Gene Transfer of Calcitonin Gene-Related Peptide Inhibits Macrophages and Inflammatory Mediators in Vein Graft Disease

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ABSTRACT

Vein graft disease is a chronic inflammatory disease and limits the late results of coronary revascularization. Calcitonin gene-related peptide (CGRP) inhibits macrophages infiltrated and inflammatory mediators, we hypothesized that transfected CGRP gene inhibits macrophages infiltrated and inflammatory mediators in vein graft disease. Autologous rabbit jugular vein grafts were incubated ex vivo in a solution of mosaic adeno-associated virus vectors containing CGRP gene (AAV2/1.CGRP) 、escherichia coli lac Z gene (AAV2/1.LacZ) or saline and then interposed in the carotid artery. Intima/media ratio were evaluated at postoperative 4 weeks, Macrophages were marked with CD68 antibody by immunocytochemistry.Inflammatory mediators were mensurated with real-time PCR.Neointimal thickening was significantly suppressed in AAV2/1.CGRP group. Macrophages infiltrated and inflammatory mediators monocyte chemoattractant protein-1 (MCP-1)、tumor necrosis factorα(TNF-α)、inducible nitricoxide synthase (iNOS)、matrix metalloproteinase-9 (MMP-9) was significantly suppressed in AAV2/1.CGRP group. Gene transfected AAV2/1.CGRP suppressed neointimal hyperplasia in vein graft disease by suppressed macrophages infiltrated and inflammatory mediators.

Key words: vein graft disease; calcitonin gene-related peptide;gene therapy

Introduction

Autologous vein is usually using a material in artery rebuild,But, it was exposed to ischemia、trauma、inflammatory reaction and received high pressure and so on , embolism、spasm were appearred in early period, neointimal thickening and atherosclerosis were appearred in later,angioma occurred by chance,the phenomenon is vein graft disease.It is affected severely clinic effect of coronary artery bypass grafting （CABG）and peripheral vascular disease operation[1,2,3].Previous data shown that inflammatory cells infiltrated and inflammatory mediators released in the vein grafts, Macrophages and inflammatory mediators stimulated hyperplasia of vascular smooth muscle cells and neointimal thickening[4,5].

Calcitonin gene-related peptide（CGRP）is a 37-amino acid biological activity neuropeptide, The nerve contained CGRP wraped in larger part vascular[6,7]. Previous studies have shown that CGRP inhibits inflammatory cells and inflammatory mediators[8,9], protects endothelial function[10,11] and inhibits vascular smooth muscle cells hyperplasia[12,13].Accordingly, we sought to evaluate the effects of CGRP, using mosaic adeno-associated virus vectors gene transfer, on development of the vein graft disease in rabbits.

Results

1. Gene Expression in Vein Grafts

Gene expression of an AAV2/1.CGRP at 4 weeks after infection and grafting was noted only in the AAV2/1.CGRP group vein grafts(Fig 1A). Both AAV2/1.CGRP group grafts and plasmid cDNA (positive control) yielded an identical cDNA product (227 bp), The AAV2/1.LacZ group and saline group and saline did not show CGRP RNA expression(Fig. 1A).

Gene expression of an AAV2/1.LacZ at 4 weeks, processed with X-gal chromagen for β-galactosidase, demonstrates excellent gene transfer with AAV2/1.LacZ (Fig. 1B). Endogenous β-galactosidase activity was not detected in saline group and AAV2/1.CGRP group vein grafts.

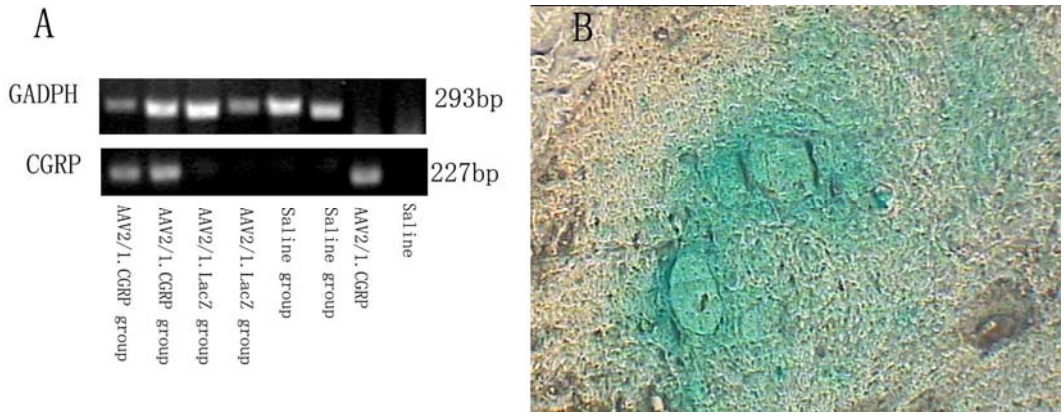

Fig. 1 Gene Expression in Vein Grafts . Fig. 1A. The expression of CGRP RNA at 4 weeks AAV2/1.CGRP group vein grafts. The total RNA extracted from AAV2/1.CGRP group, AAV2/1.LacZ group, and saline group vein grafts. AAV2/1.CGRP as postive control,Saline as negative control. AAV2/1.CGRP group and AAV2/1.CGRP are depicted here with positive. PCR cDNA products are visualized on agarose gels stained with ethidium bromide. **Fig. 1B**, Cross-sectional view of an AAV2/1.lac Z group vein grafts. Dark blue inclusions demonstrate β-galactosidase staining (original magnification, ×200).

2. CGRP Reduces Vein Graft Disease in rabbits

The intimal and medial area in EVG-stained cross sections was measured at 4 weeks(Fig. 2A). AAV2/1.CGRP group significantly suppressed intimal hyperplasia as compared to AAV2/1.LacZ

group and saline group (37% and 39% less, respectively; P<0.001, Fig. 2B). Moreover, the anti-inflammatory effect of CGRP, as infiltration of macrophages into the graft wall is also considered to play an important role in the response to vein graft disease. Immunohistochemical study demonstrated that infiltration of macrophages was markedly increased in specimens in AAV2/1.LacZ group or Saline group, while recruitment of macrophages was significantly inhibited by transfection of AAV2/1.CGRP(Fig. 3).

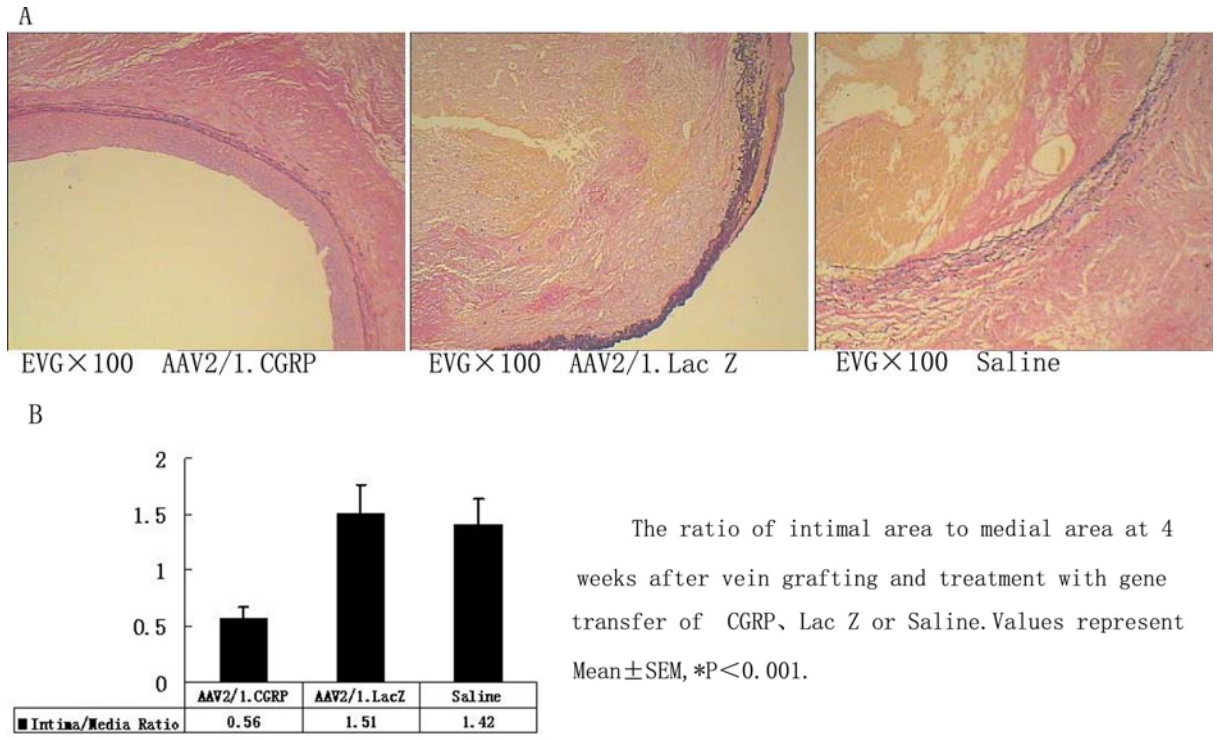

Fig. 2 Effect of transfection of CGRP on neointimal hyperplasia in vein graft at 4 weeks after surgery. Fig.2A.Representative histological sections of vein graft stained with elastic van Gieson's stain (magnification×100). **Fig. 2B**.Intimal and medial area in cross-sections and ratio of intimal to medial area.

