

Original Article

Hemocoagulase atrox reduces vascular modeling in rabbit carotid artery adventitia

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Abstract: Objective: This study aimed to compare the effects of hemocoagulase atrox and cauterization hemostasis on intimal hyperplasia and explore the effect of hemocoagulase atrox on vascular modeling in rabbit carotid artery adventitia. Methods: A total of 27 rabbits were randomly divided into 3 groups (0d, 14d, 28d). They were anaesthetized using an intramuscular injection of phenobarbital sodium (1 ml/kg). The left and right common carotid arteries were exposed and capillary hemorrhaged after blunt dissection of the adventitia layers of common carotid arteries. Nine rabbits in each group were again randomly divided into 3 groups, in which animals were respectively treated with hemocoagulase (2 U/ml), cauterization (power = 40 w) and saline (as control). Groups of animals were euthanized at 0, 14 and 28 days after surgery. The samples were equally divided in the middle of the adventitia removal section to obtain equal parts for histologic, immunohistochemical and molecular biologic analysis. The vascular repair after adventitial stripping was observed by HE staining, Masson staining and transmission electron microscopy. The expression of carotid MCP-1, PCNA, TGF- β 1, α -SMA and VEGF were measured at different time points by RT-PCR and immunohistochemical staining. Results: HE staining and Masson staining showed that hemocoagulase atrox had a significantly stronger effect on reducing intimal hyperplasia than the cauterization after 14 and 28 days. The results of RT-PCR showed that the expression of MCP-1, TGF- β 1, α -SMA and VEGF in hemocoagulase atrox-treated animals were lower than that of cauterization-treated animals. Conclusion: Our results suggested that hemocoagulase atrox as a topical hemostatic is safety and efficiently and it can accelerate adventitia restoration and decrease intimal proliferation.

Keywords: Adventitia, hemocoagulase, hemostasis, cauterization, vascular remodeling

Introduction

Perioperative bleeding and postoperative wound bleeding are common complications in surgical procedure. Reducing intraoperative and postoperative blood loss in clinical practice has being taken seriously attention [1]. Drug and physical hemostasis are commonly used to stop bleeding in clinic operation [2]. Physical hemostasis, such as cauterization, was heavily used in surgery would inevitably injure adventitia and activated adventitial fibroblasts. Cytokines and chemokines were produced which induced vascular remodeling [3].

Hemocoagulase atrox isolated from Brazil spearhead Agkistrodon is a serine protease that clots fibrinogen. It can significantly diminish blood loss and transfusion requirements in surgeries [4]. Accumulating data have demon-

strated that hemocoagulase atrox has the ability to reduce local bleeding, but not associate with any increase in the prothrombin level and consequently constitutes no danger of thrombosis [5]. In this study, we will compare the effects of hemocoagulase atrox and cauterization hemostasis on vascular remodeling.

Materials and methods

Animals and operative procedure

New Zealand White Rabbits (NZW rabbits, 3.0~3.5 kg; n = 44), 20 weeks of age, regardless of gender, were bought from Experimental Animal Center of Anhui Medical University. The experiments on laboratory animals were approved by the Authorization No. 48/2009 issued by the Ministry of Health of the People's Republic of China according to the law of The

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Guidance of Treat Experimental Animal, for the protection of animals against suffering, and in accordance with the Project of Experiments and the statement of the Ethical Committee.

27 rabbits were randomly divided into 3 groups (0d group, 14d group and 28d group) and 9 rabbits in each group. All of them were anaesthetized using an intramuscular injection of phenobarbital sodium (1 ml/kg). The left and right common carotid arteries were exposed through a vertical midline cervical incision and separated with surrounding tissue by using aseptic rubber pads. Carotid arteries were wrapped around using collagenase II (2 mg/ml, life technologies, USA) soaked gauze and digested for 15 min. Then the digestion was stopped by using 0.9% physiological saline wash 3 times. Blunt dissection the adventitia of common carotid arteries and cause capillary hemorrhage. Nine of them will receive 2 U/ml hemocoagulase atrox (Nuokang bio-pharmaceutical inc., China) for topical hemostatic, 9 of them will receive cauterization (power = 40 w) for topical hemostatic. Another 9 rabbits will use 0.9% physiological saline as control. After stop bleeding, the incision was sutured. The operative procedure was performed with an aseptic technique. Groups of animals were euthanized 0, 14 and 28 days after surgery. The specimens were equally divided in the middle of the adventitia removal section to obtain equal parts for histologic, immunohistochemical and molecular biologic analysis.

