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Original Article

Changes in expression of CD68, MMP-2, and ICAM-1 and the significance in choke vessels after cross-boundary flap

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Abstract: Objective: The aim of this study was to investigate expression of Cluster of Differentiation 68 (CD68), Matrix metalloproteinase-2 (MMP-2), and Intercellular Adhesion Molecule 1 (ICAM-1) in the evolution of choke vessels, examining the clinical significance. Methods: Sixty rats were randomized into experimental and control groups (30 rats in each group). Rats in the control group were not treated, while those in the experimental group were used to establish flap models. This study detected changes in expression of CD68, MMP-2, and ICAM-1 in choke vessels by quantitative reverse transcription-PCR and Western blot analyses. Pearson's correlation analysis was performed to explore the correlation between mRNA and protein expression of these indicators. Results: Following model establishment, congestion was observed in the veins of the flaps. After surgery, the average diameter of veins in the choke region of rats had increased until day 3. Moreover, peak levels of mRNA expression of CD68, MMP-2, and ICAM-1 were observed at day 3, followed by a gradual decrease. Moreover, mRNA expression of these indicators in the experimental group was significantly higher than the control group at all time points (P<0.05). At each time point, protein expression of CD68, MMP-2, and ICAM-1 in the experimental group was significantly higher than that in the control group (P<0.05). Pearson's correlation analysis also showed that, at day 3, a positive correlation among protein expression of CD68, MMP-2, and ICAM-1 was observed in the choke region of experimental group rats (P<0.05). Conclusion: CD68, MMP-2, and ICAM-1 expression shows positive correlation. After removing the cross-boundary flap, upregulation of CD68, MMP-2, and ICAM-1 may be involved in angiogenesis in the choke region.

Keywords: Choke vessels, CD68, matrix metalloproteinase-2, intracellular adhesion molecule-1, changes in expression

Introduction

Social development and improved quality of life have contributed to an increased number of traffic accidents, resulting in large defects in the skin or tissues of patients [1]. For skin defects or exposure of deep tissues caused by other factors, surgical transplantation of the flap has been regarded as the most efficient method [2]. The flap refers to a tissue block consisting of epidermis, dermis, and subcutaneous tissues. Flap transplantation can remarkably improve the quality of life for patients [3]. Currently, among all flap transplantation methods, perforator flap transplantation is the most widely used method. This method ensures the blood supply to the flap tissues by repairing other local perforator vessels of small diameters. Connection of the blood supply among different sites forms a consecutive vessel network, in which the minor unit is known as the perforating branch [4]. Research has shown [5] that perforating branches, which typically form tree-like branches, are connected in the choke region in a gradually decreasing area. Physiologically, the choke region undergoes closure. Any blockage occurring in the adjacent perforating arteries causes novel effective circulation to gradually develop from the corresponding anastomotic branches to compensate for the insufficient blood supply, thereby averting ischemia or necrosis in perforating flaps [6].

Matrix metalloproteinase (MMP)-2 is a type of gelatinase A. It is extensively expressed in

Table 1. Primer sequences

Gene	Upstream primer	Downstream primer
CD68	5'-CACGCAGCACAGTGGACATTCT-3'	5'-TGGGGCAGGAGAAACTTTGCC-3'
MMP-2	5'-GCTGATACTGACACTGGTACTG-3'	5'-CAATCTTTTCTGGGAGCTC-3'
ICAM-1	5'-CAGTGACCATCTACAGCTTTCCGG-3	5'-GCTGCTACCACAGTGATGATGACAA-3'
GAPDH	5'-GAAGGTGAAGGTCGGAGTC-3'	5'-TGGGGCAGGAGAAACTTTGCC-3'

humans and can degrade multiple collagenases and constituents of the basal membrane. Moreover, studies have demonstrated that MMP-2 plays a key role in the proliferation and migration of vascular smooth muscle cells and is involved in angiogenesis in a variety of tumors [7]. In recent years, cross-boundary flap transplantation has become widely clinically applied, in which communication and anastomosis control the blood supply. Inflammation is a major factor affecting angiogenesis and vascular remodeling in the choke region [6]. Cluster of differentiation 68 (CD68) and intracellular cell adhesion molecule-1 (ICAM-1) are major inflammatory cytokines. ICAM-1 is mainly distributed on the surface of neutrophils, monocytes, and endothelial cells, but shows low expression under normal conditions. However, during inflammatory response, ICAM-1 can facilitate the phenotype transformation of monocytes into CD68-labeled macrophages by dissociating monocytes from the vessels to participate in repair and reconstruction of vessels [8, 9].