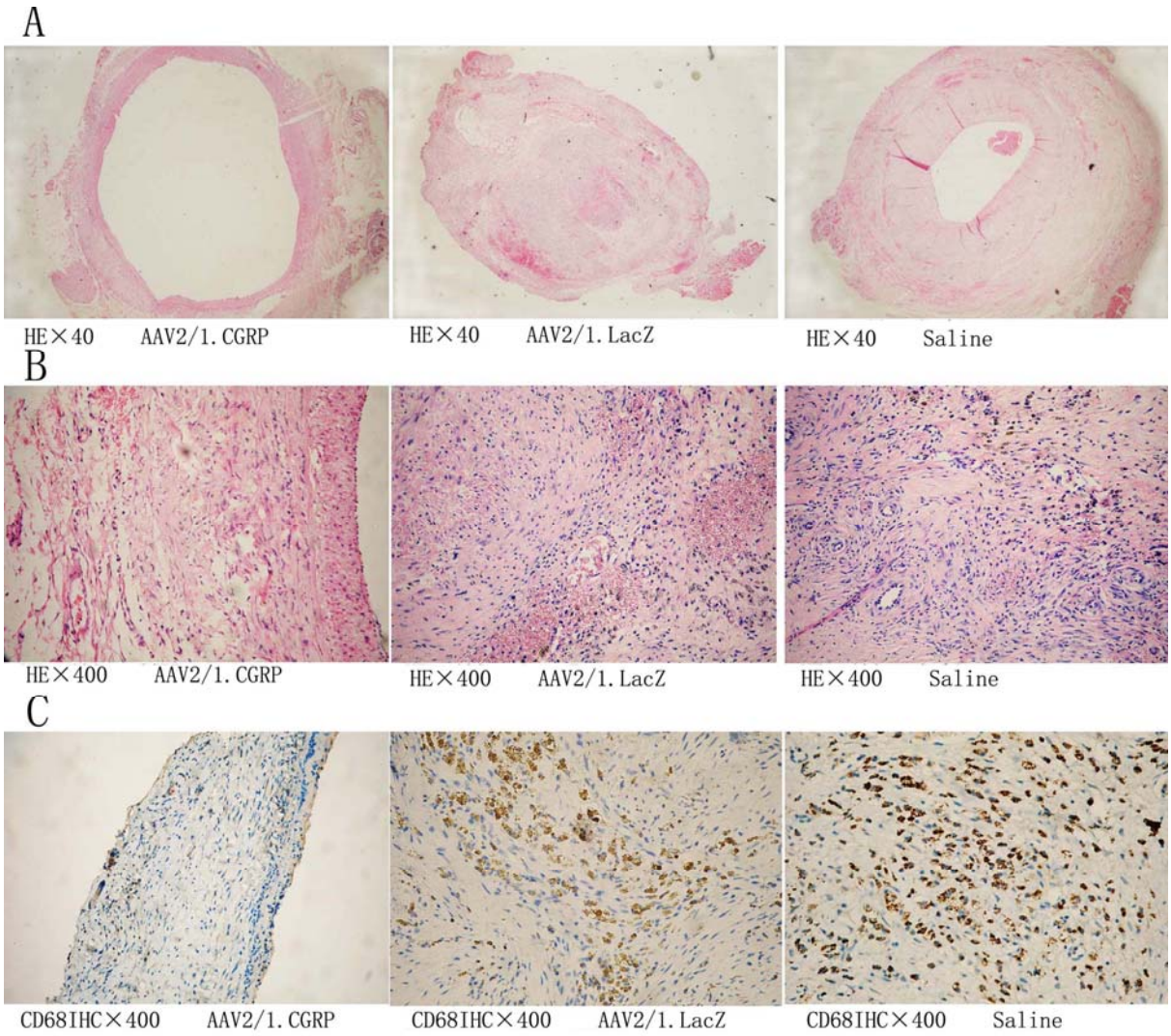

Fig. 3 Histopathological and immunohistochemical studies findings in rabbit vein grafts. In AAV2/1.CGRP group vein grafts, The muscularis and intima are intact; In AAV2/1.LacZ group and Saline group,the intima hyperplasia or untill complete occlusion(Fig. 3A). In AAV2/1.CGRP group vein grafts, There is minimal inflammation; But In AAV2/1.LacZ group and saline group, inflammatory cells infiltrated all layers of the vein grafts(Fig. 3B). Immunohistochemical studies with a monoclonal antibody CD68 specific for macrophages demonstrated minimal macrophages immunostaining was noted in AAV2/1.CGRP group vein graftss; high-level expression of macrophages in the endothelium and adventitia of AAV2/1.LacZ group and saline group vein grafts(Fig. 3C).

3. Inflammation Mediating Molecules mRNA Expression

Expression levels of inflammation mediating molecules in vein graft samples of all rabbit groups were higher than those of AAV2/1.CGRP group (Fig. 4). The AAV2/1.CGRP gene transfection significantly inhibited inflammation mediating molecules MCP-1、TNF-α、iNOS 、MMP-9 mRNA in vein grafts. The most significantly up-regulated inflammation mediating molecules mRNA was MMP-9, which displayed higher mRNA levels in the AAV2/1.LacZ group and saline group controls.

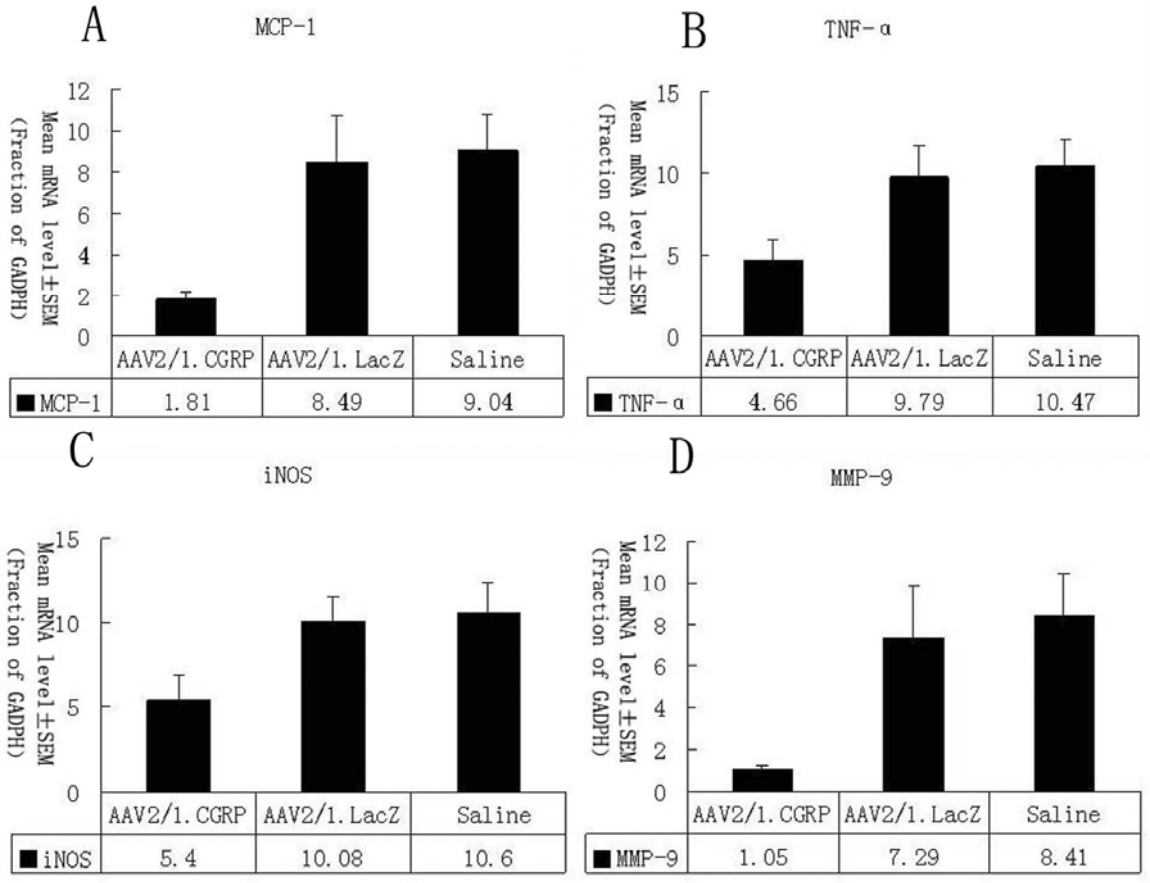

Fig. 4 Mean inflammation-mediating molecules mRNA levels in the vein grafts. All vein grafts were tested for inflammation mediating molecules mRNA levels by quantitative real-time PCR using GAPDH as internal standard. Each specific inflammation mediating molecules is indicated on the figure. *$P < 0{\cdot}05$ AAV2/1.CGRP group *versus* AAV2/1.LacZ group and saline group(n=7 per group).

Discussion

In the past, Kilian et al[15] showed transfected native rabbit jugular veins with recombinant AAV vector,Moreover, low immunogenicity of AAV may also allow long-term transgene expression; Sen et al[16] showed that AAV1 have advantages over AAV2 for vascular gene delivery at low titres; Stachler et al[17] showed that mosaic AAV1 particles in a 50-100-fold enhancement in endothelial cell gene transfer and suggest that mosaic virions hold significant promise for targeted gene delivery to the vasculature.In the present study we showed that recombinant mosaic AAV2/1 facilitates an effective gene transfection in an autologous rabbit vein grafts model. Our data have shown that AAV2/1.CGRP was transfected effectively to vein grafts(Fig. 1),AAV2/1 gene transfer of CGRP significantly reduced the ratio of intimal to medial area(Fig. 2) and dramatically reduced macrophage recruitment(Fig. 3) in vein grafts at 4 weeks after surgery.Our genic transfection means is a specific interest for the surgical perspective,Because the preparation and instillation of the viral solution can be performed easily in a bypass operation(Fig. 5).