Tissue harvesting and preparation

At 0, 14 and 28 days after surgery, the rabbits were sacrificed by intravenous sodium pentobarbital overdose (100 mg/kg). The two sides of carotid arteries were removed and divided into three parts. One part was fixed in 4% paraformaldehyde and embedded in paraffin. The second part was fixed in 2.5% glutaraldehyde and stored at 4 °C for electron microscopy analysis. The 3rd part was store in liquid nitrogen for total RNA preparation.

Histological staining

The samples embedded in paraffin were cut into 4 µm sections and stained with hematoxylin-eosin and Masson's trichrome. Each section was photographed using a Nikon eclipse 80i microscope. The following parameters were

measured: the thickness of the intima from the endothelial surface to the inner border of the tunica media, the thickness of the media from the inner border of tunica media to the border between tunica media and adventitia, and the thickness of the media-intima complex from the endothelial surface to the media-adventitia border. Data was collected from 32 to 84 measurements per rabbit in 12-28 microscopic fields using the Image Pro-Plus 6.0 Software. The circumference of vessel lumen (L_1), internal elastic lamina (L_2), external elastic lamina (L_3) and vascular wall (L_4) were calculated. To avoid the influence of folding artifacts, the area of vessel lumen (LA), the area of internal elastic lamina (IELA), the area of external elastic lamina (EELA), thickness of the intima (I), thickness of the Medial (M) and thickness of the neoadventitia (A) were calculated as follows: $LA = L_1^2/4\pi$, $IELA = L_2^2/4\pi$, $EELA = L_3^2/4\pi$, $I = (L_2 - L_1)/2\pi$, $M = (L_3 - L_2)/2\pi$, $A = (L_4 - L_3)/2\pi$ [6]. The vascular remodeling index (RI) and lumen stenosis (LS) were calculated as follows: $RI = EELA \text{ after surgery}/EELA \text{ before surgery}$; $LS = (IELA - LA)/IELA = 1 - LA/IELA = 1 - (L_1 - L_2)^2$. The mean value and the Standard Error of Mean (mean ± SEM) were calculated for each section and for each group.

Electron microscopy analysis

For electron microscopy analysis, arteries were fixed in 0.1 mol/L phosphate-buffered saline (PBS, pH 7.4) containing 2.5% glutaraldehyde and postfixed in 0.1 mol/L PBS containing 1% osmium tetroxide. Then these samples were dehydrated in a graded series of alcohol and embedded in Epon. Ultrathin sections (100 nm) were stained with uranium acetate and lead citrate and observed with a transmission electron microscope (TEM, Philips 201, USA).

Immunohistochemical staining

Immunohistochemistry was performed on 4 µm-thick paraffin sections using a three-step indirect method. The slides were deparaffinized in xylene and rehydrated in graded ethanol. After deparaffinization and rehydration, the slides were cooked in a microwave oven using 0.01 M citrate buffer pH 6.0 for target retrieval. Endogenous peroxidase was blocked by 0.3% H_2O_2 in 70% methanol for 30 min. The primary antibodies (MCP-1, α -SMA, TGF- β 1 and VEGF) were applied for 30 min at RT. Detection of the

Table 1. Primers for real time RT-PCR used in this study

Primer	Sequence	Size (bp)	Gene No.
MCP-1 sense	5'-TCATAGCAGTCGCCTTCAGC-3'	295	NM_001082294.1
MCP-1 antisense	5'-GTGTGTTCTTGGGTTGTGGAATAA-3'		
VEGF sense	5'-ACGGACAGACAGACAGACACC-3'	176	XM_002714697.1
VEGF antisense	5'-CCCAGAAGTTGGACGAAAAGT-3'		
PCNA sense	5'-GGTCTTCCAACCTCTGCCACTA-3'	215	XM_002713899.1
PCNA antisense	5'-GGTTTTCTCTTTGCCTTCCCTA-3'		
TGF-β sense	5'-CGGCAGCTGTACATTGACTT-3'	271	XM_002722312.1
TGF-β antisense	5'-AGCGCACGATCATGTTGGAC-3'		
GAPDH sense	5'-ACGGTGCACGCCATCACTGCC-3'	266	NM_001082253.1
GAPDH antisense	5'-GCCTGCTTCACCACCTTCTTG-3'		

Table 2. General data and surgical conditions of each groups ($\bar{x} \pm s$, n = 9)

Groups	BD (kg)	OT (min)	LPWS (cm)	DCAAR (mm)
Od	2.18±0.21	121±22.28	2.30±0.32	2.100±0.261
14d	2.16±0.10	118±18.16	2.47±0.24	2.050±0.288
28d	2.13±0.22	120±18.24	2.38±0.38	2.133±0.242

BD: body weight; OT: operation time; LPWS: length of operative wound surface; DCAAR: diameter of carotid arteries after adventitia removal.

monoclonal antibody was performed using biotinylated horse anti-mouse IgG (Vector laboratories, Burlingame, CA, USA) diluted 200× for 30 min. The specimens were then incubated with RTU Vectastain Elite ABC Reagent for 30 min. Finally, the specimens were stained with DAB coloration solution (Dako, Glostrup, Denmark) for 5 min, and were counterstained with Harris's hematoxylin before they were embedded in Entellan (both from Merck, Germany). The images of stained sections were obtained using an Olympus IX70 microscope (Olympus, Tokyo, Japan). All parameters were measured by computer-assisted color image analysis (Image-Pro Plus, version 6.0) [7].