Thus, the current study examined changes in expression of CD68, MMP-2, and ICAM-1 in choke vessels after obtaining cross-boundary flaps, aiming to provide a theoretical basis for clinical flap transplantation.

Materials and methods

Animals

In this study, 60 Sprague-Dawley rats (Center of Medical Experiment Animals, Guangdong, China), with an average weight of 280±10 g, aged 8 weeks, were fed *ad libitum* by the designated staff for 1 week. The environment included a temperature of 22±2°C and humidity of 55±10%.

Major reagents and materials

RNA reverse transcription kit (AT101-03, TransGen Biotech, Beijing, China), Real-time PCR kit (AQ111-03, TransGen Biotech), TRIzol Reagent (15596018, Invitrogen, Carlsbad, CA, USA), primers for CD68, MMP-2, and ICAM-1, synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) (**Table 1**), anti-CD68 monoclonal antibody (AF2320, Shanghai Beyotime Biotechnology Institute, Shanghai, China), anti-MMP-2 monoclonal antibody (AF1420, Shanghai Beyotime Biotechnology Institute), anti-ICAM-1 monoclonal antibody (AF1774, Shanghai Beyotime Biotechnology Institute), BCA protein quantification kit (P0009, Shanghai Beyotime Biotechnology Institute), horseradish peroxidase-conjugated goat anti-rat IgG (ZDR-5307, Beijing Zhongshan Goldbridge, Beijing, China), GAPDH Mouse Monoclonal Antibody (AF0006, Shanghai Beyotime Biotechnology Institute), and ABI 7500 PCR apparatus (Applied Biosystems, Foster City, CA, USA) were used.

Establishment of animal models

Sixty rats were randomized into experimental and control groups, with 30 rats in each group. In the control group, there were 17 male rats and 13 female rats. In the experimental group, there were 13 male rats and 17 female rats. Rats in the experimental group were not treated. Rats in the experimental group were anesthetized using 2% pentobarbital sodium (30 mg/kg), followed by hair and skin preparation. A ligature was placed between two iliac peaks as the lower boundary. A ligature was placed between two subscapular angles as the upper boundary. The median line was the inner boundary, while 2.5 cm from the inner boundary was the outer boundary. A rectangular flap, with a width of 2.5 mm and length of 11 cm, was prepared by cutting through the skin and subcutaneous tissues to the superficial layer of the deep fascia. The capillary network was preserved. Beneath the deep fascia, subcutaneous tissues were isolated. Following flap resection, bleeding was stopped and the choke region was identified by a light test. For each experiment, 12 rats were examined in both the

experiment group and control group. At days 0, 1, 3, 5, and 7, mRNA and protein expression of CD68, MMP-2, and ICAM-1 was examined in the choke tissues of rats in the experimental group and corresponding tissues of rats in the control group.

Detection of mRNA expression of CD68, MMP-2, and ICAM-1

Choke vessel tissues collected from the rats were prepared for extraction of total RNA using TRIzol Reagent. Purity, concentration, and integrity of total RNA was determined by UV spectrometry and agarose gel electrophoresis. Total RNA was then subjected to reverse transcription, in strict accordance with manufacturer instructions, then cDNA was stored for further analysis. The PCR system contained the following: 2× TransStart® Top Green qPCR SuperMix 10 μL + 0.4 μL upstream primer + 0.4 μL downstream primer + 2 μL cDNA + 0.4 μL ROX Reference Dye II 50×, diluted to 20 μL using nuclease-free water. PCR conditions were as follows: pre-denaturation at 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. For each sample, three replicative wells were used. qRT-PCR was performed three times. Results were normalized to the level of GAPDH and analyzed using the $2^{-\Delta\Delta ct}$ method.