MCP-1 is a potent monocyte chemotactic and activating factor belonging to the C-C family of intercrine cytokines,which is synthesized by endothelial cells, smooth muscle cells, fibroblasts, and monocytes/ macrophages, all of which are present in vein grafts[18,19]. Upregulation of MCP-1 gene expression in vein grafts results in the recruitment of monocytes and tissue macrophages to the vein wall, which leads to intimal hyperplasia (IH). The correlation between monocyte/ macrophage infiltration and IH suggests a critical role for these cells in IH development[19,20]. Previous studies have shown anti-MCP-1 gene therapy inhibits vascular smooth muscle cells proliferation and attenuates vein graft thickening both in vitro and in vivo[21,22,23]. Li et al[8]report intrinsic and extrinsic peptide CGRP inhibits IL-1β-induced MCP-1 secretion in an autocrine/paracrine mode and suppresses the IL-1β-evoked ROS-NF-κB cascade via cAMP signaling. It is also known that CGRP suppressed the number of macrophage colonies formed[24]. In the present study, Our data shown that AAV2/1.CGRP-treated suppressed MCP-1 mRNA and macrophage(Fig. 3, Fig. 4A), It is possible mechanism that CGRP-treated vein graft disease by suppressed MCP-1 and macrophages.

Macrophages express inducible nitric oxide synthase (iNOS) and tumour necrosis factor

alpha (TNF-α) and multiple metalloproteinases[4,25,26] , A number of previous studies have suggested that an essential role for MMP-9 activation in the development of vascular SMC proliferation, migration and neointima formation[27,28]. TNF-α-induced MMP-9 production and promoter activity[29], effectively suppresses TNF-alpha-induced VSMC migration through the selective inhibition of MMP-9 expression[30,31]. Likewise, iNOS increase the expression of matrix metalloproteinase-9 in vascular smooth muscle, iNOS is related with the development of atherosclerosis[32,33,34].TNF- α and iNOS generated by macrophages can be inhibited by CGRP[35,36].Our study shown that CGRP inhibit macrophages infiltration and inflammation mediating molecules TNF-αand iNOS, especially MMP-9(Fig. 3, Fig. 4B、C、D),Accordingly,We deduced that the macrophages and inflammation mediating molecules are played an essential role in the development vein graft disease, CGRP suppresses vein graft disease by inhibits macrophages infiltration and inflammation mediating molecules.

In conclusion, Our study demonstrated that AAV2/1.CGRP gene transfer via ex vivo inhibited macrophage infiltration and inflammation mediating molecules mRNA of MCP-1、TNF-α、iNOS and MMP-9,dramatically reduced vessel wall thickening in vein graft disease. These findings highlight the importance of AAV2/1.CGRP gene transfer in clinic treatment vein graft disease.

Materials and methods

1.Rabbit Vein Graft Model and ex Vivo Gene Transfer

25 Adult male New Zealand white rabbits (2.5 ~ 3.0kg) were anesthetized with an intramuscular injection of pentobarbital (3mg/kg; sigma), Neither mechanical ventilation nor supplemental intravenous fluid administration was required. Under general anesthesia and using a sterile technique, the right jugular vein and carotid artery were exposed through a midline incision. An approximately 3.0 cm segment of the jugular vein, lacking valves, was isolated by ligation of the proximal and distal vein for the autologous reversed-vein graft. A small venotomy was made in the distal vein. About a 300μL volume of AAV2/1.CGRP(at 5×10^{10}vg/ml, n=8), AAV2/1.LacZ (at 5×10^{10}vg/ml, n=9) or Saline (n=8) was injected into the lumen of the isolated vein and

maintained expanded.Then resected the segment of the jugular vein and put it into a tube contained 2ml AAV2/1.CGRP(at 5×10^{10}vg/ml) or AAV2/1.LacZ(at 5×10^{10}vg/ml) or Saline in 30 minutes at room temperature（Fig 5）.

After systemic heparinization (1000 IU), the ipsilateral common carotid artery was dissected and clamped. End-to-side interpositional grafting of the jugular vein segment to the carotid artery was performed using 9-0 nylon continuous running suture. Arterial blood flow was restored to the carotid artery and vein graft. The neck incision was closed, and the animals were placed under a warming light until fully recovered from anesthesia. After recovery, the rabbits were maintained on a light-dark (12-12 hour) cycle at 24° C and fed a normal diet and water as desired. Antibiotic prophylaxis was provided with penicillin 4×10^{5}U intramuscularly 2 times daily in 3 days. Animal care complied with the principles of laboratory animal care **(**China requirements of environment and housing facilities for laboratory animals,GB/T 14925-94; China laboratory animals- rabbits formula feeds, GB 14924.4-2001).

Fig. 5 The method of gene transfection

2.Harvest and Processing of Tissue Samples

The AAV2/1.CGRP group 、AAV2/1.LacZ group and Saline group dyed 1、2、1 rabbits within 4 weeks, Dead rate is 16%(4/25). After induction of anesthesia and after confirming patency of the vessels studied, animals were euthanized with an overdose of anesthetic. Vein grafts harvested at 4 weeks (n=7 per group), A 2.5mm long middle segment of vein grafts(n=7 per group) was fixed with 10% neutral-buffered formalin to do histological study; A 2.5mm long middle segment of

vein grafts(n=2 per group) and residual vein grafts(n=7 per group) were immediately snap-frozen with liquid nitrogen, then stored at - 70°C for later study.

3.Histological Study

The fixed in 10% neutral buffered formalin vein grafts processed for routine paraffin embedding. Two sections were taken from the midportion of the vein grafts. Cross-sections (5 μm) were stained with both hematoxylin and eosin (HE) and elastic van Gieson's stain (EVG) in a standard manner. Intimal and medial areas were calculated by quantitative morphometric analysis with a computerized sketching program. The ratio of intimal to medial area was also calculated.

4. X-gal Staining

β-Galactosidase, an index of successful AAV1⁄2.lacZ infection, was documented with X-gal chromagen in specimens,2 Samples AAV2/1.CGRP group and 2 samples saline group as negative controll. 6 Samples tissue samples were taken from -80℃ refrigeratory, Whole tissue X-gal chromagen staining was done according to exponent（GENMED）, a segment of vein was also fixed in 10% formalin and embedded in paraffin after X-gal chromagen staining. 8μm-thick cross section samples were cut from the paraffin blocks, then observed at ×400 magnification for evidence of production of β-galactosidase. Tissue cell stained positive for β-galactosidase is blue.

5. Immunohistochemical Study

Paraffin-embedded vein graft sections were stained for macrophage using a mouse monoclonal anti–CD68 antibody (Dako),For negative control experiments, the primary antibody was omitted. Immunohistochemical staining was performed using the immunoperoxidase avidin–biotin complex system. Then, 5-μm sections were deparaffinized, rehydrated before blocking endogenous peroxidase activity with 3% hydrogen peroxidase and preincubated with 5% normal horse serum in sodium phosphate-buffered saline (PBS) for 30 min. Diluted primary antibodies (1:30) were then applied to the sections, and these sections were incubated overnight at 4 °C. With intervening washing in PBS, they were serially incubated with biotinylated anti-mouse IgG in PBS and avidin-biotinylated horseradish peroxidase complex in PBS for 30 min, Immune complexes were

localized using 0.05% 3,3′-diaminobenzidine (DAB), and slides were counterstained with hematoxylin.

6. **Reverse Transcription–Polymerase Chain Reaction of CGRP**

Total RNA was isolated from rabbit vein graft tissues by use of Trizol reagent (Invitrogen), then reverse-transcribed using a one-step reverse transcription–polymerase chain reaction (RT-PCR) kit (Invitrogen) with oligo(dT) primers. Then, the sequence of CGRP was amplified by PCR (annealing at 58°C for 45 seconds, extension at 72°C for 1minute, 30 cycles), The CGRP primers refer to table1. Also, Glyceraldehyde-3- phosphate dehydrogenase (GAPDH) (The primers design was refered data of Zucker ,et al[14]) was amplified as a reference use of a primer pair specific to rabbit GAPDH at the same reaction condition for 30 cycles.

7.Real-Time Polymerase Chain Reaction

RT-PCR reactions were performed on all rabbits' mRNA at the same time for each individual inflammation-mediating molecules tested. First-strand cDNA synthesis was performed from 1.3μg of total RNA in 20 μL volumes with oligo(dT) priming, using Superscript First-strand Synthesis System (Invitrogen). PCR amplification was performed with Power SYBR Green PCR Master Mix (ABI),The molecules primers refer to table1.The reaction was incubated at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 15 seconds at 95°C, 1 minute at 60°C with use of a ABI 7000 sequence detection system. In all assays, the specificity of the final amplified products was verified by heat dissociation curve analysis with the use of software supplied by the manufacturer.

Table 1. Primers Used for Inflammation-Mediating Molecules mRNA Assessment

Molecules	Sequence(5′-3′)	PCR product size (base pairs)
CGRP	ATGGGCTTCCAAAAGTTCTCC CTGGAGCCCTCTCTCTCTTG	227
iNOS	CTGCTTTGTGCGGAGTG CACCCGAACACCAGGAT	174
TNF-α	GGCTCAGAATCAGACCTCAG GCTCCACATTGCAGAGAAGA	297
MCP-1	CCAGCCAGATGCCGTGAAT AGATCCCCTTGGCCAGTTTG	155
MMP-9	GCAGGGTAGGGGGTATGGA	630

GADPH	ACAGGGCTTGGCTTTGGA TCACCATCTTCCAGGAGCGA CACAATGCCGAAGTGGTCGT	293

8.Statistical Analysis

All results are expressed as mean±SEM. One-way ANOVA was used to compare differences between groups. A value of $P < 0.05$ was considered to indicate a statistically significant difference, with Bonferroni correction for multiple comparisons.

