European Review for Medical and Pharmacological Sciences 2016; 20: 311-316

# Study of a novel coating strategy for coronary stents: evaluation of stainless metallic steel coated with VEGF and anti-CD34 antibody *in vitro*

C.-L. SONG, Q. LI, J.-C. ZHANG, J.-P. WANG, X. XUE, G. WANG, Y.-F. SHI, H.-Y. DIAO, B. LIU

*Department of Cardiology, the Second Hospital of Jilin University, Nanguan District, Changchun, China*

**Abstract. – OBJECTIVE: To access the cytotoxicity and the effect on the endothelial progenitor cell (EPC) differentiation of stainless steel sheets simultaneously coated with VEGF and anti-CD34 antibody.**

**MATERIALS AND METHODS: 316L stainless steel sheets (diameter 6 mm, thickness 1 mm) were divided into the D-H (Bare metal), D-(H-V)10 (VEGF-coated metal) and D-(H-V)10-A (VEGF and anti-CD34 antibody co-coated metal) groups. The cytotoxicity effect of the three groups was measured using MTT assay. Percentage of EPC positive for CD34, CD133 and KDR were detected by flow cytometric assay. Endothelial cells positive for CD31 and VE-Cadherin were also detected by flow cytometric assay.**

**RESULTS: The percentages of isolated cells positive for CD133, CD34 and KDR were 89.9%, 91.3%, and 90.4%, respectively, suggesting that the EPCs were successfully isolated. MTT results showed that the stainless steel sheets coated with VEGF and anti-CD34 antibody have less toxicity on seeded EPCs than single VEGF coating or bare metal. We further found that with VEGF and anti-CD34 antibody co-coating could significantly promote the differentiation of EPCs *in vitro* when compared with that of single VEGF coating and bare metal.**

**CONCLUSIONS: Our study provided a preliminary evaluation of metallic steel sheet coated with VEGF and anti-CD34 antibody *in vitro*. Our findings suggest that simultaneously coating the stents with VEGF and anti-CD34 antibody might be a novel research direction for facilitating re-endothelialization in order to reduce ISR after stent implantation.**

*Key words:*

*Coronary artery disease, Drug-eluting stents, In-stent restenosis, VEGF, Anti-CD34 antibody, Endothelial progenitor cells, Cytotoxicity, Differentiation.*

## Introduction

Coronary artery disease (CAD) is one of the most common cardiovascular diseases with high rates of mortality and morbidity, mainly due to the fact of blood vessel narrowing caused by atherosclerosis[1]. Metallic stent implantation is a standard percutaneous therapy for coronary artery disease. However, intracoronary stenting often leads to neointimal hyperplasia and restenosis. The in-stent restenosis (ISR) rate of a simple bare metallic stent (BMS) is 10%-30%[2,3]. The most widely accepted hypothesis for the ISR is the "response-to-injury"[4], in which lack of endothelial coverage in the vessel wall are thought to stimulate a remodeling process with inward migration and proliferation of medial smooth muscle cells (SMCs). Thus, a variety of drugs has been employed for the prevention of SMC growth and proliferation, such as rapamycin and paclitaxel.

Drug-eluting stents (DESs) is a peripheral or coronary stent with a drug slowly released from a polymer coating or loaded directly onto the stent[5]. They have been designed mainly to minimize percutaneous coronary intervention (PCI) related vascular inflammation and cellular proliferation and thus reduce ISR[6]. Currently, applications of the DESs, such as rapamycin drug-eluting stents and paclitaxel-eluting stents, have greatly reduced the prevalence of restenosis through preventing or significantly reducing migration and proliferation of medial SMCs, which at the same time has been reported to delay the formation of a functional endothelial lining over the stent[7]. However, controversial results suggest that the incomplete endothelialization of the stent surface may lead to the increased long-term incidence of thrombosis and ISR[8,9].

Therefore, accelerating endothelial repair is a promising research area that may reduce the stent restenosis and long-term thrombosis after stent implantation. Recently, circulating endothelial progenitor cells (EPCs) have been identified as a key factor for re-endothelialization. The EPC capture stents have been developed using immobilized antibodies targeted at EPC surface antigens, such as CD34[10]. Vascular endothelial

*Corresponding Author:* Bin Liu, Ph.D; e-mail: liubin_china999@163.com

growth factor (VEGF) has been extensively studied and used to promote revascularization and re-endothelialization by stimulating migration and maturation of endothelial progenitor cells[11,12].

Our research team has previously developed a novel coating approach for coronary stents using the combination of VEGF and anti-CD34 antibody[13]. By using the specific method developed, our previous study indicated that stainless steel sheets could be successfully coated with VEGF and antibodies against CD34. In this study, we aim to further evaluate the toxicity and the effect of VEGF and anti-CD34 antibody co-coated 316 stainless steel on EPCs differentiation. Our results showed that the combined coating of stainless steel with VEGF and anti-CD34 antibody had less cytotoxicity compared with VEGF-coating or bare metal, and could promote the differentiation of EPCs *in vitro*.

