Influence of HMGB1 and MSCs transplantation on rat cardiac angiogenesis with acute myocardial infarction

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Abstract: To observe whether HMGB1could enhance the paracrine effect of MSCs when the Mesenchymal stem cells (Mesenchymal stem cells, MSCs) are pre-proccessed by High Mobility Group Box-1 (High Mobility Group Box-1, HMGB1). And to observe whether it can further increase the quantity of local angiogenesis in myocardial infarcts on the rat model with acute myocardial infarction, HMGB1 was combined with MSCs transplantation. MSCs in rats were cultivated with adherence and centrifugation method. Receptors of TLR4and RAGE in HMGB1 were tested. The MSCs were interfered by HMGB1 with different concentration gradient respectively, then the expression of VEGF was tested with ELISA method. SD male rats were divided into four groups: the model group, the MSCs transplantation group, the HMGB1 injection group, the HMGB1 injection plus MSCs transplantation group (n = 24), preparing rat model with acute myocardial infarction. The serum VEGF concentration levels were detected on the 3rd day, 7th and 28th day with ELISA method. On the 28th day after post operation the density of angiogenesis in infarction area was detected by immunohistochemal. (1) MSCs owned the expression of TLR4 and RAGE. (2) the secretion of VEGF increased significantly after the intervention of HMGB1 with concentration of 12.5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL and 200ng/ml on MSCs compared with the control group. While the concentration was 400ng/ml or 800ng/ml, the secretion of VEGF decreased compared with the control group (P < 0.05). (3) detection of the serum VEGF on the 3rd or7th day after post operation was arranged: The results showed that: HMGB1 injection plus MSCs transplantation group > MSCs transplantation group >HMGB1 injection group >model group (P < 0.05). (4) the quantity of CD31 stained angiogenesis in HMGB1 injection plus MSCs transplantation group increased obviously. Combining MSCs transplantation, contributed to new angiogenesis of rats with acute myocardial infarction in myocardial infarction area and its near area in rats with acute myocardial infarction.

Keywords: HMGB1, MSCs, Myocardial infarction, Vascular endothelial growth factor.

INTRODUCTION

Mesenchymal stem cells (Mesenchymal stem cells, MSCs) arecommon-used seed cells for the treatment of cardiovascular disease. They not only own the common features as stem cells, but also are equipped with the immune tolerance, and the ability to be transfectioned by foreign gene etc. MSCs transplantation can promote the micro-angiogenesis in infarction and near area, improving cardiac function with myocardial infarction (Liu *et al*., 2011). High Mobility Group Box-1 (High Mobility Group Box -1, HMGB1) is a key regulator to the whole and local inflammation released by both inflammatory and necrotic cells (Schlueter *et al*., 2005; Kohno *et al*., 2009). Schlueter *et al* (2005) have proposed that exogenous HMGB1 can exert angiogenic effects through secretion of angiogenic factors, including vascular endothelial growth factor (VEGF). VEGF is a potent angiogenic factor and is up-regulated in many tumors and its contribution to tumor angiogenesis is well defined (Duffy, Bouchier-Hayes and Harmey, 2004). In the cardiac angiogenesis process, HMGB1 can act on the receptor for advanced glycation end-products (RAGE) on the surface of human cardiac

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fibroblasts (cFbs) so as to promote the expression of the growth factors such as VEGF (Meng *et al*., 2004). Also, Biscetti *et al*. (2010) pointed out HMGB1 was reported to promote angiogenesis after peripheral ischemia in diabetic mice via a VEGF-dependent mechanism. Therefore, this experiment was based on the establishment of the rat model with acute myocardial infarction, through the expression of HMGB1 and combining with MSCs transplantation as well, to observe the expression changes of local promote angiogenesis factors of infarcted myocardium and the density changes of local angiogenesis, to verify whether increasing exogenous HMGB1 can improve local micro-environment and promote the survival of stem cells and angiogenesis effect. It was expected to provide solutions to the problems that the number of stem cells decreased and died quickly in infarction area caused by transplantation homing.

MATERIAL AND METHODS

Experiment subject
SD rats with weight 100-150 g for cell culture. 96 male SD rats with an age of 2-3 months and weight of 200-250 g, were divided into 4 groups: The model group, the

MSCs transplantation group, the HMGB1 injection group, the HMGB1 injection + MSCs transplantation group (n = 24). They were provided by the Laboratory Animal Center in The Third Military Medical University Daping Hospital, conforming to the national standard experimental animals. Certificate No: SCXK (YU) 2012-0005.