Acknowledgments

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References

1 Schachner T. Pharmacologic inhibition of vein graft neointimal hyperplasia. J Thorac Cardiovasc Surg. 2006;131(5):1065-1072.

2 Chandiwal A,et al. Gene therapy for the extension of vein graft patency: a review.Vasc Endovascular Surg. 2005;39(1):1-14.

3 Sutherland D, Block PC. Images in clinical medicine. Aneurysm of a saphenous-vein bypass graft. N Engl J Med. 2001;344(15):1139.

4 Boyle JJ. Macrophage activation in atherosclerosis: pathogenesis and pharmacology of plaque rupture. Curr Vasc Pharmacol. 2005;3(1):63-68.

5 Hinokiyama K, et al. Vein graft harvesting induces inflammation and impairs vessel reactivity. Ann Thorac Surg. 2006;82(4):1458-1464.

6 Gherardini G, et al.Comparison of vascular effects of calcitonin gene-related peptide and lidocaine on human veins. J Reconstr Microsurg. 1996;12(4):241-245.

7 Brain SD, Grant AD. Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol Rev. 2004;84(3):903-934.

8 Li W, et al. Calcitonin gene-related peptide inhibits interleukin-1beta-induced endogenous monocyte chemoattractant protein-1 secretion in type II alveolar epithelial cells. Am J Physiol Cell Physiol. 2006;291(3):C456-465.

9 Gomes RN, et al.Calcitonin gene-related peptide inhibits local acute inflammation and protects mice against lethal endotoxemia. Shock. 2005;24(6):590-594.

10 Shichiri M, et al. Induction of max by adrenomedullin and calcitonin gene-related peptide antagonizes endothelial apoptosis. Mol Endocrinol. 1999; 13(8):1353-1363.

11 Ye F, et al. Involvement of endothelial cell-derived CGRP in heat stress-induced protection of endothelial function. Vascul Pharmacol. 2007;46(4):238-246.

12 Kadowitz and J. R. Jeter, et al.Antiproliferative effects of calcitonin gene-related peptide in aortic and pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol. 2005;288(1):L202-211.

13 Deng W, et al.Inhibition of vascular smooth muscle cell proliferation in vitro by genetically engineered marrow stromal cells secreting calcitonin gene-related peptide. Life Sci. 2006;78(16):1830-1838.

14 Zucker KE, et al.Temporal expression of inflammatory mediators in brain basilar artery vasculitis and cerebrospinal fluid of rabbits with coccidioidal meningitis.Clin Exp Immunol.2006;143(3): 458-466.

15 Kilian EG, et al.Adeno-associated virus-mediated gene transfer in a rabbit vein graft model.Circ J.2008;72(10): 1700-1704.

16 Sen S, et al.Gene delivery to the vasculature mediated by low-titre adeno-associated virus serotypes 1 and 5. Gene Med. 2008;10(2):143-151.

17 Stachler MD, Bartlett JS. Mosaic vectors comprised of modified AAV1 capsid proteins for efficient vector purification and targeting to vascular endothelial cells. Gene Ther. 2006;13(11): 926-931.

18 Bidzhekov K, Zernecke A, Weber C. MCP-1 induces a novel transcription factor with proapoptotic activity. Circ Res. 2006;98(9):1107-1109.

19 Stark VK, Hoch JR, Warner TF, Hullett DA. Monocyte chemotactic protein-1 expression is associated with the development of vein graft intimal hyperplasia. Arterioscler Thromb Vasc Biol. 1997;17(8):1614-1621.

20 Wolff RA, et al.Macrophage depletion reduces monocyte chemotactic protein-1 and transforming growth factor-beta1 in healing rat vein grafts. J Vasc Surg. 2004;39(4):878-888.

21 Saiura A, et al.Antimonocyte chemoattractant protein-1 gene therapy attenuates graft

vasculopathy. Arterioscler Thromb Vasc Biol. 2004;24(10):1886-1890.

22 Schepers A, et al. Anti-MCP-1 gene therapy inhibits vascular smooth muscle cells proliferation and attenuates vein graft thickening both in vitro and in vivo. Arterioscler Thromb Vasc Biol. 2006;26(9):2063-2069.

23 Tatewaki H, et al.Blockade of monocyte chemoattractant protein-1 by adenoviral gene transfer inhibits experimental vein graft neointimal formation. J Vasc Surg. 2007;45(6):1236-1243.

24 Owan I, Ibaraki K. The role of calcitonin gene-related peptide (CGRP) in macrophages: the presence of functional receptors and effects on proliferation and differentiation into osteoclast-like cells. Bone Miner. 1994;24(2):151-164.

25 Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005;352(16):1685-1695.

26 Sisto M, et al.Inducible nitric oxide synthase expression in Leishmania-infected dog macrophages. Comp Immunol Microbiol Infect Dis. 2001;24(4):247-254.

27 Wong AP, et al.Expansive remodeling in venous bypass grafts: novel implications for vein graft disease. Atherosclerosis. 2008;196(2):580-589.

28 Porter KE, Turner NA. Statins for the prevention of vein graft stenosis: a role for inhibition of matrix metalloproteinase-9. Biochem Soc Trans. 2002;30(2):120-126.

29 Moon SK,Cha BY, Kim CH. ERK1/2 mediates TNF-alpha-induced matrix metalloproteinase-9 expression in human vascular smooth muscle cells via the regulation of NF-kappaB and AP-1: Involvement of the ras dependent pathway. J Cell Physiol. 2004;198(3): 417-427.

30 Yu YM, Lin HC, Chang WC. Carnosic acid prevents the migration of human aortic smooth muscle cells by inhibiting the activation and expression of matrix metalloproteinase-9. Br J Nutr. 2008;100(4):731-738.

31 Lee SO, et al.Wogonin suppresses TNF-alpha-induced MMP-9 expression by blocking the NF-kappaB activation via MAPK signaling pathways in human aortic smooth muscle cells. Biochem Biophys Res Commun. 2006; 351(1):118-125.

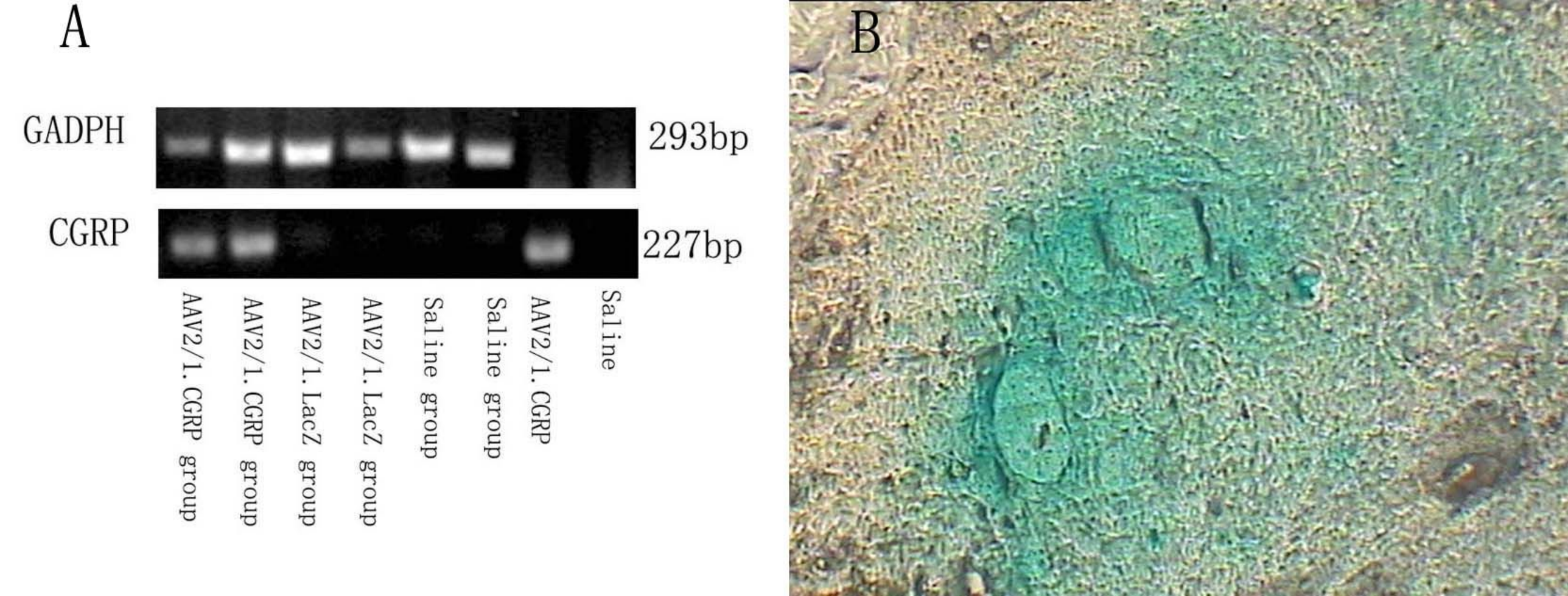
32 Egi K, et al.Inhibition of inducible nitric oxide synthase and superoxide production reduces matrix metalloproteinase-9 activity and restores coronary vasomotor function in rat cardiac allografts. Eur J Cardiothorac Surg. 2004;26(2):262-269.

33 Marcet-Palacios M, et al. Nitric oxide and cyclic GMP increase the expression of matrix metalloproteinase-9 in vascular smooth muscle. J Pharmacol Exp Ther. 2003;307(1):429-436.

34 Hayashi T, et al. Selective iNOS inhibitor, ONO1714 successfully retards the development of high-cholesterol diet induced atherosclerosis by novel mechanism. Atherosclerosis. 2006;187(2):316-324.

