

Effects of granulocyte colony-stimulating factor on rabbit carotid and porcine heart models of chronic obliterative arterial disease

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Abstract. Previous studies suggest that granulocyte colony-stimulating factor (G-CSF) can promote bone marrow derived progenitor cells to mediate cardiovascular repair, potentially reversing mechanical dysfunction in chronic ischaemic heart disease and post myocardial infarction. Two models were used in the present study both using a surgical ameroid constrictor to induce arterial stenosis. The first model used the carotid artery of rabbits. They were divided into high fat diet (inducing atherosclerosis) or normal fat diet (control) groups. Each was subdivided into surgical exposure group without constrictor, ameroid constrictor receiving normal saline or receiving G-CSF 15 $\mu\text{g/kg/day}$. Endothelial markers of endothelial nitric oxide synthase and endothelin 1 were increased by the use of ameroid constrictor in both atherosclerotic and non-atherosclerotic mice, however were not further altered by G-CSF. Scanning electron microscopy indicated that ameroid constrictor application altered endothelial morphology from an oval shape to a round shape and this was more prominent in the atherosclerotic compared with the non-atherosclerotic group. G-CSF injection increased the number of endothelial cells in all groups. The second model used the left coronary artery of pigs. They were equally divided into following groups, receiving normal saline (control), G-CSF 2.5 $\mu\text{g/kg/day}$ (low dose), 5 $\mu\text{g/kg/day}$ (medium dose) and 10 $\mu\text{g/kg/day}$ (high dose) for 5 days. G-CSF at a low or high dose worsened intimal hyperplasia however at a medium

dose improved it. In conclusion, G-CSF had no effect in a rabbit carotid artery model of atherosclerosis. Its effects on the porcine heart were dose-dependent; arterial disease worsened at a low or high dose, but improved at a medium dose.

Introduction

Stem cell-based therapy has been demonstrated as a good option for cardiac repair following myocardial infarction (MI). Previous clinical trials have demonstrated efficacy using a traumatic method of bone marrow aspiration, culture of the bone marrow stem cells and intracoronary infusion of this end-product (1). Another less invasive option is to use granulocyte colony-stimulating factor (G-CSF) to mobilize bone marrow-derived progenitor cells to undertake tissue repair (2). The advantage of this technique is that it is less traumatic, requiring only subcutaneous injections. However, the results on its therapeutic efficacy have thus far been conflicting. In the FIRSTLINE-AMI study, subcutaneous injection of G-CSF shortly after successful primary percutaneous coronary intervention (PCI) in patients suffering from acute ST elevation MI was demonstrated to mobilize mononuclear blood stem cells (3). This resulted in enhanced resting wall thickening of the infarcted myocardium, improvement of wall motions and systolic function without apparent re-stenosis. Further studies have demonstrated a good safety profile without reports of adverse effects (2,4,5). However, the REVIVAL-2 study demonstrated that delayed application of G-CSF 5 days after successful PCI did not significantly reduce infarct size or improve left ventricular function (6). Furthermore, usage of G-CSF in acute MI may be a double-edged sword, due to the fact that it may paradoxically reduce the migratory capacity of bone marrow-derived progenitor cells into the ischaemic myocardium and thus there is a need to optimize the cytokine profile with other agents to ensure their successful migration (7).

Animal studies have proven useful in the study of the properties of G-CSF. A previous study used a porcine model of ischaemic heart disease to demonstrate improvement in cardiac contractile function in chronic myocardial ischaemia after G-CSF administration (8). Whilst its efficacy in reversing mechanical dysfunction in the heart has been observed, the

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potential benefits in chronic obliterative arterial disease remain to be fully investigated. Thus, the present study aimed to investigate the effects of G-CSF in a novel rabbit model of chronic obliterative arterial disease, which was generated by clamping the carotid artery using a surgical ameroid constrictor. These results were compared with those of an established porcine model of ischemic heart disease generated by clamping of the left coronary artery also using this constrictor.

Materials and methods

Animal model 1. A novel rabbit model of chronic obliterative arterial disease was produced by clamping the right carotid artery using an ameroid constrictor. The current study was approved by the Animal Welfare Ethics Committee of the Tongji Hospital of Tongji University. A total of 36 New Zealand rabbits were used (male, six months old male weight 2-3 kg; SPF grade Ivl; Experimental Animal Centre, Tongji Hospital). The animals were housed at $22\pm 1^\circ\text{C}$ and 50-60% relative humidity with a 12-h light/dark cycle. They were divided into 2 groups, with one group being fed a high fat diet for two months to induce atherosclerosis (AS), whereas the other group was fed a normal fat diet, acting as the control group (CON). Each group was further subdivided into three groups: Sham group (carotid arteries surgically exposed but no ameroid constrictors were applied; SHAM), ameroid constrictor group receiving hypodermic saline (0.5 ml daily for 5 days; NS) and ameroid constrictor group receiving G-CSF ($15\ \mu\text{g}\cdot\text{kg}^{-1}$ daily; Shanghai 3D Biotechnology Co., Ltd.). The 6 groups were therefore: AS-SHAM, AS-NS, AS-G-CSF; CON-SHAM, CON-NS and CON-G-CSF.