RT-PCR

Total RNA were extracted from samples store in liquid nitrogen with TRIZOL (Invitrogen, USA) and reverse-transcribed with the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) as per the manufacturer's instructions. PCR was performed by using corresponding primers listed in **Table 1**.

Statistical methods

Non-parametrical one-way Kruskal-Wallis ANOVA was used for a statistical evaluation of the

histology and histo-morphometry. Two-way ANOVA and the Tukey studentized range method were used for the data analyses of the thickness of the intima, media, intima/media complex and the media/intima ratio. The variables were tested for normality by Shapiro and Wilk's statistics. Since normality was rejected, the logarithmic transformation was applied. The effects of time, drug, and the interaction between time and drug were

tested, and $P < 0.05$ was considered to be significant.

Results

Operative sequelae

The severe complications such as hemorrhage, shock and excess anesthesia were not happen in operative procedure. There were no significant differences ($P > 0.05$) in body weight, operation time, operative wound surface and diameter of carotid arteries after adventitia removal of all groups' rabbits (**Table 2**).

Histology assay

The adventitia was completely removed observed by H&E and Masson staining. The smooth muscle cell morphology has been destroyed and the structure of collagen fiber was loosely in tunica media in adventitia removal carotid arteries using cauterization for topical hemostatic. But the tissue structure of tunica media in adventitia removal carotid arteries using hemocoagulase atrox for topical hemostatic is the same as control (**Figure 1A** and **1B**). In 14d and 28d groups, the adaptation process of adventitia removal led to the formation of multiple smooth muscle cells layers,

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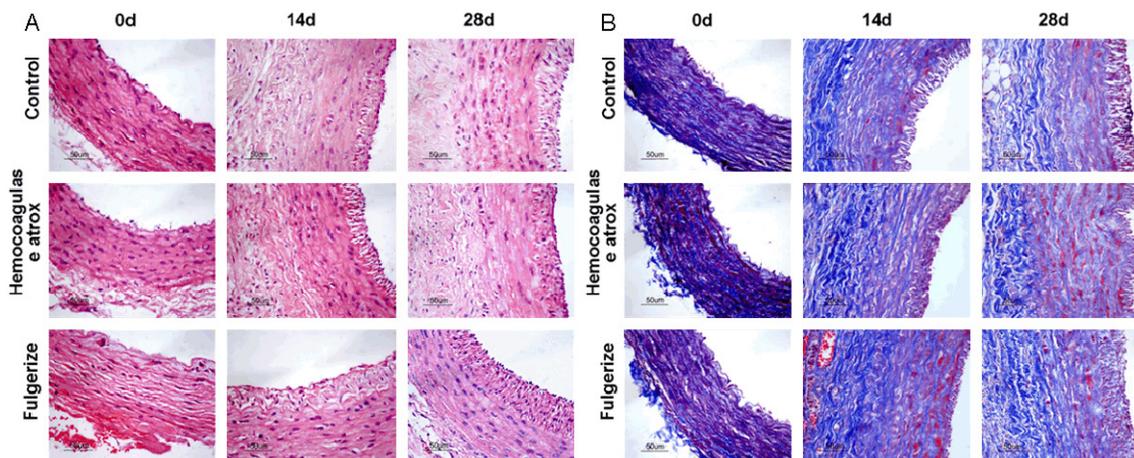


Figure 1. The carotid arteries samples which removed adventitia in NZW rabbits from 0, 14, 28-day postsurgery. A: Hematoxylin-Eosin staining, B: Masson staining.