35 Feng Y, Tang Y, Guo J, Wang X. Inhibition of LPS-induced TNF-alpha production by calcitonin gene-related peptide (CGRP) in cultured mouse peritoneal macrophages. Life Sci. 1997; 61(20):PL 281-287.

36 Taylor AW, Yee DG, Streilein JW. Suppression of nitric oxide generated by inflammatory macrophages by calcitonin gene-related peptide in aqueous humor. Invest Ophthalmol Vis Sci. 1998;39(8):1372-1378.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GADPH
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AAV2/1. CGRP group
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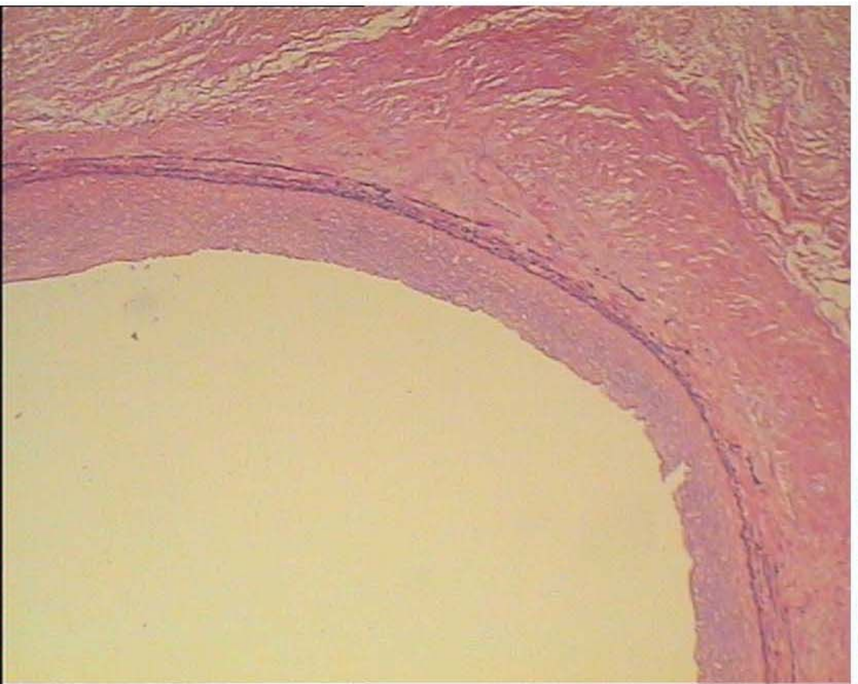
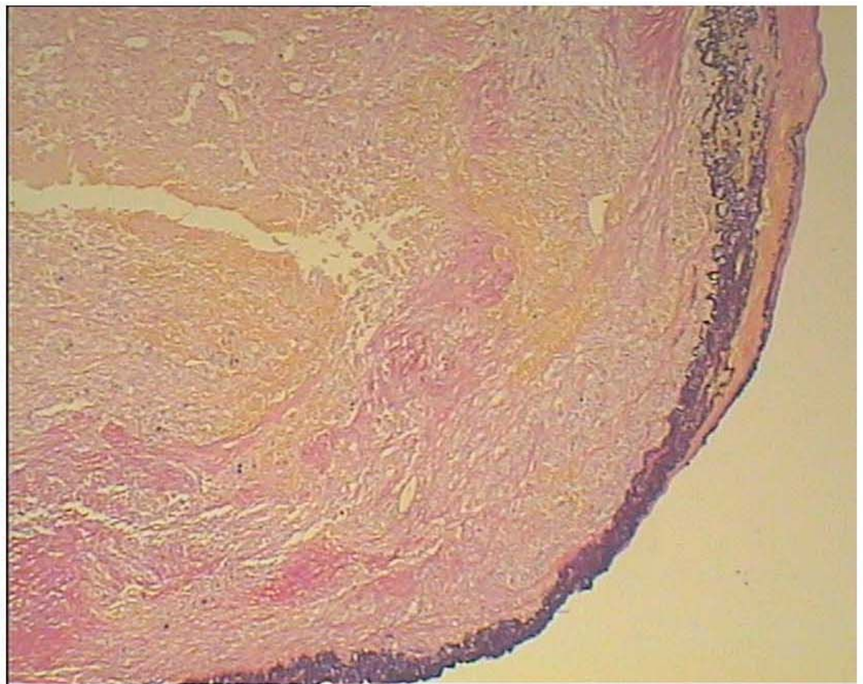
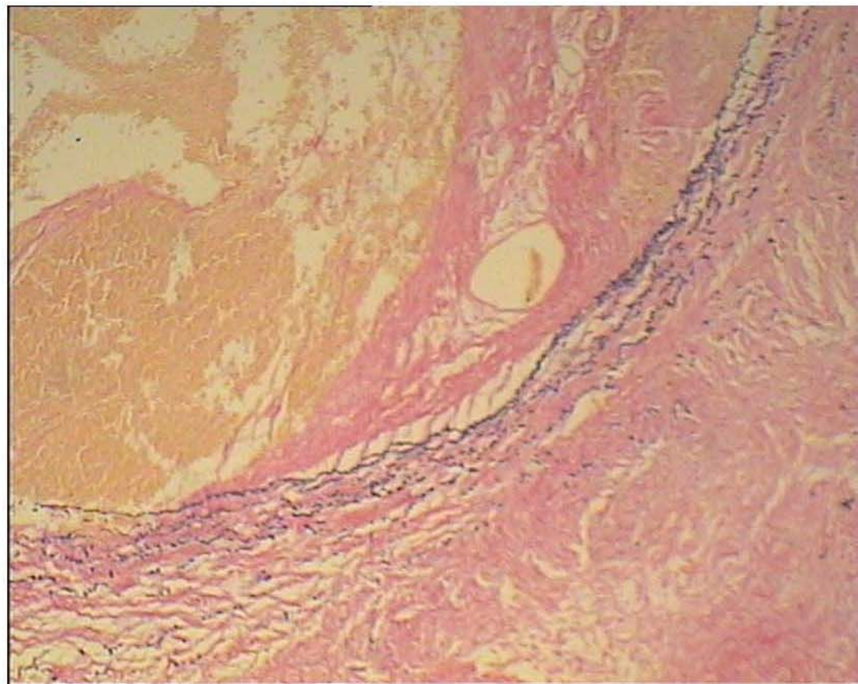


EVG×100 AAV2/1.CGRP



EVG×100 AAV2/1.Lac Z



EVG×100 Saline

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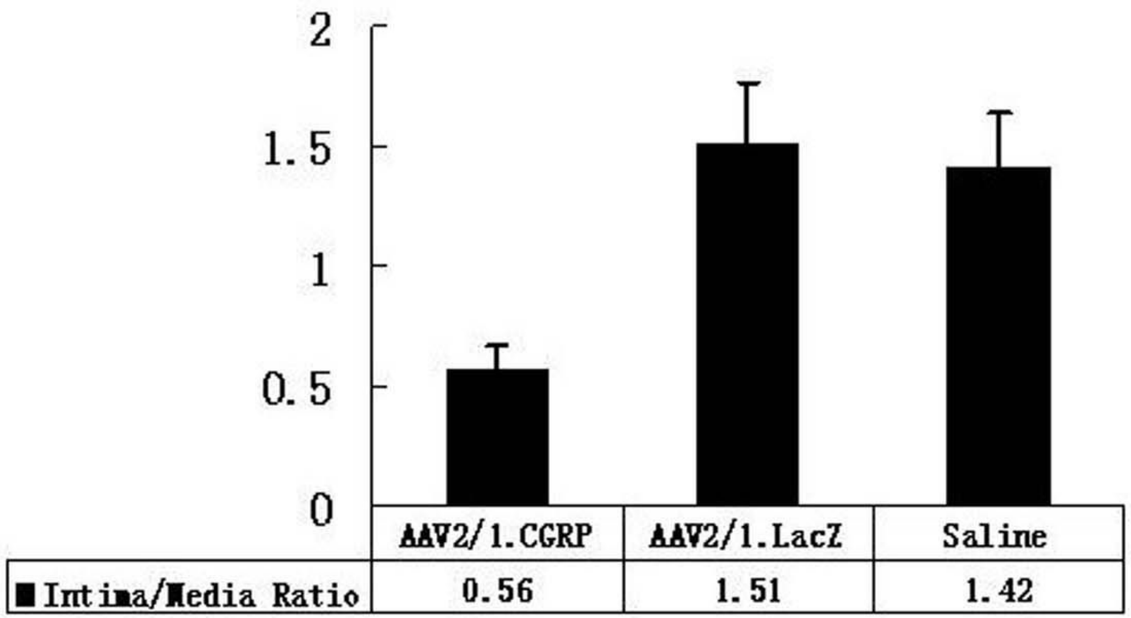
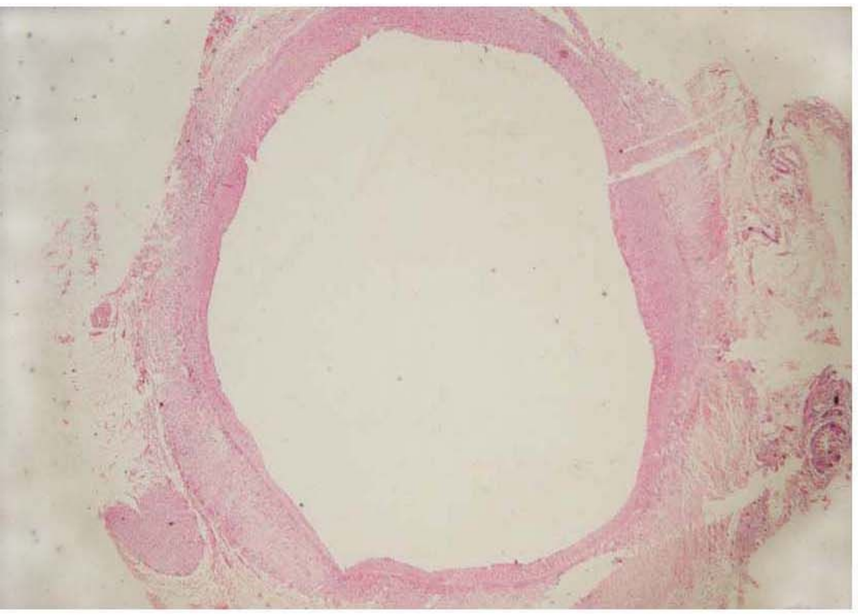


The ratio of intimal area to medial area at 4 weeks after vein grafting and treatment with gene transfer of CGRP、Lac Z or Saline.Values represent Mean±SEM,*P＜0.001.