Methods
The rat MSCs were cultivated and identified *in vitro*. SD rats with weight of 100-150 g and age of 3 weeks were selected with bilateral femur and tibia divided in aseptic condition. The primary cells were obtained with adherence and centrifugation method (Garvican *et al.*, 2014). Then they were divided form generation to generation. This experiment adopted the third or the fourth generation. The surface markers of MSCs were detected and identified using flow cytometry instrument and antibodies of APC anti-ratCD29, anti-ratCD45 PE, PE anti-ratCD90. Whether the receptors of TLR4 and RAGE in HMGB1 were expressed or not in MSCs was detected by cell immunity fluorescence.

The detection of the secretion level of VEGF after the intervention of HMGB1 on MSCs (enzyme linked immunosorbent assay, ELISA): The third generation of MSCs cells were selected out to inoculate into the 24-hole plate, adding the culture medium with HMGB1, adjusting the concentration of HMGB1 respectively to 0ng/ml, 12.5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 200 ng/mL, 400 ng/mL and 800 ng/mL. And the 0 ng/mL HMGB1 was taken as the control group. The secretion level of VEGF in each culture medium was tested respectively after 12 h, 24 h and 48 h culture.

The establishment of the rat model with myocardial infarction: Take the abdomen anesthesia with 10% chloral hydrate according to the 3 mL/kg. Using the small animal breathing machine assisted the positive pressure ventilation with 3 mL/100g of the ventilator tidal volume, 100 times/min of the respiratory frequency and the breathing ratio 1: 3. Open the chest on the left edge of the 3rd-4th breastbone. Take the region 2 mm below the crossing point of the pulmonary arterial cone and the left atrial appendage of heart as the jointing point with the ligation of the left anterior descending coronary artery. Based on the successful model, inject 1.0*106 of MSCs into the intramyocardial, inject 100ng HMGB1 (10 μg/mL) once into the front and the back of the intraperitoneal of the left anterior descending coronary artery of ligation, put the rats into the tidy rat cage after the disinfection of the sternal closure, put them into the constant temperature animal room with 26°C to feed regularly.

The concentration level of VEGF was tested with the ELISA method on the 3rd, 7th and 28th day respectively. It should be done according to the specification in the ELISA kit.

The newborn capillaries in immunologic tissue were detected with immunohistochemistry. The rat paraffin sections of each group killed on the 28th day were selected out to be tested CD31 positive cells of immunologic tissue with immunohistochemistry, which was based on to be counted the number of capillaries. The first antibody: Rabbit Anti-rat CD31 (1:50, 1:100, 1:200); the second antibody Goat Anti-rabbit IgG. It was counted with the Weidner micro vascular counting method.

STATISTICAL ANALYSIS

SPSS17. 0 software package was used to take statistical analysis. Quantitative data was expressed by using Mean ± Standard Deviation (x ± s). The data among groups was analyzed by using single factor variance and tested by using T Test. P 0. 05 was statistically significant.

RESULTS

The culturing and identifying of MSCs
The dense round cells contained various primitive marrow cells could be seen during the culturing process of the original generation. On the 3rd day, the culture medium was changed in which adherent cells could be seen as well as few cells changed from the round to the polygon or to the short rod-like, distributed in colony. To the 7th day or so, all the cells were in spindle shape, and in eddy current shape. Part of cells fused, paving 80%-90% of the bottle bottom. To the third generation, the cells were seen under the inverted microscope growing into the spindle shape gradually with balance and distributed evenly. During this time, cell proliferation rate was significantly faster than the original generation. To the 4th and after, cell growth became slow, with an aging trend (fig. 1).