Table 3. The results of cross-sectional thickness and collagen fibers density of carotid artery in each groups ($\bar{x} \pm s$, n = 6)

Groups	Neoadventitia (mm)	Medial (mm)	Intimal (mm)	Collagen fibers density (%)	
0d	control	/	0.0615±0.0061	/	5.28±1.126
	HA	/	0.0671±0.0092	/	5.84±1.139
	FG	/	0.0610±0.0065	/	5.08±1.166
14d	control	0.0653±0.0051	0.0994±0.0208	0.0903±0.0032	29.82±5.181
	HA	0.0668±0.0058	0.1010±0.0254	0.0911±0.0040	31.16±4.977
	FG	0.0726±0.0040 ^{Δ#}	0.1384±0.0286 ^{Δ#}	0.1046±0.0020 ^{Δ#}	38.71±5.984 ^{Δ#}
28d	control	0.0941±0.0075	0.1011±0.0205	0.0977±0.0093	14.38±3.013
	HA	0.0997±0.0050	0.1193±0.0220	0.1024±0.0092	15.36±3.497
	FG	0.1118±0.00947 ^{▲*}	0.1445±0.2528 ^{▲*}	0.1258±0.0164 ^{▲*}	23.39±5.080 ^{▲*}

^ΔP<0.05 vs. 14d control group; [#]P<0.05 vs. 14d HA group; [▲]P<0.05 vs. 28d control group; ^{*}P<0.05 vs. 28d HA group. HA: hemocoagulase atrox group; FG: cauterization group.

which resulted in thickening of the intima, media and neoadventitia. These were further led to the rises of RI and LS index. The intima measurements after 28 days showed the highest intima thickness value in all groups (**Figure 1A**). In hemocoagulase atrox topical hemostatic samples the thickness of the intima was reduced compared to the cauterization topical hemostatic samples (0.1024±0.0092 vs. 0.1258±0.0164, $P<0.05$). The hemocoagulase atrox had a significantly stronger effect reducing intimal hyperplasia than the cauterization after 14 and 28 days. The increase in media thickness (**Figure 1A**) was significantly reduced by using the hemocoagulase atrox as topical hemostatic after 14 days of adventitia removal, compared to the cauterization topical hemostatic samples (0.1010±0.0254 vs. 0.1384±0.0286, $P<0.05$, **Tables 3, 4** and **Figure**

1A). The relative density of collagen fibers was increased both in hemocoagulase atrox treated and cauterization treated animals, but hemocoagulase atrox treated animals lower than cauterization treated animals (**Table 3** and **Figure 1B**).

Transmission electron microscope observation

In 0d group, endothelial cell integrity, internal elastic membrane continuous, uniform thickness and smooth muscle cells with normal morphology were observed both in hemocoagulase atrox treated animals and controls (**Figure 2A**). Endothelial cells showing fusiform, cytoplasmic vacuoles, internal elastic plate swelling, hydropic degeneration of smooth muscle cells in medial and the cell structure is unclear in cauterization treated animals (**Figure 2B-D**). In 14d

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Table 4. The results of lumen cross-sectional area; remodeling index and lumen stenotic ratio of each groups ($\bar{x} \pm s$, n = 6)

Groups		Lcsa (mm ²)	RI	LS
0d	control	1.410±0.1689	/	/
	HA	1.407±0.1958	/	/
	FG	1.357±0.1715	/	/
14d	control	1.1750±0.1263	0.852±0.1179	0.222±0.0274
	HA	1.1172±0.1404	0.845±0.1161	0.236±0.0271
	FG	0.9450±0.1418 ^{Δ, #}	0.679±0.1075 ^{Δ, #}	0.272±0.0287 ^{Δ, #}
28d	control	0.9200±0.1481	0.780±0.0737	0.295±0.0438
	HA	0.9150±0.1691	0.753±0.0739	0.298±0.0303
	FG	0.6817±0.1848 ^{Δ, *}	0.618±0.0989 ^{Δ, *}	0.363±0.0393 ^{Δ, *}

^ΔP<0.05 vs. 14d control group; [#]P<0.05 vs. 14d HA group; ^ΔP<0.05 vs. 28d control group; ^{*}P<0.05 vs. 28d HA group. Lcsa: lumen cross-sectional area; RI: remodeling index; LS: lumen stenotic ratio; HA: hemocoagulase atrox group; FG: cauterization group.

group, smooth muscle cells were observed in the neoadventitia and migrate to the intima. All of the cells showed the characteristics of myofibroblasts (**Figure 2E**). The number of these cells in hemocoagulase atrox treated animals is more than that of cauterization treated animals. In 28d group, smooth muscle cells were observed in intima and showed normal morphology in hemocoagulase atrox treated animals and myofibroblasts differentiate into fibroblasts in neoadventitia. However, myofibroblasts can also be observed in neoadventitia and intima in cauterization treated animals (**Figure 2F**).

Hemocoagulase atrox administration decreases macrophage infiltration

The results of RT-PCR indicated that comparing with control animals, the expression of MCP-1 mRNA in cauterization treated animals after 14d and 28d increased dramatically; while there was no statistically significant difference of MCP-1 expression between hemocoagulase atrox treated animals and control animals (**Figure 3A**). In addition, the MCP-1 positive cells were lightly increased after 14 days in hemocoagulase atrox treated animals, and return to normal level after 28 days detected by using immunohistochemical staining, and significantly lower than that of cauterization treated animals at same time points (**Figure 4A**). In cauterization treated animals the MCP-1 positive cells were mostly distributed in neoadventitia and circumambient connective tissue (**Figure 4A**).