Detection of protein expression of CD68, MMP-2, and ICAM-1

Total protein was extracted from choke vessel tissue samples stored at -80°C using RIPA reagent, then protein concentrations were detected. According to protein concentrations, the same amount of protein was mixed with 5× SDS loading buffer and added to the wells of a gel for SDS-PAGE. This was followed by separation at 80 V for the stacking gel and 120 V for the separating gel. Proteins in the gel were transferred to polyvinylidene difluoride membranes, followed by ponceau staining to clarify the protein bands. Membranes were immersed into phosphate-buffered saline containing Tween 20 (PBST) for 5 minutes, followed by 5% skim milk at 4°C overnight to block non-specific binding. Monoclonal CD68, MMP-2, and ICAM-1 antibodies, diluted with PBST containing 1% skim milk, were used to probe proteins on the membranes using Tris-buffer saline supplemented with Tween 20 (TBST). The immunoblots were incubated with secondary horseradish peroxidase-conjugated goat anti-rat antibodies (1:5000) for 1-2 hours, followed by 5 washes with TBST for 5 min/wash. Residual liquid on the membranes was removed using filter paper and immunoblot bands were developed using electrochemiluminescence reagent in a dark room for 5 minutes. The bands were scanned and analyzed using Quantity One software. Relative expression level of protein=gray value of target proteins/gray value of GAPDH.

Outcome measures

CD68, MMP-2, and ICAM-1 in the vascular evolution in the choke region of rats was observed using the skin window [10]. Changes in mRNA and protein expression of CD68, MMP-2, and ICAM-1 in rats were observed on days 0, 1, 3, 5, and 7. Correlation between mRNA and protein expression of CD68, MMP-2, and ICAM-1 was also detected.

Statistical analysis

Data collected from this study were subjected to statistical analysis using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). Figures were prepared using GraphPad Prism 7 (GraphPad, Inc., La Jolla, CA, USA). Enumeration data are presented as rates (%) and were compared using the Chi-squared test. Measurement data are expressed as means ± standard deviation (SD). Normally distributed data were analyzed between two groups using *t*-test, while analysis of variance was performed for comparison among groups. Correlation between mRNA and protein expression of CD68, MMP-2, and ICAM-1 was identified by Pearson's correlation analysis. $P<0.05$ suggests that differences are statistically significant.

Results

Growth of flap veins in rats

Following model establishment, congestion was observed in the veins of flaps, with the peak level of vessel dilation in the choke region reached on day 3. After surgery, a gradual increase was observed in the average diameter of veins in the choke region of rats. The maximum diameter was observed at day 3, followed by a gradual decrease (**Table 2**).

Table 2. Changes in vein diameter in rats

Vascular diameter	0 d	1 d	3 d	5 d	7 d
Vein (mm)	0.09±0.05	0.12±0.06	0.18±0.06	0.11±0.05	0.12±0.08
t value		10.513	13.601	10.402	10.510
P value		0.01	0.001	0.01	0.01

Table 3. Expression of CD68 in the two groups of rats

Time	Control group	Experimental group	t value	*P* value
0 d	1.012±0.025	1.642±0.125#	12.106	<0.001
1 d	1.015±0.020	1.722±0.184#	9.357	<0.001
3 d	1.020±0.022	2.015±0.233	10.414	<0.001
5 d	1.017±0.029	1.837±0.157	12.581	<0.001
7 d	1.016±0.018	1.734±0.163#	10.725	<0.001

Note: # Indicates a difference from 3rd day (P<0.05).

Table 4. Expression of MMP-2 in the two groups of rats

Time	Control group	Experimental group	t value	*P* value
0 d	1.035±0.042	1.425±0.152#	6.056	<0.001
1 d	1.029±0.031	1.597±0.168#	8.144	<0.001
3 d	1.031±0.035	1.955±0.196	11.368	<0.001
5 d	1.025±0.037	1.732±0.177#,##	9.577	<0.001
7 d	1.026±0.033	1.520±0.161#,###	7.363	<0.001

Note: # Represents a difference from 3rd day (P<0.05), ## Indicates that there is a difference from the 0th day (P<0.05), ### Indicates that there is a difference from the 5th day (P<0.05).