(A)   (B)

(C)   (D)
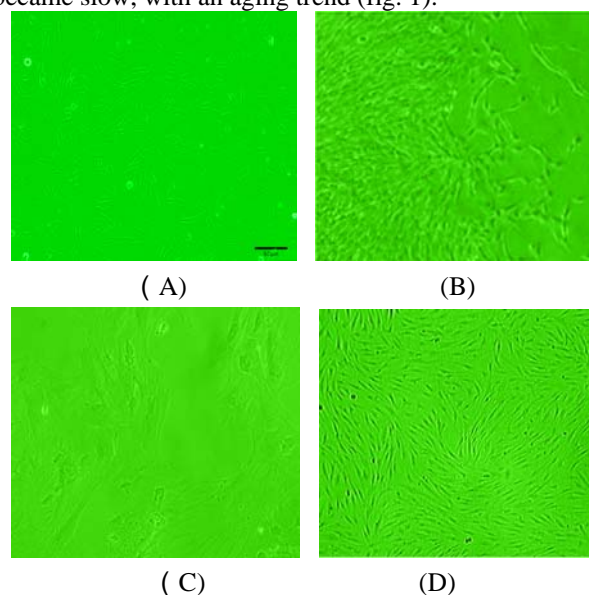
A Cells of the one-day-culture of the original generation (×200)
B MSCs of the five-day-culture of the original generation (×40)
C MSCs of the ten-day-culture of the second generation (×100)
D MSCs of the 25-day-culture of the third generation (×200)

Fig. 1: MSCs culture

To the 3rd or the 4th generation of MSCs, cells (6.0 × 106) in good condition were selected out to be detected the surface markers with flow cytometry instrument. The results showed that the positive rates of CD45, CD29, CD90 were 2.7%, 99.1% and 91.6% respectively. It showed that the cultivated cells were MSCs (fig. 2).
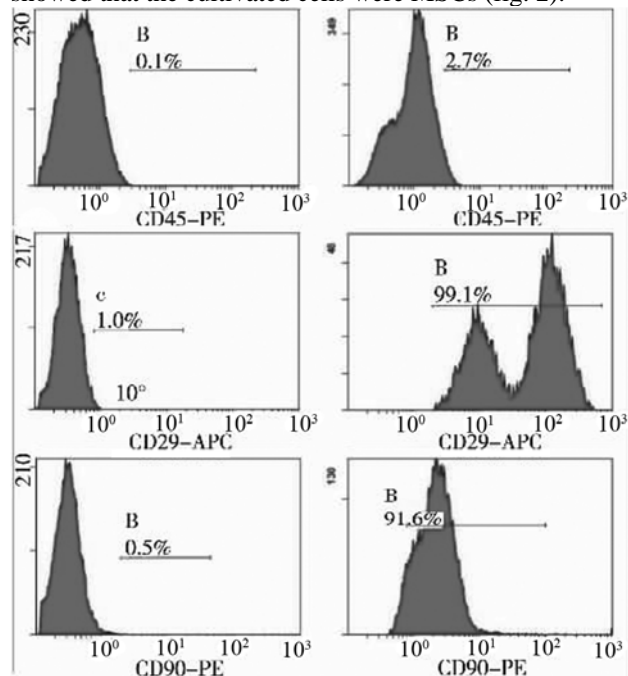
Flow cytometry instrument analysis showed that most of the MSCs cells expressed CD29 and CD90, while a small number of cells expressed CD45.

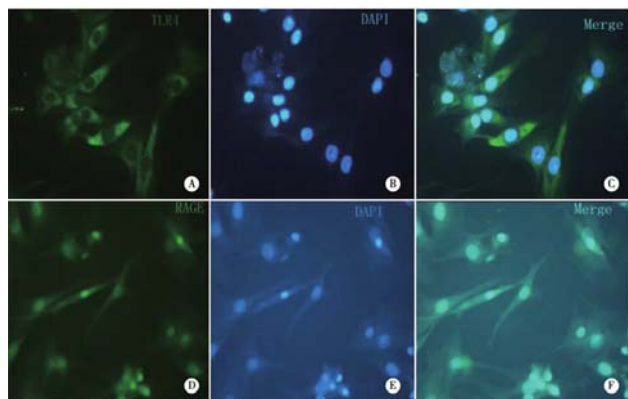**Fig. 2**: The detection of MSCs with Flow Cytometry Method

Fig. 3: Immunocytochemistry detection of the expression of HMGB1 receptor in MSCs

The detection of receptor expression of HMGB1 in MSCs

The P_3MSCs in good condition were selected out to be inoculated into the 6-hole plate with the cellular immune fluorescence detection. It was showed that the TLR4 was mainly expressed in the cytoplasm of the MSCs while the RAGE in the nuclei (fig. 3).