A



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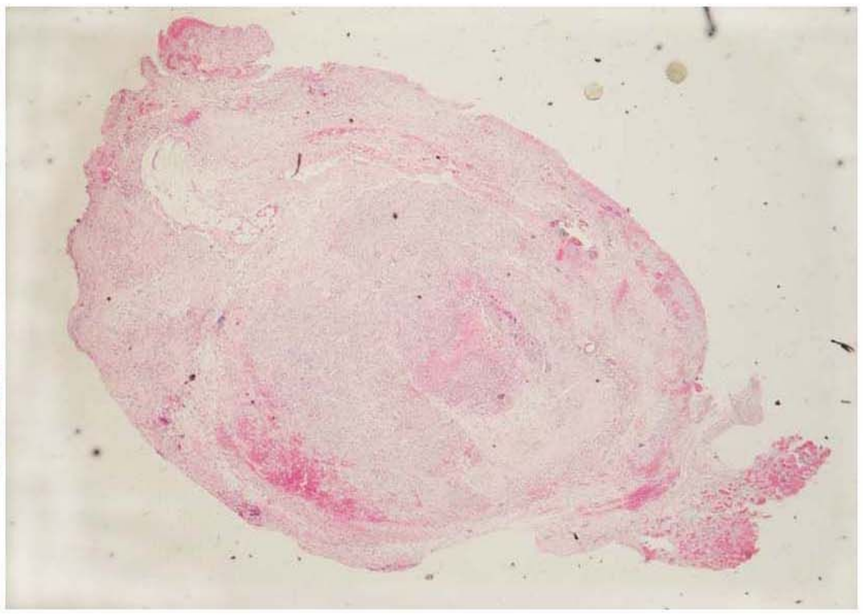
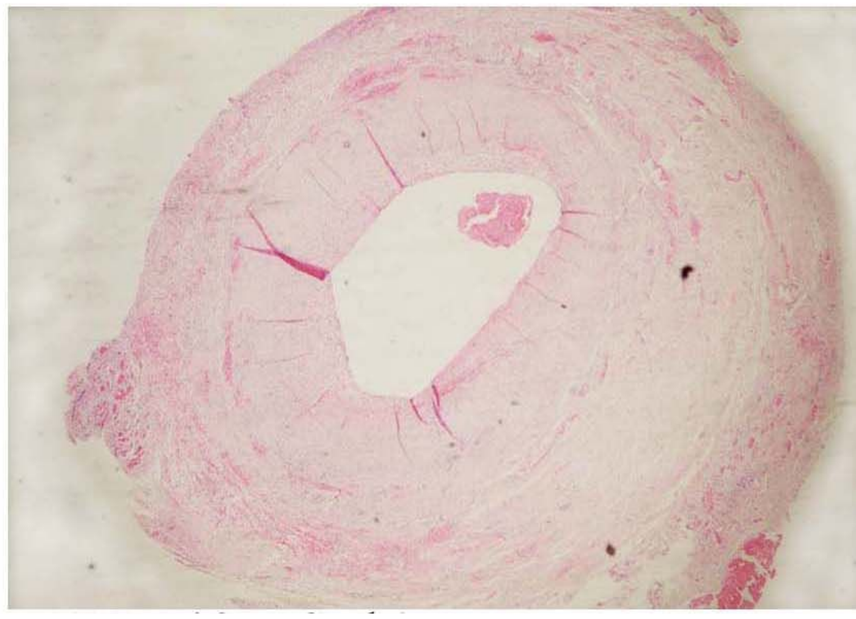
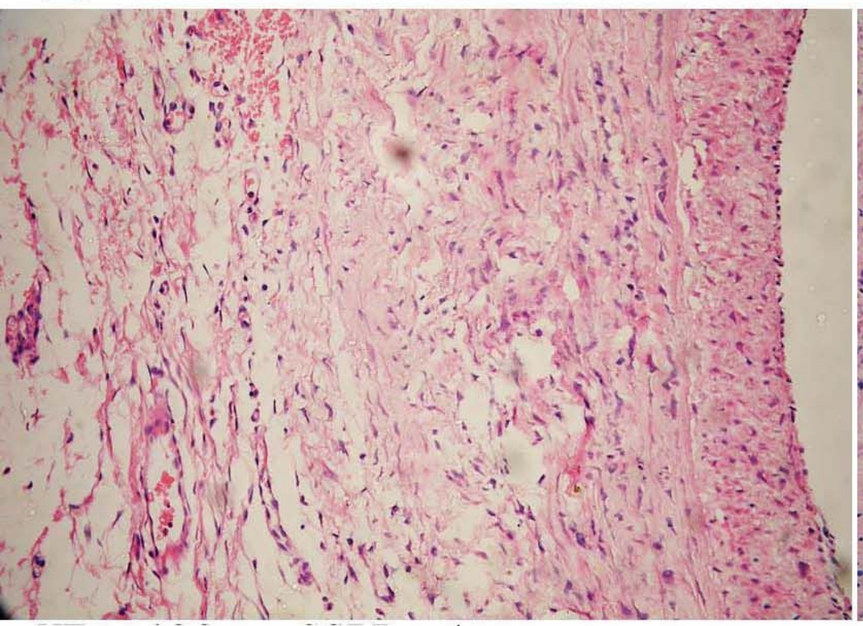