Table 5. Expression of ICAM-1 in the two groups of rats

Time	Control group	Experimental group	t value	*P* value
0 d	1.011±0.032	1.751±0.184#	9.706	<0.001
1 d	1.015±0.022	1.932±0.201#	11.109	<0.001
3 d	1.009±0.025	2.335±0.255	12.677	<0.001
5 d	1.016±0.024	2.024±0.212#,##	11.573	<0.001
7 d	1.012±0.027	1.911±0.193#	11.300	<0.001

Note: # Represents a difference from 3rd day (P<0.05), ## Indicates that there is a difference from the 0th day (P<0.05).

mRNA expression of CD68, MMP-2, and ICAM-1 in the choke region of rats

Moreover, this study detected mRNA expression of CD68, MMP-2, and ICAM-1 in corresponding tissues of rats at different times. An increasing trend was observed in the mRNA expression of CD68, MMP-2, and ICAM-1. Peak levels were observed on day 3, followed by gradual decreases. Compared to the control group, mRNA expression of these indicators was significantly higher in the experimental group at all time points (*P*<0.05) (**Tables 3**-**5** and **Figure 1**).

Protein expression of CD68, MMP-2, and ICAM-1 in the choke region of rats

The current study also measured protein expression of CD68, MMP-2, and ICAM-1 in the two groups of rats. In the control group, CD68, MMP-2, and ICAM-1 were all expressed at low levels in corresponding tissues. In the choke region of the experimental group, protein expression was increased over time. The peak level was reached on day 3, followed by a gradual decrease. At each time point, protein expression of CD68, MMP-2, and ICAM-1 in the experimental group was significantly higher than that in the control group (*P*<0.05) (**Tables 6**-**8** and **Figure 2**).

Correlation between mRNA and protein expression of CD68, MMP-2, and ICAM-1 in the choke region of rats in the experimental group

Pearson's correlation analysis was conducted to examine the correlation between mRNA and protein expression of CD68, MMP-2, and ICAM-1 in the choke region of rats in the experimental group on day 3. A positive correlation was found between protein expression of CD68, MMP-2, and ICAM-1 in the choke region of rats (*P*<0.05) (**Table 9** and **Figure 3**).

Discussion

Recent statistical analysis of traffic accidents [11] has shown that, in the USA, more than 2.3 million patients suffer from traffic accidents. These are concomitant with extensive injuries. Most of these injuries require surgical treat-