Hemocoagulase atrox administration accelerates adventitia restoration and decreases vascular proliferation

The TGF-β1, α-SMA and VEGF positive cells were increased in all animals after 14 days. However, 28 days later the positive cells were return to normal level in hemocoagulase atrox treated animals and control animals, while there were also many positive cells in cauterization treated animals, especially

observed in neoadventitia and intimal. The number of positive cell in hemocoagulase atrox treated animals was fewer than that of cauterization treated animals but more than that of control animals at the same time points (**Figures 4B, 5**). Furthermore, the expression of TGF-β1, α-SMA and VEGF mRNA was detected at elevated levels in cauterization treated animals after 14d and 28d (**Figure 3**). These results showed that hemocoagulase atrox can accelerate adventitia restoration and decrease vascular proliferation in carotid arteries adventitia removal rabbit model (**Figures 3-5**).

Discussion

Perioperative bleeding and post operative wound bleeding are very important issues considered by surgeons. A bad stop bleeding intervention will cause hematoma in operative site, delay incision healing, increase the chance of infection, reduce the patient's quality of life and poor prognosis and even endanger the lives of patients [8]. Drug hemostasis and physical hemostasis were commonly used to stop bleeding in surgical procedures [9]. The hemocoagulase atrox is obtained from Brazil spearhead *Agkistrodon* after segregation and purification. It can turn fibrinogen into fibrin monosomic I, which is further turned into fibrin multimer I, so it makes blood plaque aggregate. The phospholipid-dependent factor X (FX) activator of hemocoagulase atrox can also make blood coagulation FX changed and turned thrombinogen into thrombin, fibrinogen into fibrin and decreased bleeding in result. Hemocoagulase atrox does

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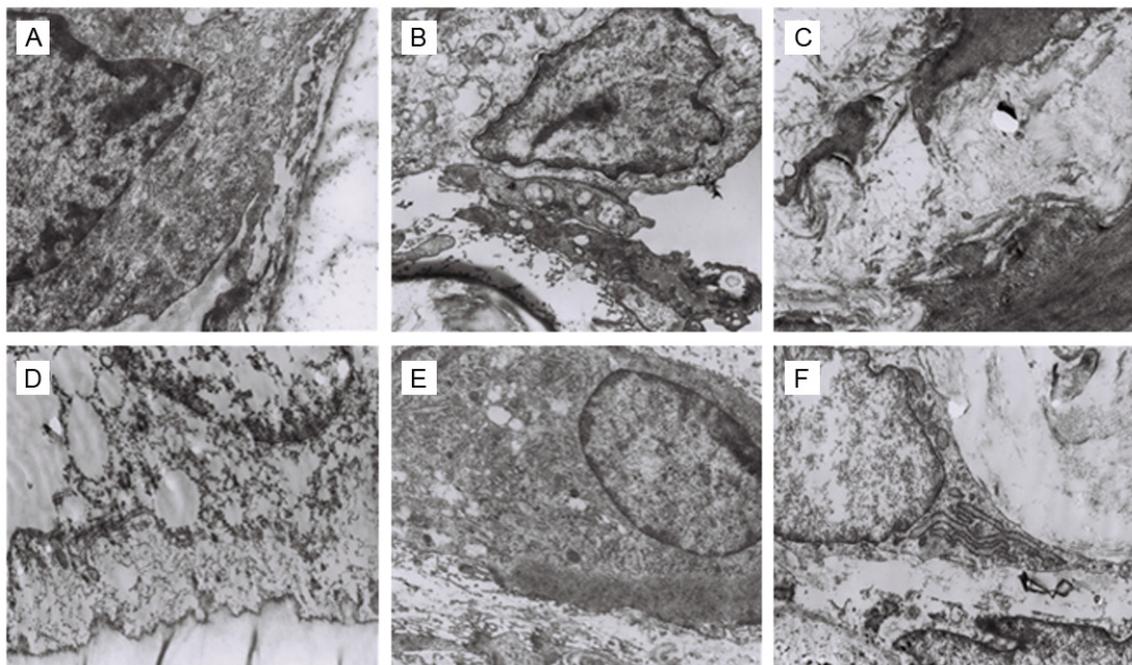