The influence of HMGB1 intervention MSCs on the secretion level of VEGF

The secretion level of VEGF in the culture supernatant at at different time points (12 h, 24 h and 48 h) after the intervention of HMGB1 with different concentration gradient on MSCs were detected with the ELISA method. The results (table 1) showed that at the same time point, the concentration of HMGB1 ranging from 12. 5 to 200 ng/mL intervention on MSCs, the VEGF secretion level significantly increased, compared with the control group, it was statistically significant (P < 0. 05 or P < 0. 01).
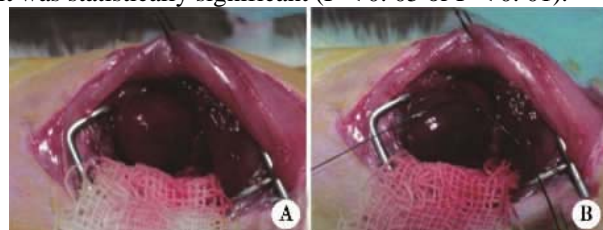
A: The anterior descending branch of heart before ligation
B: The anterior descending branch of heart after ligation
Fig. 4: Establishment of rat model with myocardial infarction

A: Normal ECG of rats
B: ECG of rats after the ligation of the anterior descending branch of heart
Fig. 5: ECG of the rats before and after myocarding infarction

And when in 25 ng/mL, it reached to the peak. However, when the concentration of HMGB1 ranged from 400 ng/mL to 800 ng/mL, with the increase of the concentration, it had inhibitory effect on the secretion, it was statistically significant (P < 0.05).
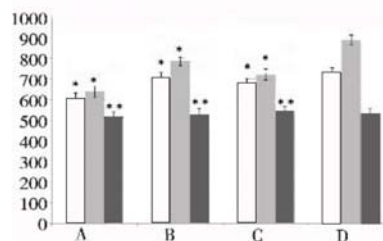

Fig. 6: VEGF levels in each group at different time points
Note: white represent concentration of VEGF on the 3rd day after postoperatiom (pg/mL), gray represent concentration of VEGF on the 7th day after postoperatiom (pg/mL), black represent concentration of VEGF on the 28th day after postoperatiom (pg/mL); A: Model group, B: MSCs transplantation group, C: HMGB1 injection group, D: HMGB1 injection+MSCs transplantation group

### The assessment on the rat model with acute myocardial infarction

After the anesthesia militating, the chest was quickly cut open to strip pericardium to observe that the rat was with ruddy myocardial and force beating pulse (fig. 5A) and with heart rate of 300 bmp/min (fig. 5A). It was seen that the color of myocardial tissue under the plane of the ligation region deepened quickly to dark brown because of the veinous backflow obstruction after few seconds of ligation. After few minutes of ligation, it could be seen that the moving intensity of the left ventricular wall decreased significantly along with the exacerbation of the ischemia of the left anterior descending branch as the time went on. The color of myocardial tissue from the apex of heart to the region below the ligation site turned gradually to pale while the non-infarcted region was rubby with a clear boundary (fig. 4B). II lead electrocardiogram showed that the J point of the QRS back of the wave obviously raised, with a typical arch elevation changes (fig. 5B), meaning the success of the establishment of the myocardial infarction model.
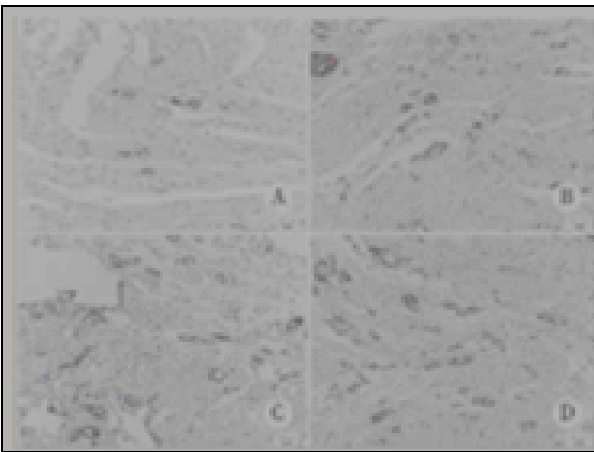
A: Control group (× 200)
B: MSCs transplantation group (× 200)
C: HMGBI injection group (× 200)
D: HMGBI injection + MSCs transplantation group (× 200)

Fig. 7: New microvascutar density of the myocardial tissue with the immunohistochemical CD31 Detection in each group