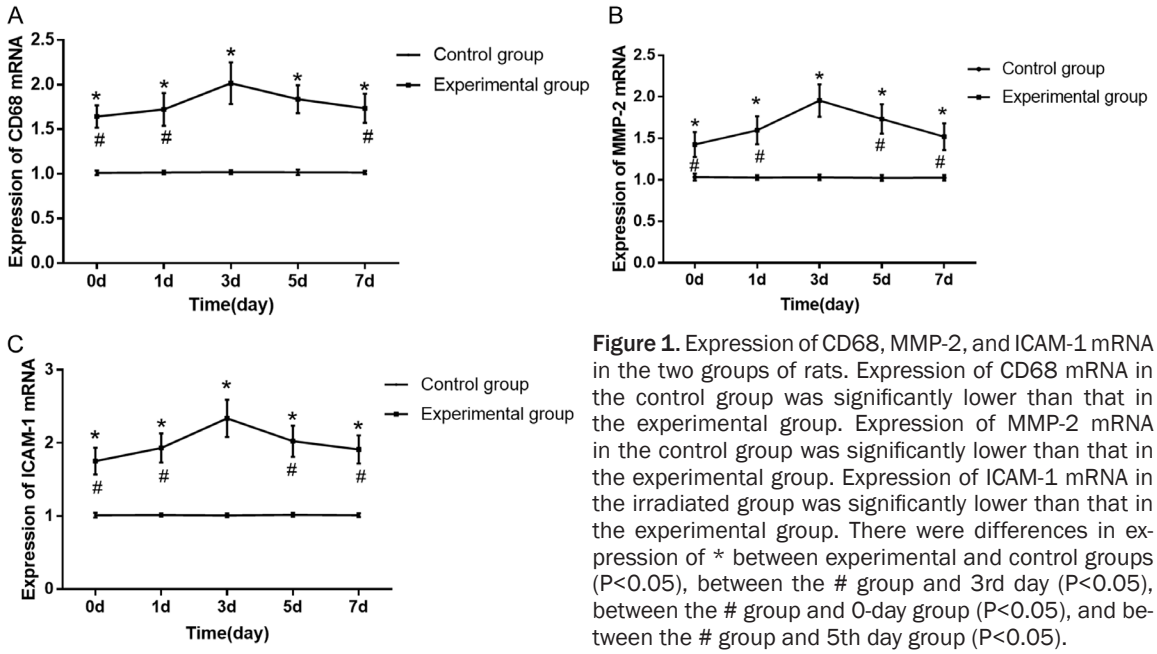

Figure 1. Expression of CD68, MMP-2, and ICAM-1 mRNA in the two groups of rats. Expression of CD68 mRNA in the control group was significantly lower than that in the experimental group. Expression of MMP-2 mRNA in the control group was significantly lower than that in the experimental group. Expression of ICAM-1 mRNA in the irradiated group was significantly lower than that in the experimental group. There were differences in expression of * between experimental and control groups (P<0.05), between the # group and 3rd day (P<0.05), between the # group and 0-day group (P<0.05), and between the # group and 5th day group (P<0.05).

Table 6. Expression of CD68 protein in the two groups of rats

Time	Control group	Experimental group	t value	*P* value
0 d	0.354±0.024	0.845±0.132#	8.946	<0.001
1 d	0.345±0.018	0.925±0.154	9.163	<0.001
3 d	0.364±0.032	1.259±0.185	11.677	<0.001
5 d	0.355±0.028	0.955±0.160	9.048	<0.001
7 d	0.361±0.030	0.886±0.128#	9.782	<0.001

Note: # Represents a difference from 3rd day (P<0.05).

Table 7. Expression of MMP-2 protein in the two groups of rats

Time	Control group	Experimental group	t value	*P* value
0 d	0.451±0.054	0.955±0.185	6.406	<0.001
1 d	0.484±0.052	1.113±0.198	7.526	<0.001
3 d	0.442±0.059	1.428±0.235	9.968	<0.001
5 d	0.465±0.061	1.241±0.211	8.654	<0.001
7 d	0.471±0.054	1.088±0.195	7.469	<0.001

Table 8. Expression of ICAM-1 protein in the two groups of rats

Time	Control group	Experimental group	t value	*P* value
0 d	0.484±0.035	0.874±0.095#	9.436	<0.001
1 d	0.499±0.038	0.942±0.112#	9.175	<0.001
3 d	0.492±0.039	1.254±0.154	11.749	<0.001
5 d	0.497±0.035	1.021±0.124	9.962	<0.001
7 d	0.485±0.037	0.958±0.105#	10.407	<0.001

Note: # Represents a difference from 3rd day (P<0.05).

ment, with flap transplantation the most widely used method for repairing extensive injuries. Clinically, severe trauma requires extensive repair by expanding the flap, in which blood supply from some vessels may cover adjacent donor sites. These flaps are known as cross-boundary flaps [12, 13]. However, after surgery, patients are more susceptible to complications, including ischemic necrosis, one of the major complications of flap transplantation. This may increase the risk of secondary surgery or even result in death, particularly for patients suffering from severe conditions [14].