Figure 2. The carotid arteries samples observed by TEM ($\times 8000$). A: Injured endothelial cells from 0 day postsurgery in cauterization group; B: Internal elastic lamina which injured by cauterization; C: The smooth muscle cell is seriously edematous and the cell organelle is unclear in cauterization group; D: The fibroblast transformed into myofibroblasts and migrated to the intima by 14-day postsurgery in hemocoagulase atrox group; E: The normal myofibroblasts in hemocoagulase atrox group; F: The smooth muscle cells shows normal morphology treated by hemocoagulase atrox after 28-day postsurgery.

not play a role in complete vessels, so it will not cause complication of DIC (disseminated intravascular coagulation). It is known that hemocoagulase atrox is used successfully in various operations for hemostasis in nervous, respiratory, digestive and circulation systems. In the past few decades, hemocoagulase atrox has been playing an important role in the management of varices hemorrhage for patients with portal hypertension. Many researches have verified the advantages of hemocoagulase atrox in biocompatibility, nontoxicity, non-carcinogenicity and absorbability [10-12]. The cauterization is also common used in various operations due to the rapid hemostasis and improving surgical efficiency. In this study, we compared the effects of hemocoagulase atrox and cauterization hemostasis on intimal hyperplasia in carotid arteries adventitia removal rabbits.

Arterial adventitia injuring induced the pathological changes of tunica-intimal and tunica-media has been proposed in 1999 by Ross R et al [13]. The fibroblasts are the main cell type in arteries adventitia. Arterial adventitia injuring

will cause the fibroblasts differentiated into myofibroblasts [14, 15]. Myofibroblasts will participate in vascular injury repair and vascular remodeling when there is seriously injured, induce intimal hyperplasia related diseases [4, 16-18]. In this study, we used carotid arteries adventitia removal rabbit model [19] to compare the effects of hemocoagulase atrox and cauterization hemostasis on intimal hyperplasia. The intima measurements (Tables 3 and 4) after 14 and 28 days showed the relative density of collagen fibers, RI and LS index in cauterization group are higher than that of hemocoagulase atrox group. The numbers of myofibroblasts in cauterization group are more than that of hemocoagulase atrox group after 28 days. This suggests that cauterization increased the level of vascular injury, and do not conducive to long-term restoration. The same findings were reported by Guang Weiji et al [20]. These results showed that using hemocoagulase atrox as a local hemostatic is safety and efficiently, local stimulation of vascular tissue of small, conducive to long-term restoration.

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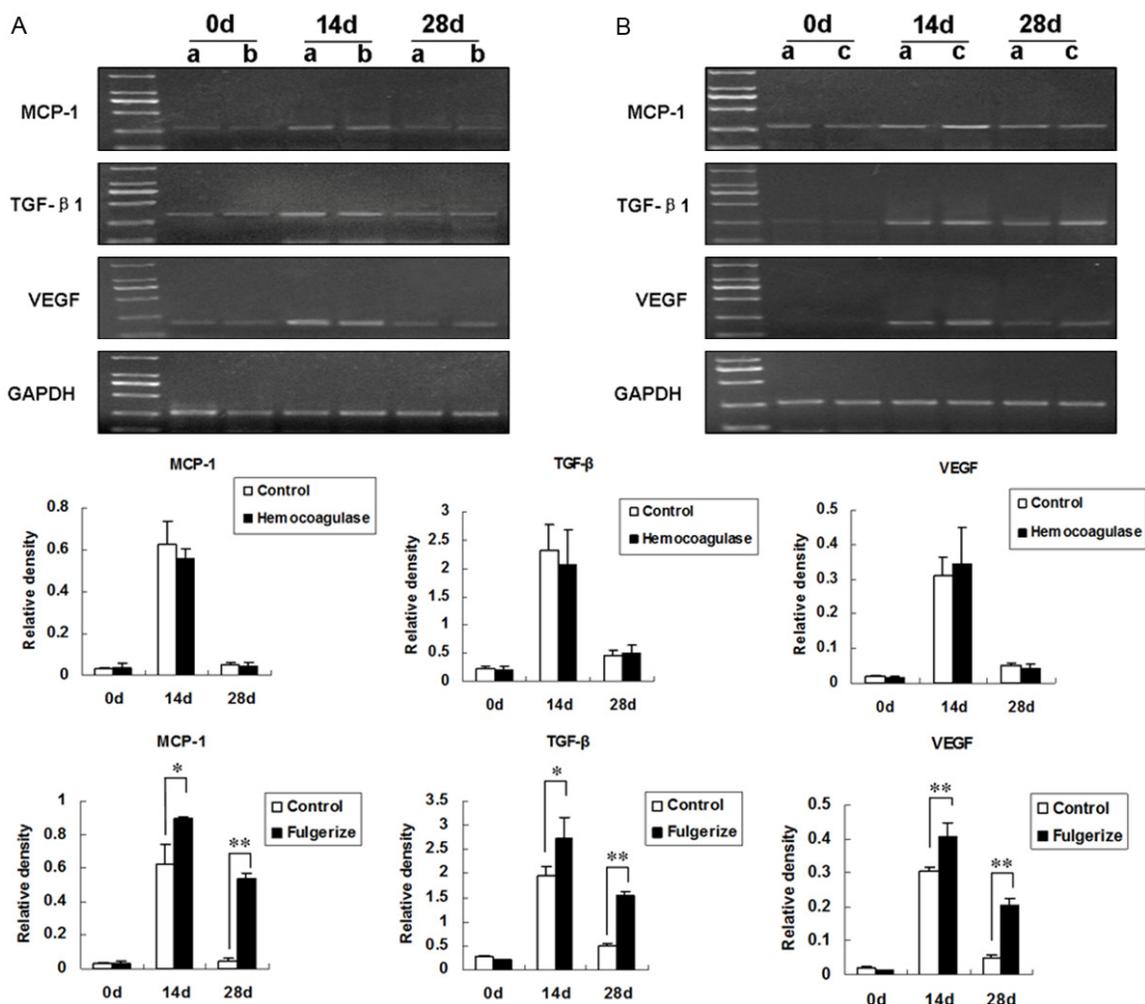