Ultrasound studies of the carotid arteries. The following parameters were measured using Doppler ultrasound Vevo770 Imaging system (VisualSonics, Inc., Toronto, ON, Canada) at four different time points (prior to surgery and 3, 5 or 7 weeks after surgery): Peak systolic flow velocity, resistance index and the end-diastolic velocity. Stenosis rate was calculated by (difference in diameters between left and right carotid arteries)/(left carotid artery).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted and purified from the proximal portion of the right carotid artery using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Quantification was achieved using a spectrophotometer. A total of $2\ \mu\text{g}$ RNA was used as a template for a RT-qPCR with random hexamers (Toyobo Life Science, Osaka, Japan) as primers. Then, $10\ \mu\text{l}$ cDNA was purified with an additional ethanol washing step. Levels of mRNA were quantified by RT-qPCR using a Rotor-Gene 2000 machine (Corbett Research, Mortlake, Australia). The results were presented as gene copy numbers relative to GAPDH. The primers were used as follows: Endothelin 1 (ET-1), upstream 5'-CTCTCTGCTGTTGGTGGCTTT-3' and downstream 5'-TGGGTTTTCCGCTCCTGT-3'; endothelial nitric oxide synthase (eNOS), upstream 5'-AGGCCTCCTGTGAGACTTTC-3' and downstream 5'-AAGGAGTCGAGGACTGGATG-3'; GAPDH, upstream 5'-CCACTTGTGAA GCTCATTTCT-3' and downstream 5'-TCGTCCTCCTCTGGTGCTCT-3'.

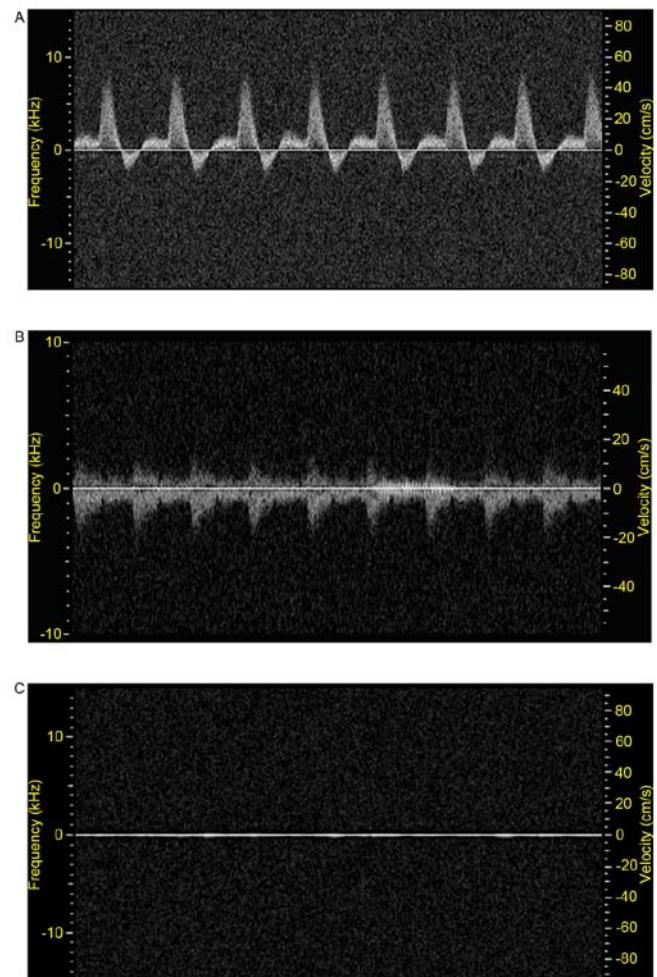


Figure 1. Pulse-wave Doppler ultrasound flow (A) before, (B) 3 weeks and (C) 5 weeks following ameroid constrictor placement around right common carotid artery. Pre-surgery, the peak blood flow velocity of right common carotid artery) was evident, however, the peak flow velocity was markedly reduced at 3 weeks after the operation, and was not observed at 5 weeks.

Scanning electron microscopy. The appearance of the endothelium of the rabbit carotid arteries close to the site of ameroid constrictor application was studied using a scanning electron microscope (HITACHI-S520; Hitachi, Tokyo, Japan).

Animal model 2. A well-established porcine model of chronic ischemic heart disease was also used in the present study, which involved clamping the left coronary artery using an ameroid constrictor. A total of 24 pigs (male, 1 year old, weight ~ 40 kg; Beijing University of Agriculture, China) The animals were housed in a cage with straw at $22\pm 1^\circ\text{C}$ and 50-60% relative humidity with a 12-h light/dark cycle and water and food *ad libitum*. They were equally divided into four groups, receiving normal saline 0.5 ml (control), G-CSF $2.5\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (low dose), $5\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (medium dose) and $10\ \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ (high dose) for 5 days. G-CSF was diluted using normal saline.