Based on differences in donor regions, cross-boundary flaps are divided into the anatomic donor region, dynamic donor region, and potential donor region. Their survival mainly depends on the blood supply to the choke tissues [15]. Previous studies [16] have shown that, in cross-boundary flap models, dimethyloxallyl glycine injections can enlarge the diameter of choke vessels and significantly

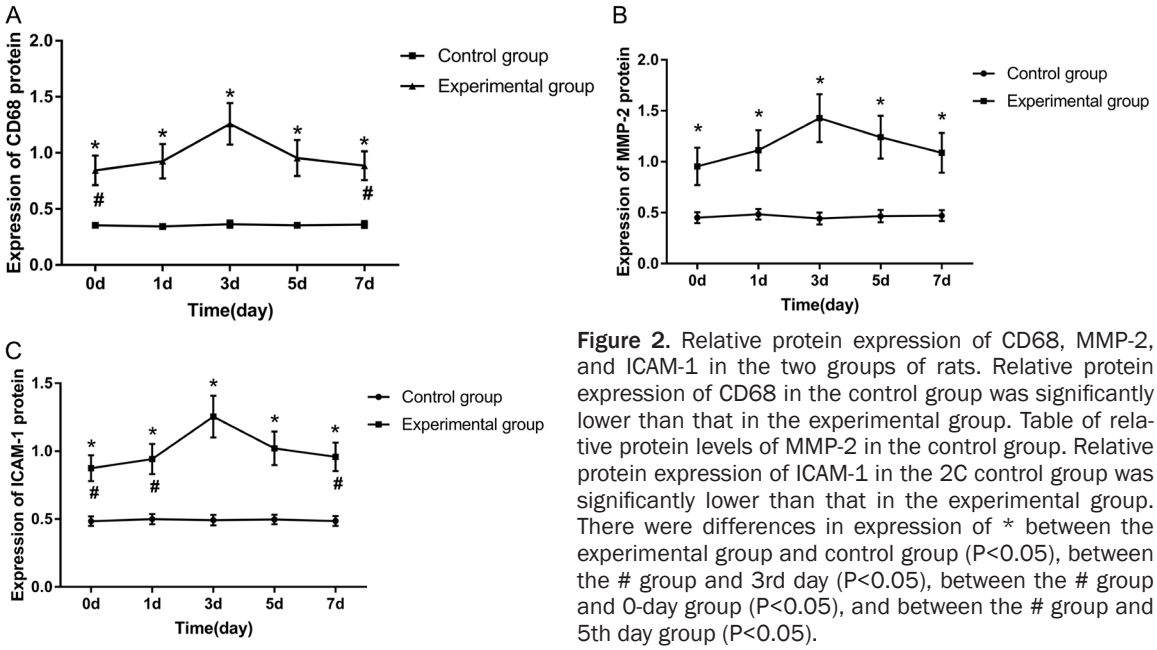

Figure 2. Relative protein expression of CD68, MMP-2, and ICAM-1 in the two groups of rats. Relative protein expression of CD68 in the control group was significantly lower than that in the experimental group. Table of relative protein levels of MMP-2 in the control group. Relative protein expression of ICAM-1 in the 2C control group was significantly lower than that in the experimental group. There were differences in expression of * between the experimental group and control group (P<0.05), between the # group and 3rd day (P<0.05), between the # group and 0-day group (P<0.05), and between the # group and 5th day group (P<0.05).

Table 9. Correlation analysis between CD68, MMP-2, and ICAM-1 mRNA and protein in the choke region of rats in the experimental group

Analysis index	r value	*P* value	Analysis index	r value	*P* value
CD68mRNA VS MMP-2mRNA	0.880	0.021	CD68 protein VS MMP-2 protein	0.882	0.020
CD68mRNA VS ICAM-1mRNA	0.956	0.003	CD68 protein VS ICAM-1 protein	0.873	0.023
MMP-2mRNA VS ICAM-1mRNA	0.919	0.010	MMP-2 protein VS ICAM-1 protein	0.886	0.019

inhibit ischemia and hypoxia. Vascular remodeling involves the inflammatory environment and various factors. ICAM-1, a major factor sustaining cell structure, immunoregulation, and inflammation [17], has been found to be involved in angiogenesis, invasion, and metastasis of tumor cells. During inflammation, ICAM-1 facilitates the transformation of monocytes into CD68-labeled macrophages to facilitate tissue repair. CD68 is the major antigen in monocytes/macrophages and a specific indicator [18]. MMPs, critical proteases in tissue remodeling, have been reported to be involved in arterial development, morphological evolvement, and traumatic repair. MMP-2, a key MMP, is extensively expressed in humans. It can be degraded into multiple collagens, gelatin, and constituents of the basal membrane, displaying a key role in the migration and proliferation of vascular smooth muscle cells [10]. However, few studies have examined expression patterns and correlation of these factors in choke vessels after obtaining cross-boundary flaps. Thus, the current study examined expression of CD68, MMP-2, and ICAM-1 in the choke region following cross-boundary flap, aiming to provide a reference for clinical practice.