The results of the vascular endothelial growth factor (VEGF) level changes showed that the serum concentration in the model group, the MSCs transplantation group, HMGB1 injection group and the HMGB1 injection + MSCs transplantation group were all in increasing trend after post operation in 3 and 7 days and on the 7th day appeared the peak and on the 28th day it decreased almost to the normal control group. And on the 3rd and 7th day the concentration of the HMGB1 injection + MSCs transplantation group was much higher than the MSCs transplantation group and the HMGB1 injection group (P < 0.05) and on the 28th day there was no statistical significance (P > 0.05) (table 2 and fig. 6).
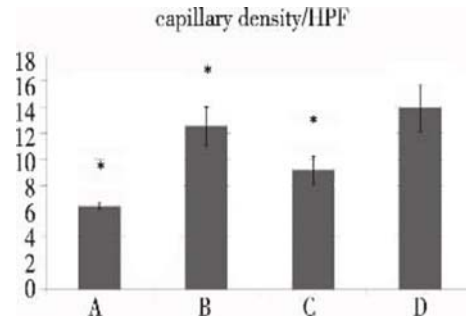
A: Control group
B: MSCs transplantation group
C: HMGBI injection group
D: HMGBI injection + MSCs transplantation group

Fig. 8: The quantitative comparison of the new microvascular density of the myocardial tissue with the immunohistochemical CD31

The detection of angiogenesis in myocardial infarction region showed that the dense of the angiogenesis in myocardial infarction region and its border in the HMGB1 injection + MSCs transplantation group (18.95 ± 1.80) increased compared with the model group (6.38 ± 0.25), HMGB1 injection group (9.16 ± 1.10) and the MSCs transplantation group (12.54 ± 1.50), (P < 0.05) (figs. 7 and 8).

DISCUSSION

MSCs transplantation treatment for myocardial infarction

This experiment still adopted the traditional MSCs culture method, with a stable cell proliferation and differentiation and good cellular immune phenotype characteristics. MSCs cultivated in this experiment were identified by flow cytometry identification. Most of the MSCs expressed CD29 and CD90, very few cells expressed CD45, meaning that the MSCs were in high purity, according to the identification standards of the characteristics of MSCs surface antigen at home and abroad (Biscetti *et al*., 2010). In previous studies, researchers showed that the MSCs might taking part in the infarction cardiac tissue repair on the following several aspects: (1) they directly differentiated into cardiomyocyte-like cells. MSCs which were activated or transplanted expressed a variety of specificity markers of the myocardial cell, and the phenotype and activity were similar to those of the myocardial cells of the developing fetus, according to the research *in vivo* and *in vitro* (Murry *et al*., 2004). (2) effects of the vascular differentiation and cell fusion. In the micro-environment of ischemia, hypoxia and inflammation, with the stimulation of the growth factors and cytokines, MSCs could gradually obtain the endothelial cell phenotype, forming capillary-like network in the extracellular matrix, expressing and secreting vascular endothelial growth factor and alkaline fibroblast growth factor (Garvican *et*

Table 1: The secretion level of VEGF at different time points after the intervention of the HMGBI on the MSCs (pg/mL) (x ± s, n = 8)

Concentration	12h VEGF Concentration	24h VEGF Concentration	48h VEGF Concentration
0	256.85±16.87	564.96±19.31	757.10±21.98
12.5	5390.36±16.6**	713.86±12.92**	849.01±25.29**
25	403.64±10.76**	772.54±15.04**	886.37±27.77**
50	357.49±13.86**	728.68±19.35**	848.56±19.23**
100	303.06±8.06**	668.11±12.45**	790.82±12.57**
200	294.06±14.82*	570.42±15.04*	778.07±23.83*
400	233.73±9.75*	526.88±17.47*	734.21±19.47*
800	208.69±1.99*	487.67±13.32*	716.93±18.76*

** $P < 0.01$ compared with 0ng/ml HMGBI, *$P < 0.05$ compared with 0ng/ml HMGBI

Table 2: VEGE level in the serum among the groups at different time points (pg/ml) (x ± s, n=24)

Group	3d	7d	28d
Control group	604.98±7.54*	639.14±7.34*	514.53±3.74**
MSCs transplantation group	705.69±5.58*	786.84±1.74*	524.35±1.37**
HMGBI injection group	678.47±3.03*	722.12±6.21*	540.24±5.55**
HMGBI injection + MSCs transplantation group	728.35±4.27	890.02±2.67	528.39±0.03

al., 2014). (3) Paracrine function. MSCs could secrete a variety of growth factors and cell factors and angiogenin and chemokines etc (Cui *et al.*, 2014).