Histology and immunohistochemistry. In the porcine model, the proximal portion of occluded coronary arteries were obtained four weeks after the operation, which allowed effects of G-CSF on histology to be determined. Hematoxylin and eosin (H&E) staining was used to examine angiogenesis and

Table I. Doppler flow parameters after 3 weeks.

Sample	Group	AS-SHAM	AS-NS	AS-G-CSF	CON-SHAM	CON-NS	CON-G-CSF
Opposite	D (cm)	2.01±0.13	2.00±0.095	2.0±0.09	1.82±0.16	1.91±0.11	1.76±0.05
	V _{max} (cm/s)	34.11±3.21	42.65±2.08	44.23±2.80	42.70±3.66	45.52±4.95	43.47±4.24
	EDV (cm/s)	11.25±1.34	17.48±1.27	13.47±1.10	18.99±2.23	19.13±2.50	14.34±1.37
	RI	0.67±0.18	0.59±0.20	0.78±0.17 ^a	0.55±0.26	0.58±0.25	0.66±0.03 ^b
Proximal	D (cm)	1.80±0.11	1.53±0.12	1.33±0.14	1.81±0.12	1.81±0.14	1.64±0.23
	V _{max} (cm/s)	33.63±3.57	28.31±3.89	25.28±7.02	45.00±6.76	54.32±5.44 ^c	33.75±9.85 ^b
	EDV (cm/s)	12.44±1.80	11.6±1.89	9.89±2.91	19.00±3.47	19.44±1.77 ^d	12.28±3.69
	RI	0.62±0.21	0.64±0.05	0.66±0.05	0.57±0.17	0.81±0.05 ^d	0.75±0.06
	Stenosis rate (%)	0	33.24±0.06	49.51±0.13	0	8.55±0.04 ^d	42.13±0.10 ^a

^aP<0.01 and ^bP<0.05 G-CSF vs. control; ^cP<0.01 and ^dP<0.05, VS between atherosclerosis or none. D, diameter; V_{max}, maximum velocity; EDV, end diastolic velocity; RI, resistance index; AS atherosclerosis; NS, ameroid constrictor group receiving saline; G-CSF, granulocyte colony-stimulating factor; CON, control.

Table II. Frequency of complete obliteration among the groups after 5 and 7 weeks.

Group	AS-SHAM	AS-NS	AS-G-CSF	CON-SHAM	CON-NS	CON-G-CSF
Week 5	0	2	5 ^a	0	1	2
Week 7	0	4 ^a	6 ^b	0	3	5 ^a

^aP<0.05 and ^bP<0.01 vs. sham operation group. AS, atherosclerosis; NS, ameroid constrictor group receiving saline; G-CSF, granulocyte colony-stimulating factor; CON, control.

quantified using a Leica Qwin V3 imaging system (Leica Microsystems GmbH, Wetzlar, Germany). Mallory stain was used to study collagen content. Immunohistochemical staining were used to examine vascular endothelial growth factor (VEGF) and tumor necrosis factor (TNF)- α , which reflected angiogenesis and inflammation, respectively.

Statistical analysis. All data were presented as mean \pm standard error. Fisher's exact test was used as appropriate using SPSS (version 11.5; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Carotid artery in rabbit model

Ultrasound assessment of the occluded right carotid artery. Ultrasound was used for assessing the functional status of the right carotid artery of rabbit hearts. Firstly, B-mode ultrasonography was used to determine real-time information on the lumen and vessel wall (9,10). Secondly, Doppler imaging was used to determine flow parameters (Fig. 1). Three weeks after the operation, the right carotid artery developed stenosis (Table I). Five weeks later, total occlusion was observed in all groups at different rates (Table II).

Assessment of endothelial function using RT-qPCR. RNA was extracted from the proximal portion of the right carotid arteries in the different groups (Fig. 2). Expression indices of

endothelial nitric oxide synthase (eNOS) in the AS-SHAM and CON-SHAM groups were 0.85±0.11 and 0.91±0.12, respectively. They were increased to 2±0.206 and 1.89±0.33 for the AS-NS and CON-NS groups. However, these values were not significantly different from the values in the presence of G-CSF (2.14±0.30 and 1.94±0.32 for AS-G-CSF and CON-G-CSF). The expression levels of ET-1 were 1.3±0.17 and 1.5±0.25 (AS-SHAM and CON-SHAM), 0.65±0.08 and 0.67±0.09 (AS-NS and CON-NS) and 0.57±0.05 and 0.59±0.05 (AS-G-CSF and CON-G-CSF). There were no significant difference between the groups treated with normal saline compared with those treated with G-CSF.