It has been shown [19] that, at days 2-3 following the establishment of cross-boundary flap models, choke vessels are rapidly dilated and vascular remodeling develops along the axis of vessel distribution. At day 7 following surgery, the diameter of choke vessels approximates that of the main vessels. However, results of this study showed that, following surgery, the flap veins were congested. The peak diameter was reached on day 3, followed by a gradual decrease. At day 7, the vessel diameter was nearly equivalent to that of the main vessels, suggesting the successful establishment of animal models. Next, this study detected mRNA and protein expression in the choke tissues of rats in the experimental group. It was found that mRNA and protein levels of CD68, MMP-2, and ICAM-1 were high in the choke tissues, with peak levels reached on day 3 after model establishment. This was followed by a gradual

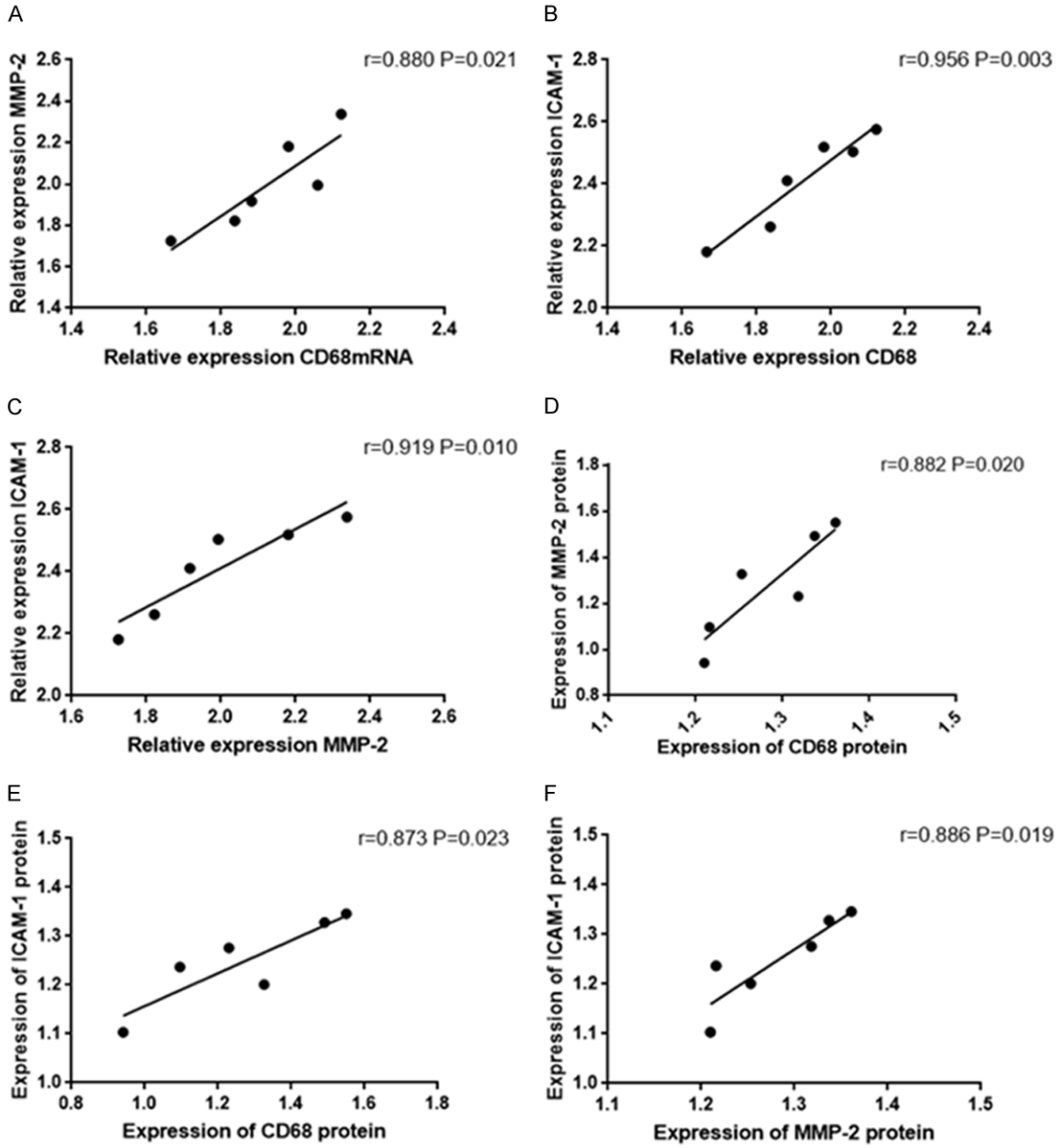

Figure 3. Correlation analysis of CD68, MMP-2, and ICAM-1 mRNA and protein expression on the 3rd day in the experimental group. (A) There was a positive correlation between expression of CD68 and MMP-2 mRNA (r=0.880, P=0.021) (B), on the third day in the experimental group. There was a positive correlation between expression of CD68 and ICAM-1 mRNA (r=0.956, P=0.003) (C). MMP-2 and ICAM-1 mRNA tables of rats in the experimental group on the third day. There was a positive correlation (r=0.919, P=0.010) (D). There was a positive correlation between expression of CD68 and MMP-2 (r=0.882, P=0.020) (E), on the third day in the experimental group. There was a positive correlation between expression of CD68 and ICAM-1 (r=0.873, P=0.023) (F), on the third day in the experimental group. There was a positive correlation between expression of MMP-2 and ICAM-1 protein on the third day in the experimental group (r=0.886, P=0.019).

decrease. However, in the control group, mRNA and protein expression of CD68, MMP-2, and ICAM-1 in the corresponding tissues of rats was significantly lower than those in rats in the experimental group. Expression was gradually augmented as the vessels enlarged. At the peak level, expression was downregulated as vessel diameter decreased, suggesting that, after flap transplantation, CD68, MMP-2, and ICAM-1 expression was correlated with enlarge-