Figure 3. The mRNA expression of MCP-1, TGF- β 1, α -SMA, and VEGF in animals treated with hemocoagulase atrox (A) or cauterization (B) by reverse transcription Polymerase Chain Reaction (* $p < 0.05$ vs. control group; ** $p < 0.01$ vs. control group). (a) control group; (b) hemocoagulase atrox group; (c) cauterization group.

Response after arterial injury and wound repair process induced pathophysiological changes, many changes in cytokine expression, cell phenotype and function were adapt to the changes in the local microenvironment involved in vascular repair [21]. MCP-1 is the strongest monocyte chemotactic factor in the inflammatory response [22]. In physiological conditions MCP-1 showed low expression, but in injured vascular the expression of MCP-1 was up-regulated and inducing local inflammation [23]. Same studies suggest that knockdown MCP-1 can effectively prevent vascular restenosis [24]. In this study we find the MCP-1 positive cells in hemocoagulase atrox treated animals were significantly lower than that of cauterization treated animals at same time points. In the vascular repair process, the TGF- β 1 inhibit ECM

degradation enzymes and plasminogen activating factor synthesis, reduce the degradation of ECM and cause increasing synthesis and deposition of ECM [25, 26]. At the same time, it will regulate cellular proliferation and differentiation through activating the smad-dependent or not dependent signal transduction pathway, promote the proliferation and transformation of fibroblasts and stimulate myofibroblast to express its marker of α -SMA, which leading to vascular remodeling and lesion stenosis [27, 28]. VEGF is highly specific endothelial cell mitogenic growth factors [29]. It was low expression in normal artery and could increase by activating phospholipase C (PLC) when it was stimulated. Then it promoted endothelial cell mitosis and resulted in cell proliferation and migration [30, 31]. In this study, the expres-

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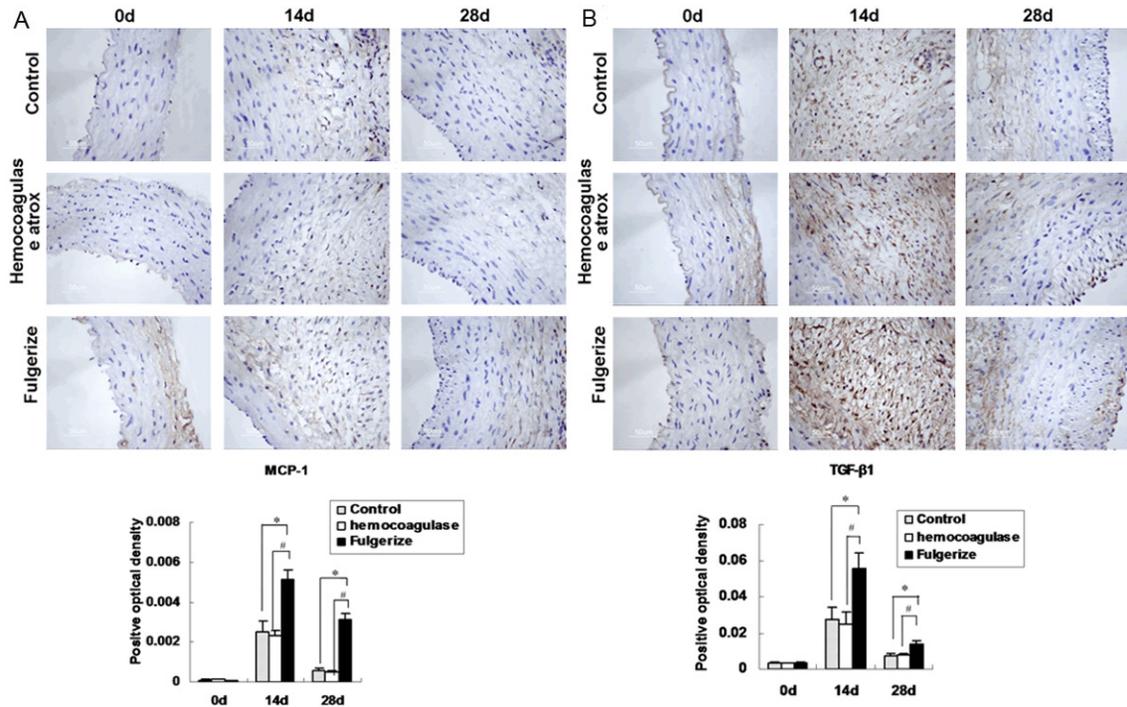