Assessment of endothelial morphology using scanning electron microscopy. The morphology of endothelial cells of the proximal carotid artery were assessed using scanning electron microscopy (Fig. 3). In the AS-SHAM and CON-SHAM groups, endothelial cells took on an oval shape, which was due to linear blood flow within the artery. When compared with the CON-SHAM group, the AS-SHAM group, the endarterium developed a rougher morphology with a smaller number of endothelial cells, and had a more shrunken image with tiny lipid droplets. With application of the ameroid constrictor, this morphology was changed to one of roundness due to the turbulent flow distal to the site of occlusion. Such a change was more prominent in the AS-SHAM than CON-SHAM group. The number of endothelial cells increased after G-CSF injections.

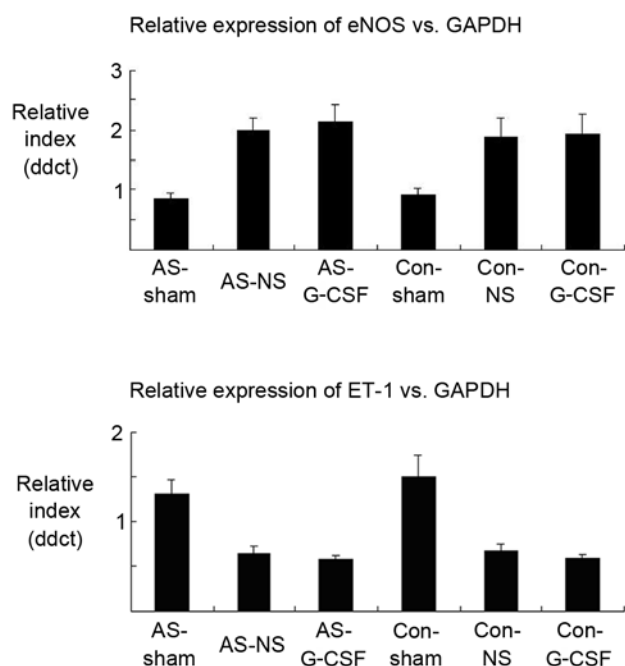


Figure 2. G-CSF administration did not result in a significant difference in eNOS and ET-1 gene expression. The proximal carotid near the constrictor or the same site was harvested for reverse transcription-quantitative polymerase chain reaction analysis from the rabbits for all groups studied. In addition, no changes after high fat diet administration were observed (n=6 per group). G-CSF, granulocyte colony-stimulating factor; eNOS, endothelial nitric oxide synthase; ET-1, endothelin 1; AS, atherosclerosis; NS, ameroid constrictor group receiving saline; Con, control.

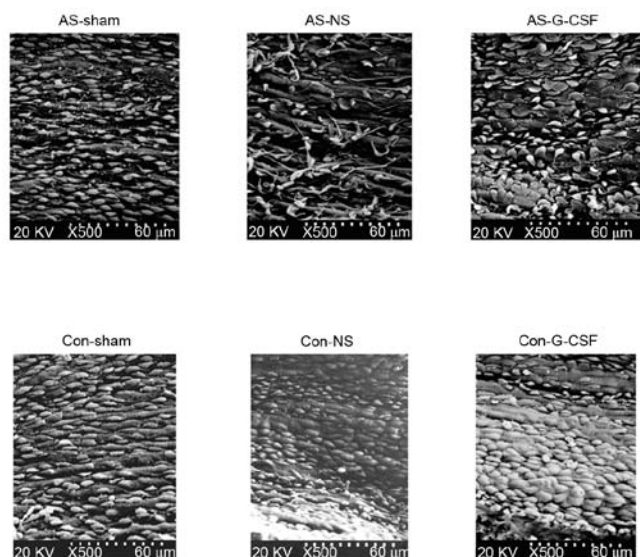


Figure 3. Endothelial cells under scanning electron microscopy. AS, atherosclerosis; NS, ameroid constrictor group receiving saline; Con, control; G-CSF, granulocyte colony-stimulating factor.

Left coronary artery in swine model

Histological studies to determine effects of varying dosages of G-CSF. Four weeks after the operation, histological studies exhibited intimal proliferation together with eccentric narrowing in the left coronary arteries (Fig. 4). H&E staining indicated varying degrees of intimal hyperplasia in the different groups. Intimal hyperplasia was more severe