ment of veins in the choke tissues. A previous study [24] indicated that MMP-2 is extensively involved in a variety of tumors, including ovarian cancer, breast cancer, and colorectal cancer, being closely correlated with angiogenesis. The current study found that MMP-2 was highly expressed in the choke region following the cross-boundary flap, which agrees with results of Zhuang et al. [20]. Additionally, this study measured inflammatory factors, finding similarly high expression of CD68 and ICAM-1. Guo et al. [21] suggested that ICAM-1 facilitates angiogenesis in tumors, as well as the invasion and mitigation of tumor cells. However, a previous study [22] showed that ICAM-1 is critical in the involvement of monocytes in expanding the choke region, verified in the current study. Li et al. [23] also showed that CD68 is closely related to angiogenesis in breast cancer. The current study detected the involvement of CD68, MMP-2, and ICAM-1 in angiogenesis in the choke region following cross-boundary flap. Correlation analysis of mRNA and protein expression of CD68, MMP-2, and ICAM-1 at day 3 showed that mRNA and protein expression of these three factors was positively correlated. Jung et al. [24] indicated that MMP-2 expression paralleled that of CD68. Additionally, ICAM-1 promotes the generation of CD68 by regulating monocytes, suggesting a regulatory relationship between ICAM-1, CD68, and MMP-2. However, the specific details require further verification.

There were some limitations to the current study. First, the absence of inhibitors of CD68, MMP-2, and ICAM-1 prevented observation of angiogenesis in the choke tissues, following inhibition of these factors. Moreover, this study was limited by the small sample size. Present results should be verified in further studies. Further studies should verify current results using additional indicators and a larger sample.

In conclusion, expression levels of CD68, MMP-2, and ICAM-1 were positively correlated. Following cross-boundary flap, upregulation of CD68, MMP-2, and ICAM-1 suggests the involvement of these proteins in angiogenesis in the choke region.

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Disclosure of conflict of interest

None.

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