 0.05;



Note: They are N group,
Figure 6. **Expression of p38MAPK and p-p38MAPK in Cardiomyocytes of Each Group**
A group, A + A + W group, and A + A + Y group at gray strips from left to right.

4.10 RT-PCR

Compared with N group, miR-155 in A group decreased significantly. Compared with A group, A + A + W group and A + A + Y group increased significantly. The above differences were statistically significant (P < 0.05). p38MAPK mRNA in A group was significantly higher than that in N group. Compared with A group, A + W group and A + Y group decreased significantly. The above differences were statistically significant (P < 0.05). See Table 7 for details.

Table 7. **Expression of miR-155 and p38MAPK mRNA in H9C2 Cardiomyocytes ($\bar{x} \pm s$, n=6)**

Group	miR-155	p38MAPK mRNA
N	0.94±0.03	0.99±0.02
A	0.34±0.02*■☆	3.83±0.03*■☆
A+A+W	0.54±0.04*▲☆	2.34±0.02*▲☆
A+A+Y	0.83±0.05*▲■	1.23±0.04*▲■

Note: Compared with the group, * p < 0.05; compared with group A group, ▲ p < 0.05;Compared with A + D group, ■ p < 0.05; compared with A + E group, ☆ p < 0.05;

5. Discussion

Exosomes are vesicular structures released by cells after fusion of poly-vesicular bodies and cytoplasmic membranes. Existing studies have found that almost all cells can actively secrete exosomes, and exosomes are naturally found in a variety of body fluids [13]-[14]. After exosomes are secreted by mother cells, they can be transported at short distance and long distance. When exosomes are actively ingested by other cells, bioactive components such as proteins and nucleic acids from mother cells contained in their vesicles can directly act on active ingestion cells. Some studies [15] have shown that exosomes mediate the transfer of miR-155 from smooth muscle cells to endothelial cells, induce endothelial cell damage and promote atherosclerosis, while overexpression of miR-155 in endothelial cells can inhibit its proliferation and migration. Wang C [16] et al. found that macrophage-derived miR-155-rich exosomes can inhibit fibroblast proliferation and promote fibroblast inflammation in mice with acute myocardial infarction, thus aggravating injury. Vicencio [17] et al. extracted exosomes which are extracted from plasma of distal ischemic preconditioning rats and infused these exosomes into isolated rat hearts after ischemia-reperfusion, which found that the infarct area of rat hearts could be significantly reduced. In the hypoxia-induced myocardial cell injury model, adding the above exosomes to co-culture can reduce cell apoptosis. The main mechanism is that HSP70 heat shock

protein on the exosomes surface interacts with TLR4 protein, activates ERK and p38MAPK signal pathways which are the main subfamilies of MAPK signal pathways, and finally promotes phosphorylation of another heat shock protein HSP27, thus protecting cardiomyocytes and preventing ventricular remodeling. From this point of view, miR-155 contained in exosomes plays an important role in cardiomyocytes proliferation, migration and inflammatory response. It has been proved that miR-155, as an important upstream non-coding regulatory gene of p38MAPK, can directly regulate the expression of MKK-3 and MKK-6 through MAP3K10 pathway, thus affecting p38MAPK and mediating a series of biological activity reactions [18]. We concluded that p38MAPK, an active protein with myocardial injury effect, and its upstream regulatory gene miR-155, are likely to act on paracellular cells through exosomes transport during the pathological process of chronic heart failure, thus producing mutual dialogue and influence. This experimental study found that: Wenyang Zhenshuai Granule-containing serum can up-regulate miR-155 and down-regulate the expression of p38MAPK, p-p38MAPK, p38MAPK mRNA in damaged myocardial cells, it was observed that exosomes could gather and enter the damaged myocardial cells after intervention by laser confocal system. In addition, it also up-regulates the expression of p-p38MAPK and p38MAPK mRNA in target cells by up-regulating miR-155. Combined with the physiological characteristics of exosomes, the previous conjecture of exosomes-mediated effect can be verified. This result is consistent with the report in literature [11]. Therefore, we come to the conclusion that Wenyang Zhenshuai Granules up-regulate miR-155 and down-regulate p-p38MAPK in target cells through exosomes to realize the protective effect on damaged cardiomyocytes. Exosome miR-155 is expected to become a new biological target for repairing damaged cardiomyocytes, and its specific mechanism of action is expected to be discussed in subsequent experiments.

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