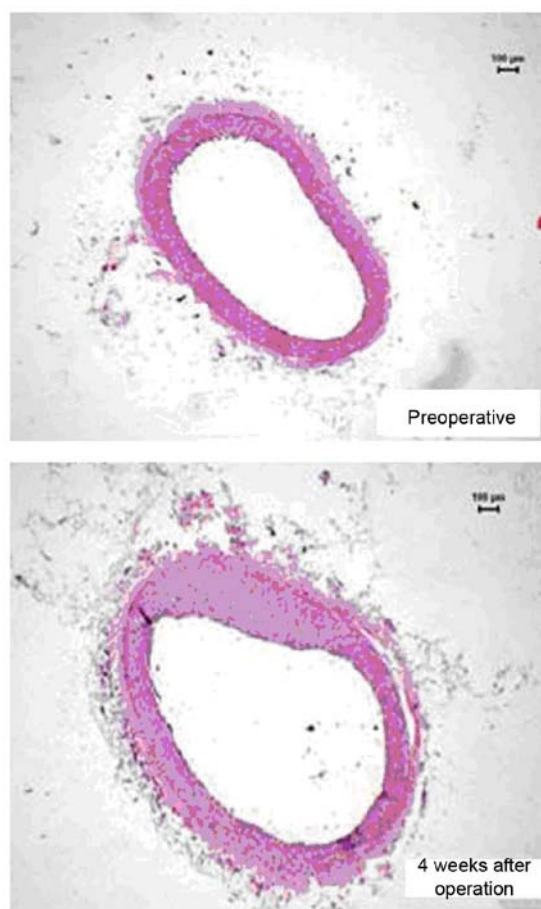


Figure 4. The proximal endangium near the constrictor (hematoxylin and eosin stain; magnification, x40).

in the low and high dose G-CSF groups compared with the control group, whereas it was less severe in the medium dose group compared with the control (Fig. 5). The areas of tunica intima and vessel lumen were analyzed using Leica Qwin Plus Graphic Processing System, which allowed proliferation area to be calculated (Fig. 6).

Mallory's trichrome staining of the proximal coronary artery indicated significantly smaller amounts of collagen in the medium and high dose groups compared with the control group. However, there was no significant difference between the low dose and control group (Fig. 7).

Effects on VEGF and TNF- α . In order to examine the role of angiogenesis and inflammation, immunohistochemistry was used to determine the expression of VEGF and TNF- α . G-CSF exerted a dose-dependent effect on VEGF and TNF- α , increasing them both at increasing concentrations (Fig. 8).

Discussion

G-CSF has exhibited some efficacy in reversing mechanical dysfunction in chronic ischaemic heart disease and following MI. A previous studies demonstrated its safety in humans, however results regarding its therapeutic effects remain conflicting (10). A previous study failed to demonstrate improvement in ventricular function post MI using G-CSF

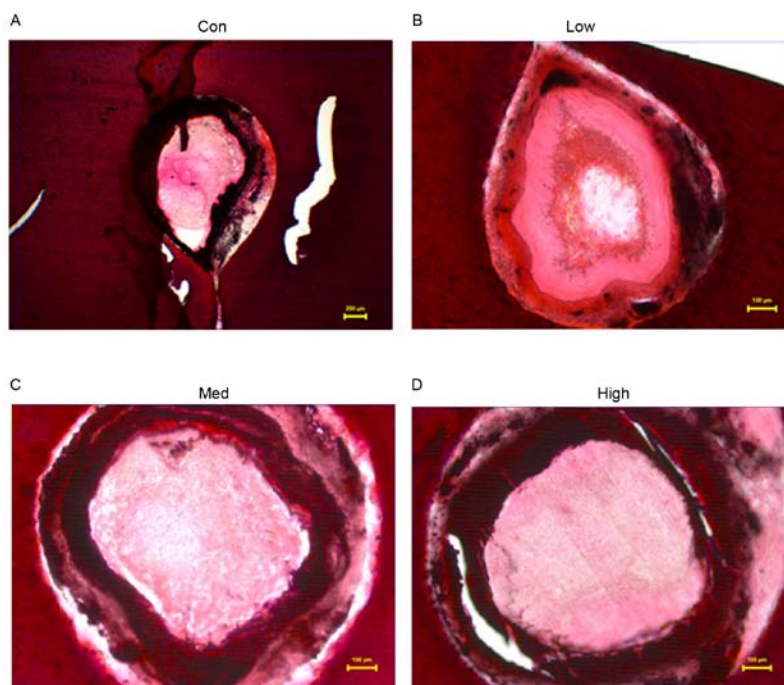


Figure 5. Intimal hyperplasia in different G-CSF treatment groups. (A) Control group (NS 0.5 ml/kg/day), (B) the low dosage group (G-CSF 2.5 mg/kg/day), (C) medium dosage group (G-CSF 5 mg/kg/day) and (D) high dosage group (G-CSF 10 mg/kg/day) (hematoxylin and eosin stain; magnification, x40). G-CSF, granulocyte colony-stimulating factor; NS, ameroid constrictor group receiving saline; Con, control.