Figure 4. Immunohistochemical staining showed the protein expression of MCP-1 and TGF-β1 in animals treated with hemocoagulase atrox or cauterization (* $P < 0.05$ vs. control group; # $P < 0.05$ vs. hemocoagulase atrox group).

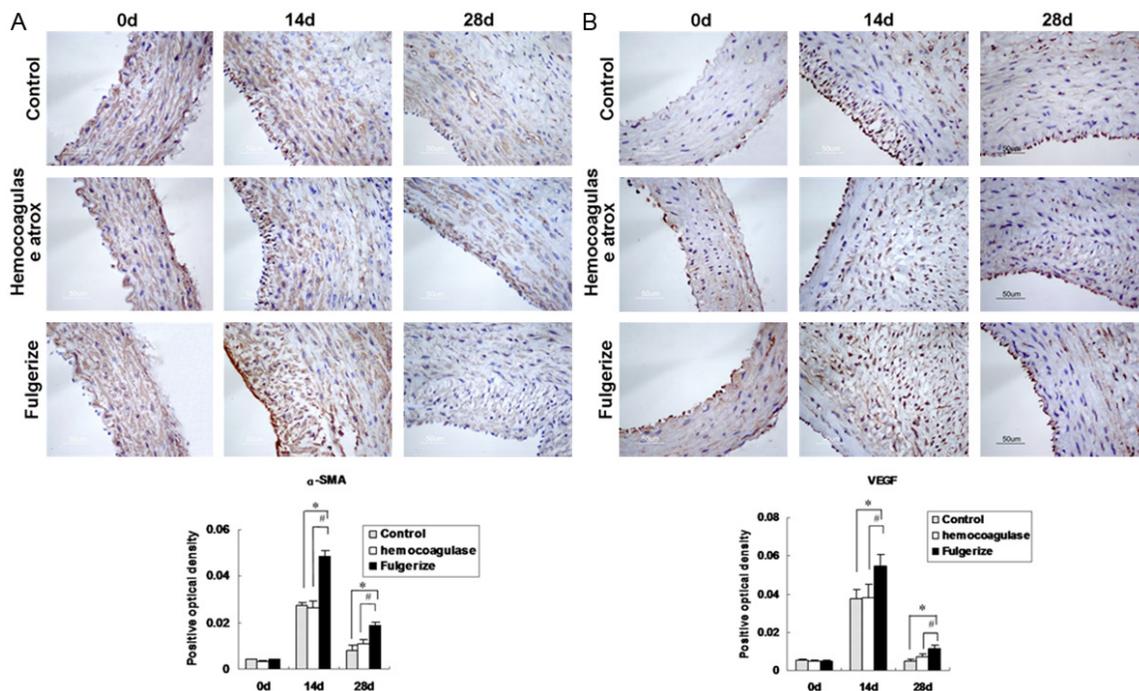


Figure 5. Immunohistochemical staining showed the protein expression of α-SMA and VEGF in animals treated with hemocoagulase atrox or cauterization (* $P < 0.05$ vs. control group; # $P < 0.05$ vs. hemocoagulase atrox group).

sion of TGF-β1, α-SMA, VEGF and PCNA in hemocoagulase atrox treated animals were

lower than that of cauterization treated animals. These results showed that hemocoagu-

lase atrox can accelerate adventitia restoration and decrease vascular proliferation in carotid arteries adventitia removal rabbit model.

Conclusion

In summary, this study showed that using hemocoagulase atrox as a local hemostatic is safety, efficiently and conducive to long-term restoration. It can accelerate adventitia restoration and decrease intimal proliferation use in topical hemostatic.

Disclosure of conflict of interest

The authors have no financial conflicts of interest.

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