HE×40 AAV2/1.LacZ

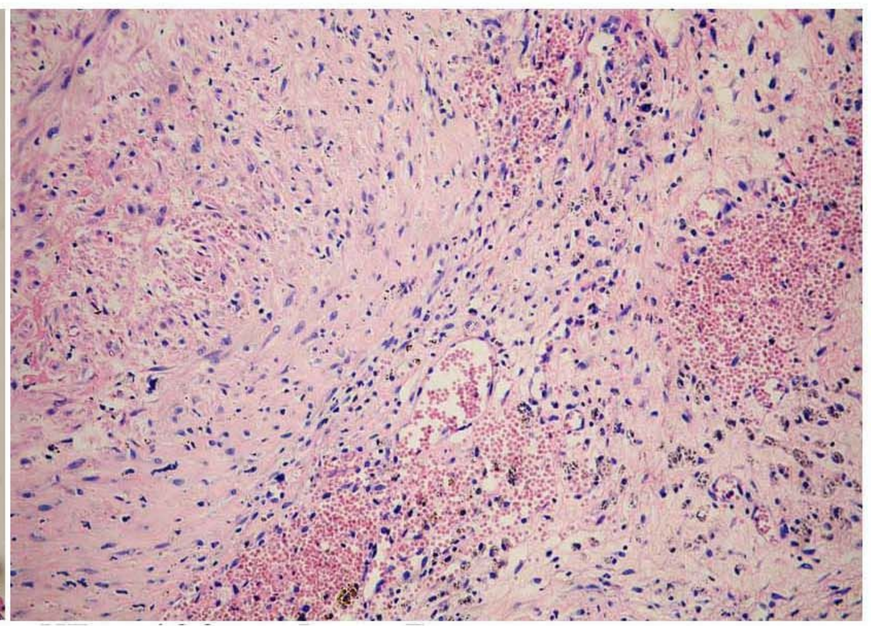


HE×40 Saline

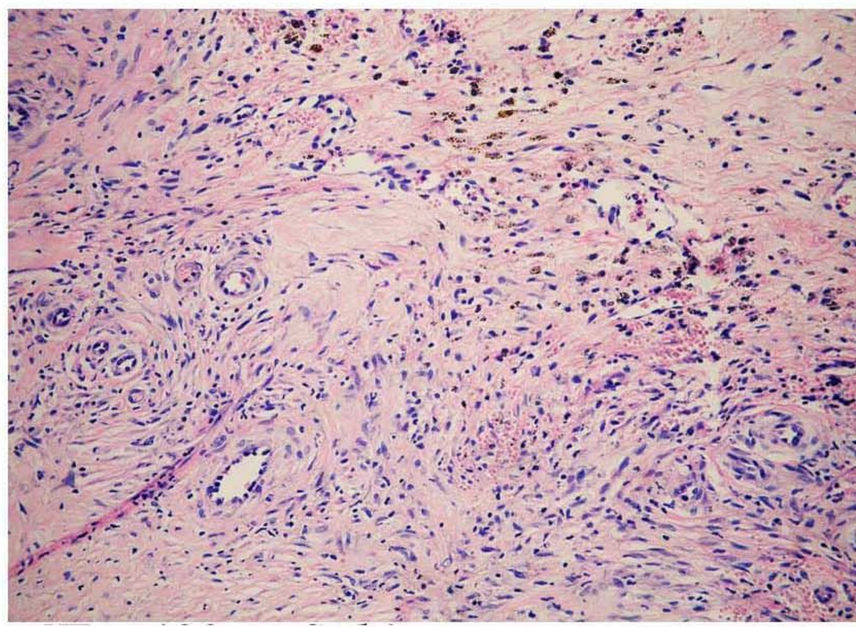
B



HE×400 AAV2/1.CGRP

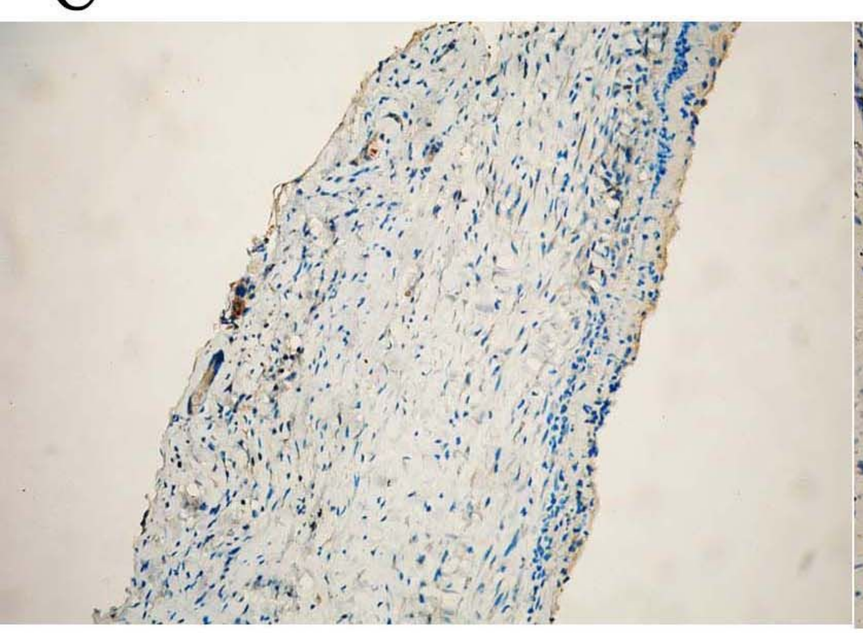


HE×400 AAV2/1.LacZ

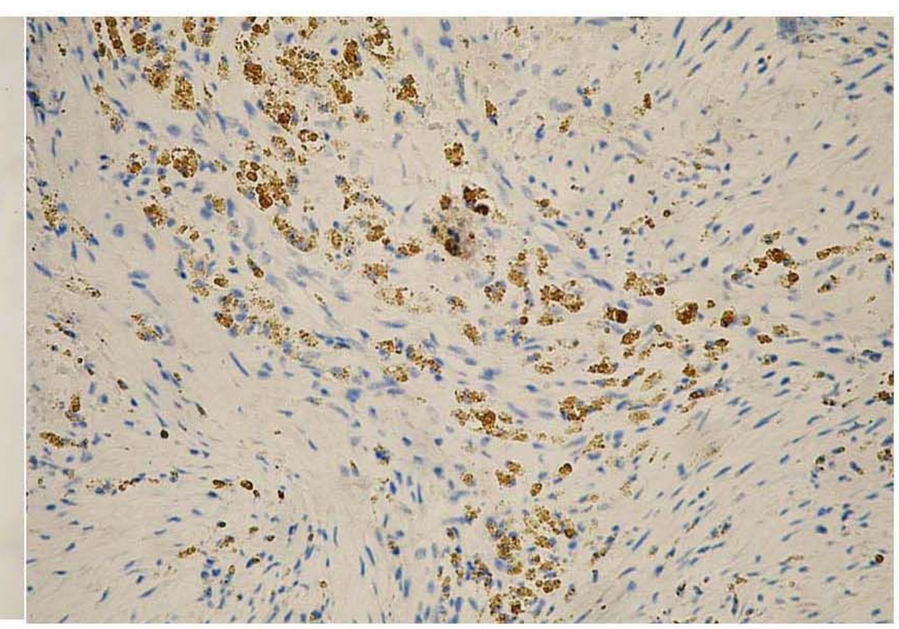


HE×400 Saline

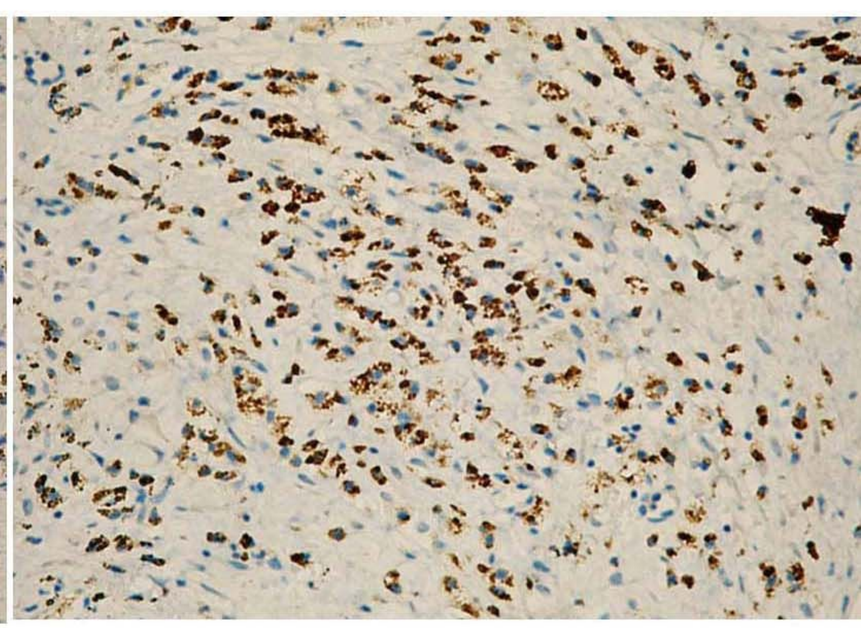
C



CD68IHC×400 AAV2/1.CGRP



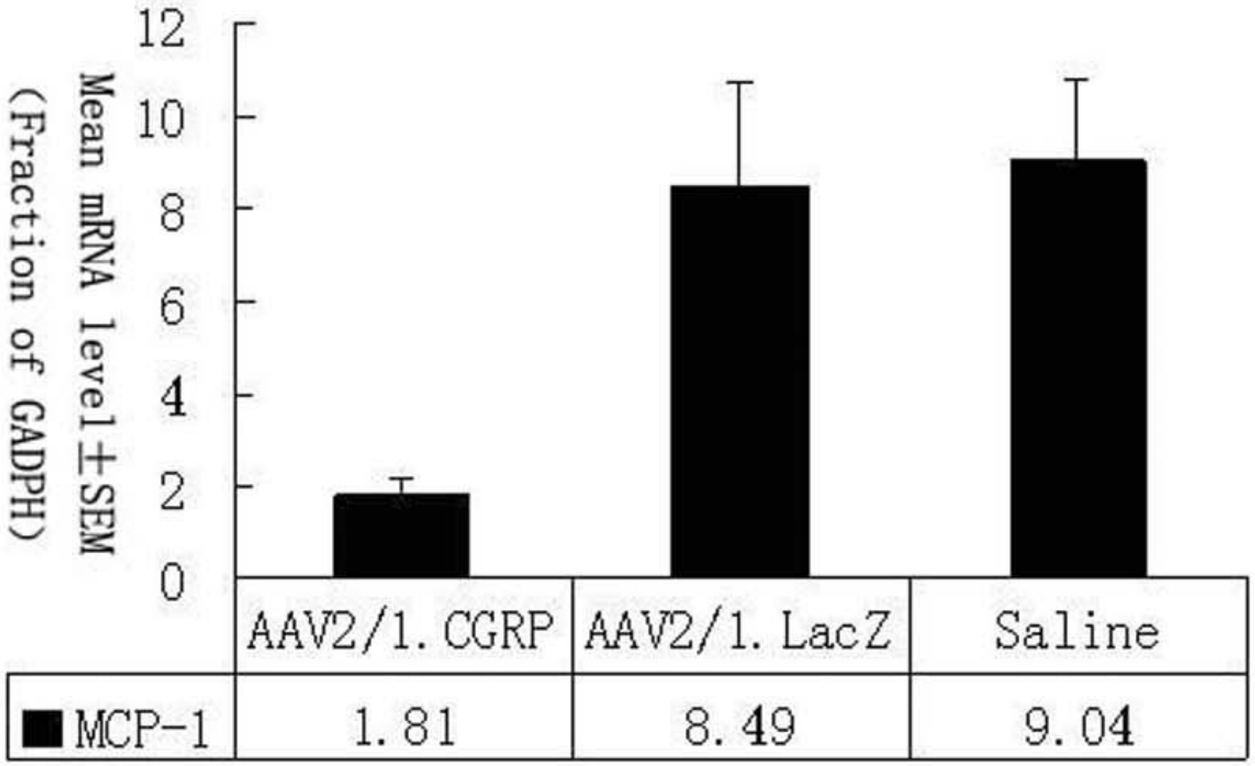
CD68IHC×400 AAV2/1.LacZ



CD68IHC×400 Saline

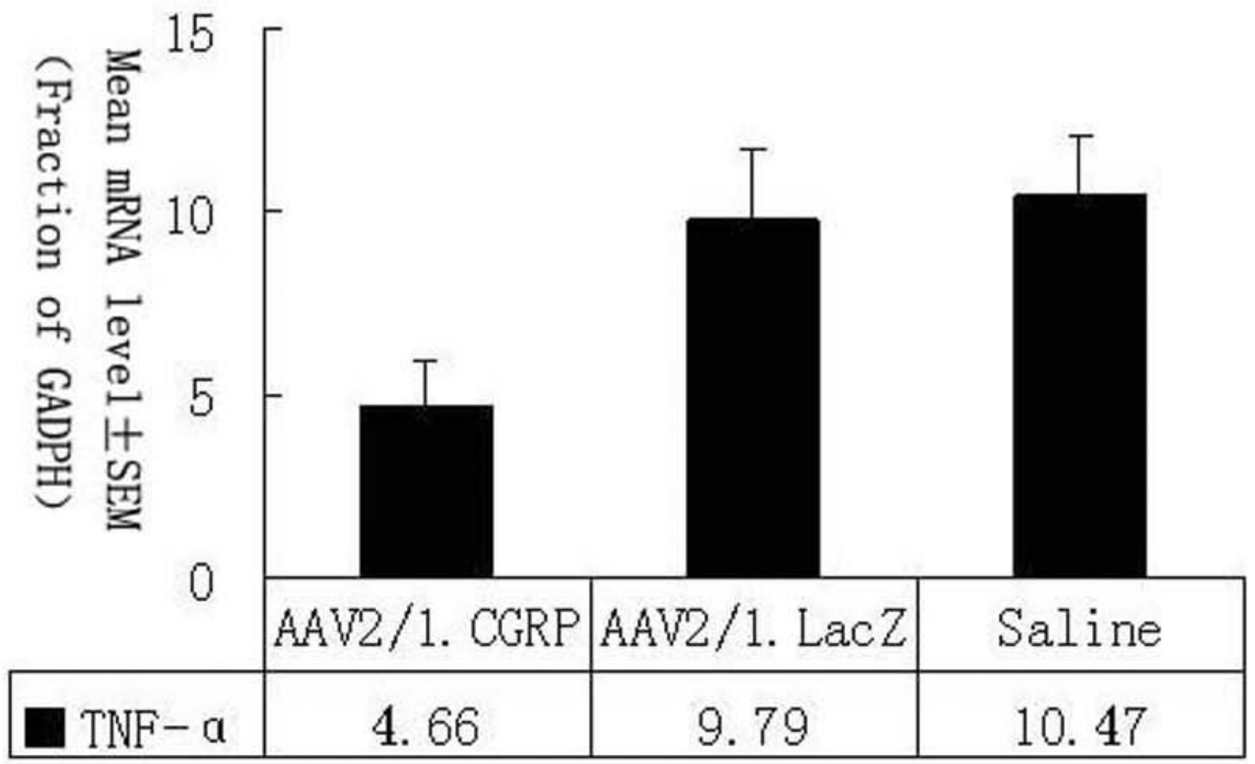
A

MCP-1



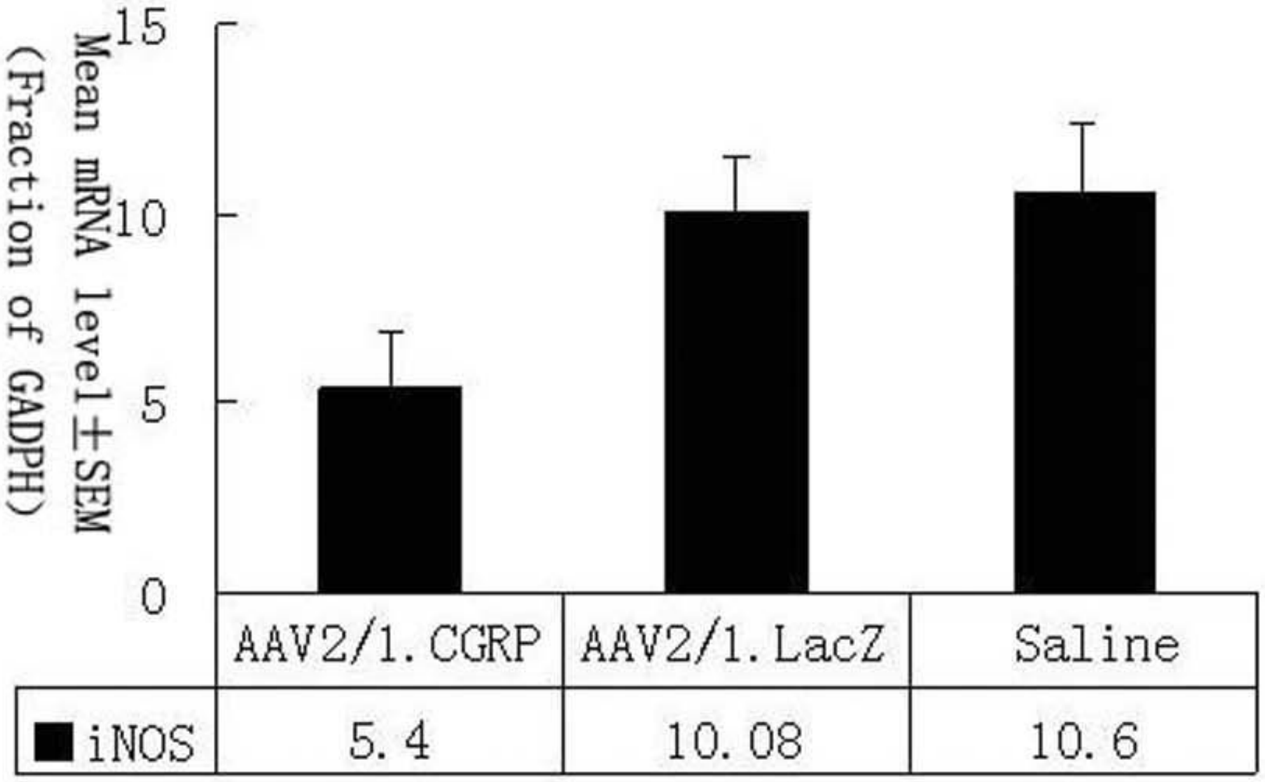