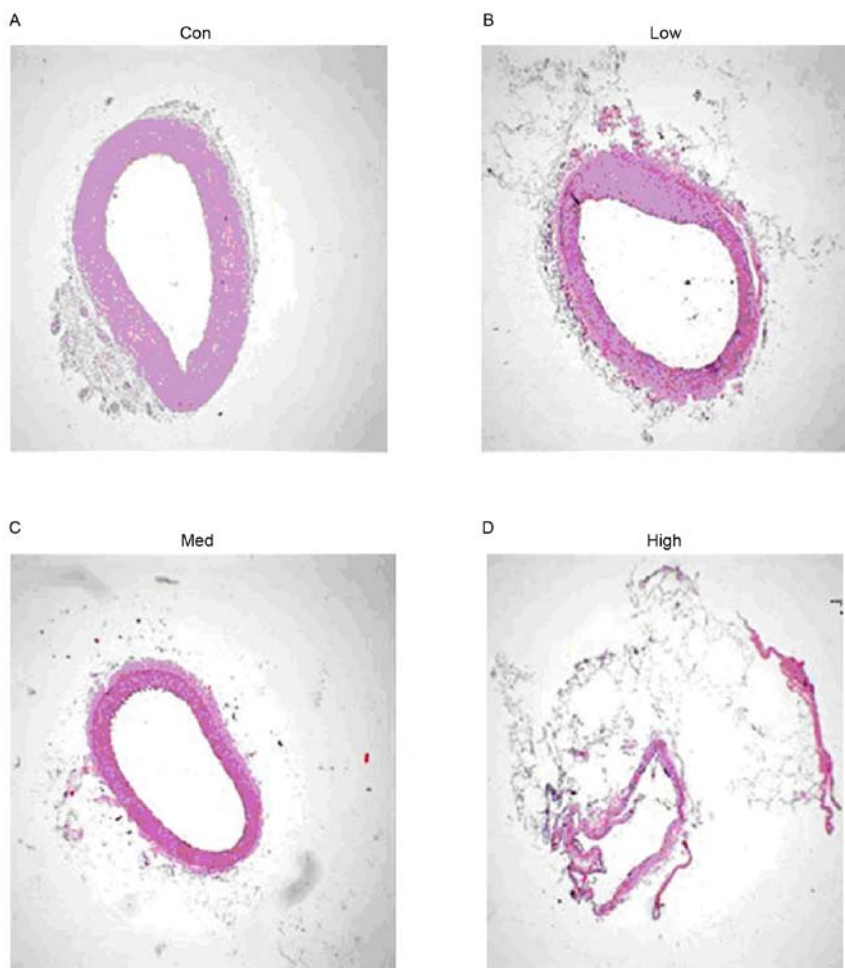


Figure 6. Local endarterium of the proximal artery near the constrictor. (A) Control group (NS 0.5 ml/kg/day), (B) the low dosage group (G-CSF 2.5 mg/kg/day), (C) medium dosage group (G-CSF 5 mg/kg/day) and (D) high dosage group (G-CSF 10 mg/kg/day); hematoxylin and eosin stain; magnification, x40; NS, ameroid constrictor group receiving saline; G-CSF, granulocyte colony-stimulating factor; Con, control.