In this study the number of angiogenesis in the infarction border region of the MSCs transplantation group increased obviously compared with the model control group, in addition, the VEGF concentration level increased with the detection by ELISA method. Combined with previous research results, the transplantation of MSCs into the rabbit model with myocardial infarction could increase the myocardial capillary growth in the infarction region to improve the heart function by improving the function of repairing related cytokines level (Liu *et al.*, 2011). It fully illustrated that the MSCs played an active role in improving vascular differentiation and the restore of the heart ventricle in the infarction area and infarction border area with acute myocardial infarction.

The influencing factors and evaluation of the rat model with myocardial infarction: The experiment adopted the cervical trachea cutting open the intubation tube (Ruan and He, 2011), the coronary artery ligation method, taking the 2 mm down along the left coronary vein of the junction between the pulmonary arterial cone and left atrial appendage of the heart as the coronary artery ligation point (Yuan *et al.*, 2012), with the ligation depth of about 1-1.5mm. It was proved that the low perfusion and tissue necrosis occurred when the color of the ligation myocardial region turned from red to pale and the ventricular wall motion descended. The II lead electrocardiogram showed that the J point obviously raised >0. 2mv after the QRS wave, with the arch elevation changing, R wave amplitude reducing, meaning the successful establishment of the model (Hou *et al.*, 2010). Thus was taken as a judge standard of myocardial infarction model.

The effects of HMGB1 on the MSCs paracrine
It was verified in some researches that the MSCs expressed the TLR4 and the RAGE mRNA of the HMGB1 receptors (Ikegami *et al.*, 2010). It was not clear that where the HMGB1 receptors exist in the MSCs exactly. It was proved that TLR4 existed in the cytoplasm of MSCs and RAGE in the nucleus of MSCs using the immune cell fluorescence detection in this experiment.

Tissue damage and (or) inflammation releasing a high concentration HMGB1 may induce chemotaxis of the MSCs, involved in the repair of stem cells (Biscetti *et al.*, 2010). Meng and others proved that HMGB1 could promote the migration of MSCs. The possible mechanism was that the TLR4 took effect in the signaling pathways of the HMGB1, TLR4 and its ligands could regulate the proliferation, differentiation and migration of MSCs, affecting the maintain of the multidirectional potentials of MSCs. The results of the experiment *in vitro* proved that HMGB1 could promote MSCs paracrine, with a two-way adjustment. When its concentration was ranging from 12. 5 ng/mL to 200 ng/mL, HMGB1 could promote the secretion of VEGF of MSCs and the 25-50 ng/mL, reached a peak. On the contrary, with the increasing of concentration, 400-800 ng/mL suppressed the secretion of VEGF. The VEGF increased with the extension of time. The experiment confirmed that the MSCs itself owned a strong endocrine function, can secrete VEGF cell growth factor etc. Meanwhile, it also confirmed that a certain concentration range of HMGB1 could promote MSCs paracrine function.

The angiogenesis density with the detection of the immunohistochemical method and the serum VEGF concentration levels with the detection of the ELISA method in the infarction region in the HMGB1 injection + MSCs transplantation group of the experiment *in vivo* all increased compared with the model group, the MSCs transplantation group and the HMGB1 injection group. In the rat models with alkali inducing corneal neovascularization (CNV), the lack of the TLR4 receptor of HMGB1 led to the decrease of the promote angiogenesis factors, using HMGB1 in part of the injured cornea macrophages which could promote corneal neovascularization by increasing the macrophage. The Results showed that the release of HMGB1 started the response of the TLR4 dependency, helping form angiogenesis (Lin *et al*., 2011). HMGB1 combined with MSCs transplantation therapy might contribute to the increase of the expression of the VEGF and the neovascularization of part of the infraction region and the improvement of the heart function compared with the single MSCs transplantation, HMGB1 injection treatment. However, after MSCs transplantation, how TLRs pathways regulate the function of the MSCs chemo taxis and paracrine and inducing, and whether it can maintain for a long time and their mechanisms still need further study.

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