B

TNF-α



C

iNOS



D

MMP-9

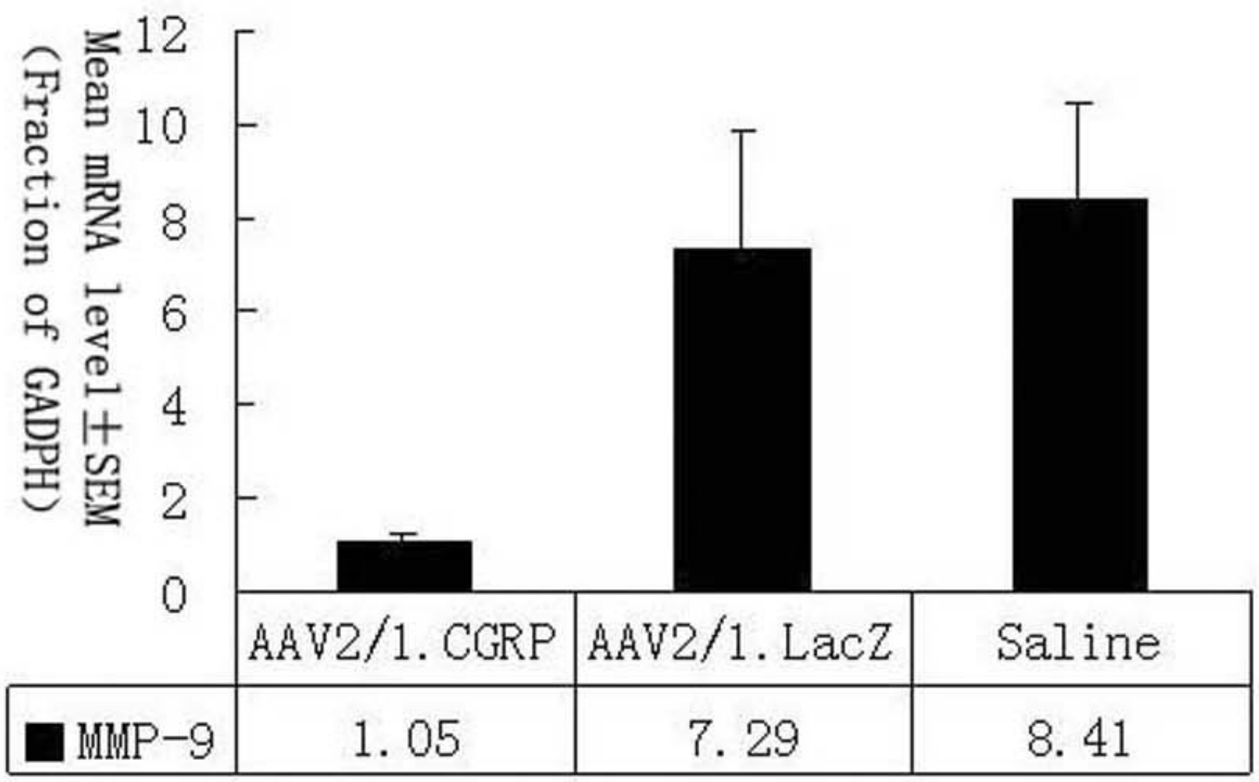


Table 1. Primers Used for Inflammation-Mediating Molecules mRNA Assessment

Molecules	Sequence(5′-3′)	PCR product size (base pairs)
CGRP	ATGGGCTTCCAAAAGTTCTCC CTGGAGCCCTCTCTCTCTTG	227
iNOS	CTGCTTTGTGCGGAGTG CACCCGAACACCAGGAT	174
TNF-α	GGCTCAGAATCAGACCTCAG GCTCCACATTGCAGAGAAGA	297
MCP-1	CCAGCCAGATGCCGTGAAT AGATCCCCTTGGCCAGTTTG	155
MMP-9	GCAGGGTAGGGGGTATGGA ACAGGGCTTGGCTTTGGA	630
GADPH	TCACCATCTTCCAGGAGCGA CACAATGCCGAAGTGGTCGT	293