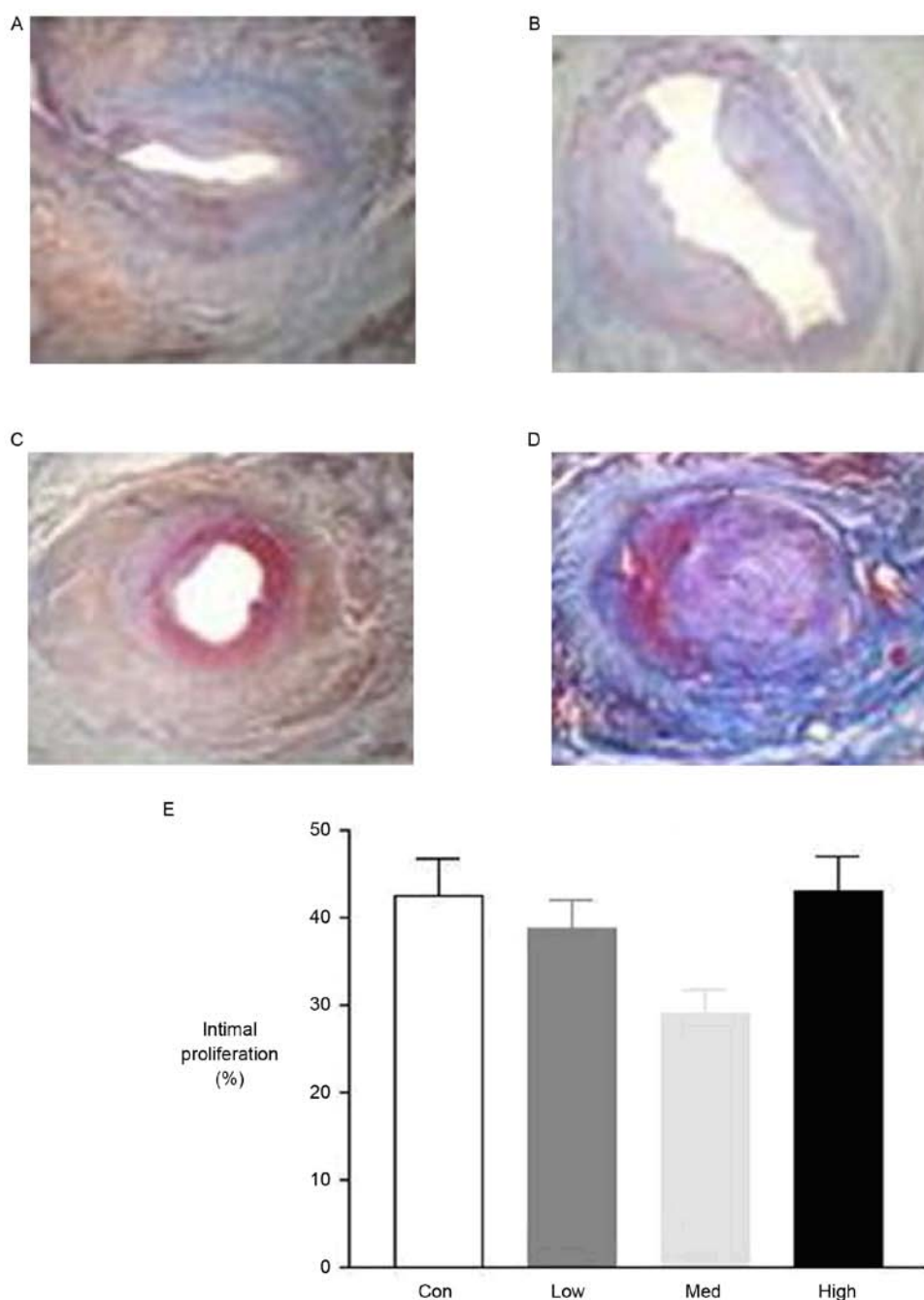


Figure 7. The pathology of different dosage groups. (A) Control group (NS 0.5 ml/kg/day), (B) the low dosage group (G-CSF 2.5 mg/kg/day), (C) medium dosage group (G-CSF 5 mg/kg/day) and (D) high dosage group (G-CSF 10 mg/kg/day); (A-C) hematoxylin and eosin and (D) Mallory's trichrome staining; magnification, $\times 400$. Quantification is presented in (E). NS, ameroid constrictor group receiving saline; G-CSF, granulocyte colony-stimulating factor; Con, control.

when compared with the control group (11), in contrast to other studies in which improved left ventricular systolic function was observed (4,5). However, G-CSF was demonstrated to reduce the migratory capacity of bone marrow-derived progenitor cells into the ischaemic myocardium (7). In addition, patients suffering from coronary artery disease responded to G-CSF by exhibiting increased numbers of endothelial progenitor cells (EPCs) and higher expression of the chemokine receptor CXCR4, which directed EPCs to ischaemic tissue (12). However, increased mobilization did not equate to reversal of damage, no significant improvement in wall motion, perfusion or exercise duration after G-CSF use was observed (13). However, intra-coronary infusion of G-CSF did mobilize

peripheral blood stem cells and improved ejection fraction in patients with acute MI although not in those with chronic ($\geq 6 \pm 1.2$ months) MI (14).

The mechanism of action of G-CSF remains to be fully elucidated. Different animal models have been useful for modelling the molecular mechanisms of human diseases ADDIN EN.CITE (15-35), resulting in translational insights (36-41). Mice and rats have been used because of their amenability to genetic modification, as demonstrated by the study of ion channel mutations on cardiovascular physiology (42-44). The effects of increased oxidative stress on endothelial function have been studied in diabetic mice and spontaneously hypertensive rats, modelling human cardio-metabolic disorders (45-57). Larger

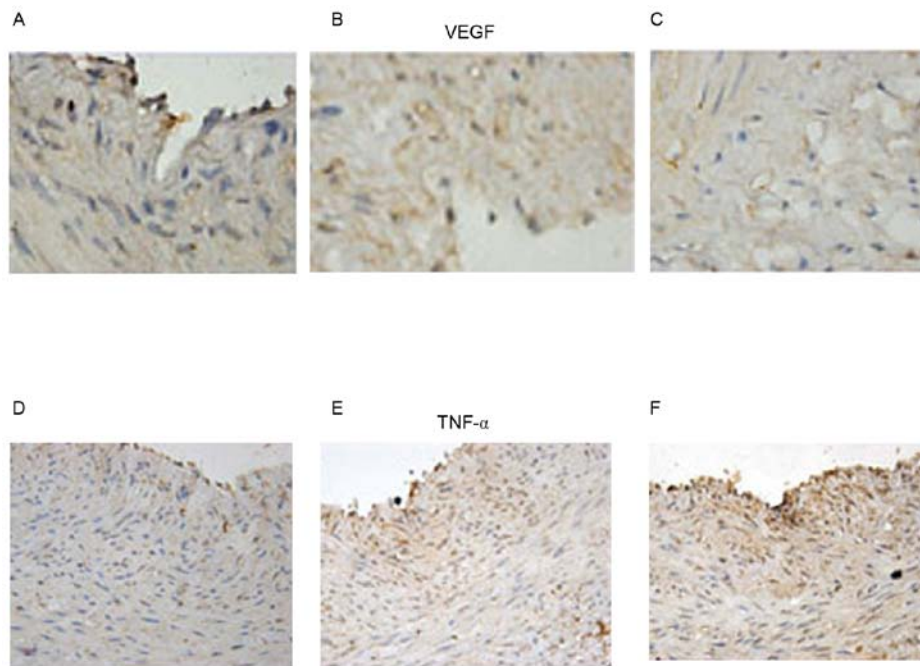


Figure 8. VEGF in the (A) low dosage group (G-CSF 2.5 mg/kg/day), (B) medium dosage group (G-CSF 5 mg/kg/day) and (C) high dosage group (G-CSF 10 mg/kg/day). TNF- α in the (D) low dosage group (G-CSF 2.5 mg/kg/day), (E) medium dosage group (G-CSF 5 mg/kg/day) and (F) high dosage group (G-CSF 10 mg/kg/day). Immunohistochemistry images, magnification $\times 1,000$. VEGF, vascular endothelial growth factor; G-CSF, granulocyte colony-stimulating factor; TNF- α , tumor necrosis factor α ; Con, control.

animals such as guinea pigs and pigs have haemodynamic parameters more similar to humans, and are therefore useful for studying flow properties, however it remains a challenge to study using methods involving large machines, such as cardiac magnetic resonance imaging (34,35). In a rat model, inhibition of neointimal formation and increased re-endothelialisation of injured arteries has been observed, potentially via a direct effect on the heart and the arteries (58). In the current study, two animal models were employed by applying ameroid surgical constrictors to the right carotid artery of rabbits or to the left coronary artery of pigs.

In the rabbit model, a high fat diet led to the development of atherosclerosis, a chronic inflammatory disease (59). The number of endothelial cells increased after G-CSF injection at a dose of $15 \mu\text{g/kg/day}$ as demonstrated using scanning electron microscopy. However, there was no change in endothelial function assessed using RT-qPCR for the endothelial markers endothelial nitric oxide synthase and ET-1. Whether or not G-CSF is protective in atherosclerosis remains controversial. At a dose of $10 \mu\text{g/kg/day}$, G-CSF has been demonstrated to worsen atherosclerosis in apolipoprotein E-deficient mice (60), however by contrast, at a high dose of $100 \mu\text{g/kg/day}$, it prevented progression of atherosclerosis in heritable hyperlipidemic rabbits and following vascular injury induced by angioplasty balloon in rabbits (61). In an iliac artery injury model of rabbits, G-CSF at a dose of $70 \mu\text{g/day}$ mobilized vascular progenitor cells, which induced neointimal overgrowth at the stented vessels and enhanced endothelial healing was observed when drug-eluting stent was used when compared with a bare metal stent (62). In a rat model, G-CSF increased the number of mononuclear cells in the circulation, increased

endothelial adhesion markers and re-endothelialization of the denuded vessels, which resulted in parallel reductions in inflammation in the vessel wall (63).

The present study in porcine hearts observed increasing intimal hyperplasia after low ($2.5 \mu\text{g/kg/day}$) or high ($10 \mu\text{g/kg/day}$) doses of G-CSF. Furthermore, the mobilization of bone marrow-derived progenitor cells using G-CSF resulted in increased endothelial expressions of VEGF and TNF- α , markers of angiogenesis and inflammation, respectively, as assessed using immunohistochemistry and western blot analysis. G-CSF has pleotropic effects, and is capable of inducing hyperplasia, angiogenesis and inflammation (62). Whether it is beneficial or harmful depends on the relative contributions to the above processes. In the present study, intimal hyperplasia was increased in chronic obliterative arterial disease, in contrast to observations in humans where G-CSF was demonstrated to be effective in acute as opposed to chronic ($\geq 6 \pm 1.2$ months ± 1.2) MI (14). Other beneficial effects of G-CSF have been reported in rats, where a neuro-protective role was demonstrated in transient middle cerebral artery occlusion (64). However, G-CSF can be ineffective or potentially harmful. For example, experiments in a swine model of ischaemia-reperfusion injury indicated that G-CSF accelerated angiogenesis, reduced fibrosis however did not improve either ejection fraction or end-diastolic volume (65). Furthermore, intramyocardial VEGF gene transfer followed by bone marrow stem cell mobilization using G-CSF was safe however did not significantly improve myocardial perfusion as assessed by single photon emission computerized tomography (66). G-CSF aggravated in-stent re-stenosis, which was partly dependent on VEGF and STAT-3, although this may be reduced by using a sirolimus drug-eluting stent (67).

In conclusion, the present study identified that: i) G-CSF did not alter endothelial function in the carotid artery of a rabbit model of atherosclerosis; ii) its effects on chronic ischaemic heart disease in pigs are dose-dependent, worsening intimal hyperplasia when used at a low or high dose, however improving it at a medium dose. Therefore, its therapeutic role warrants further attention.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

The manuscript was written with contributions from all authors. All authors have given approval to the final version of the manuscript. ZH performed the research and experiments. ZC, YW, JJ, GT, WX, JG and BS contributed to data analysis. ZH and ZC were responsible for the overall project design and manuscript organization.

Ethics approval and consent to participate

The present study was approved by the Animal Welfare Ethics Committee of the Tongji Hospital of Tongji University and the approval number was 2017-DW-006.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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