## Materials and Methods

### Materials

$VEGF_{165}$, fetal bovine serum (FBS), glutamine, penicillin-streptomycin and Alexa Fluor® 488 Goat Anti-Rabbit IgG were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Epithelial growth factor (EGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), M199 medium and human fibronectin (FN) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Human umbilical cord blood used for isolation of epithelial progenitor cells was from the Xinhua Hospital (Shanghai, China). Primary antibodies, like rabbit anti-human CD34 antibody, rabbit anti-human VEGF primary antibodies, mouse anti-human KDR, rabbit anti-human CD31 and mouse anti-human VE-cadherin were purchased from Abcam (Cambridge, UK). Round 316L stainless steel sheets (diameter 6 mm, thickness 1 mm) were used in this study, and three coating groups were studied, including the D-H group (heparinization), D-$(H\text{-}V)_{10}$ group (steel sheets coated with VEGF) and D-$(H\text{-}V)_{10}$-A (simultaneously coated with VEGF and anti-CD34 antibody) group.

### Preparation of the coated steel sheet

The coated steel sheet was prepared according to our previous report[13]. Briefly, round 316L stainless steel sheets (diameter 6 mm, thickness 1 mm) were divided into three different groups according to coating procedures: D-H group, coated with heparin; D-$(H\text{-}V)_{10}$ group, coated with $VEGF_{165}$; D-$(H\text{-}V)_{10}$-A group, coated stepwise with $VEGF_{165}$ and rabbit anti-human CD34.

### Isolation of endothelial progenitor cells (EPC)

Human umbilical cord blood (50 mL) was diluted 1:1 with phosphate buffered saline (PBS) containing 0.02% EDTA, added to a Ficoll gradient, and centrifuged at 2,000 rpm/min for 20 min. Carefully aspirate the monocyte layer and wash with PBS containing 0.02% EDTA for two times. Discard supernatant and the cell pellet were resuspended in M199 medium supplemented with 20% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were counted, placed into 6-well plates, coated with human fibronectin at a density of $2\times 10^6$ cells/$cm^2$, and incubated at 37°C, 95% humidity, and 5.0% carbon dioxide. Refresh medium after 48 hours to remove non-adherent cells. Refresh medium every three days afterwards. Incubate for 1-2 weeks.

### Identification of endothelial progenitor cells (EPC) by flow cytometry

Harvest cells at 12 days of incubation by 0.05% trypsin and wash with PBS containing 0.5% bovine serum albumin (BSA) for two times. Resuspended cells were incubated for 30 min at 37°C with rabbit anti-human CD34 and CD133, and mouse anti-human KDR and a non-specific antibody IgG, respectively. Wash off the unbound antibody using 0.5% PBS containing 0.5% BSA. Cells were fixed in 4% paraformaldehyde and analyzed by a FACSAria flow cytometer (BD Biosciences, Framklim Lakes, NJ, USA).

### Methyl thiazolyl tetrazolium (MTT) assay

Coated steel sheets from the D-H, D-$(H\text{-}V)_{10}$ and D-$(H\text{-}V)_{10}$-A group were placed into 48-well plates, respectively. The isolated EPC cells were seeded at a density of 5,000 cells/well. At 0, 24, 48 and 72 hours, add 50 µl MTT reagent (5 mg/mL) and incubate for 4 hours at 37°C. Add 500 µL DMSO and vortex for 10 min. 100 µL of the supernatant was aspirate for the detection of absorbance at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The growth curve was generated accordingly.

### Measurements differentiation of EPCs by flow cytometry

The isolated EPC cells were incubated in DMEM supplemented with 20% FBS, $VEGF_{165}$

(20 ng/mL), epithelial growth factor (EGF, 1 ng/mL), insulin-like growth factor-1 (IGF-1, 2 ng/mL), basic fibroblast growth factor (bFGF, 4 ng/mL) and glutamine (300 μg/mL). Refresh medium every 2 or 3 days for two weeks. The expression of KDR, CD31 and VE-cadherin were detected by flow cytometry after staining with mouse anti-human KDR, rabbit anti-human CD31 and mouse anti-human VE-cadherin.

### Statistical Analysis

Statistical significance was assessed by comparing mean values (± standard deviation) using the two-tailed Student's *t*-test for independent groups. The probability value of $p<0.05$ was considered to be statistically significant.

## Results

### Morphologic characteristics and identification of EPCs

Isolated cells were seeded into the FN-coated 6-well plates and the morphology of adherent cells at cultivation day 7 was shown in Figure 1A. Three markers have been previously used to characterize the functional endothelial precursor cells: CD133, CD34, and the vascular endothelial growth factor receptor-2 (kinase insert domain receptor, KDR)[14,15]. We performed flow cytometry to detect the three EPC surface markers in the isolated cells. Our results showed that 89.9% of the isolated cells were positively stained for CD133, 91.3% positively stained for CD34 and

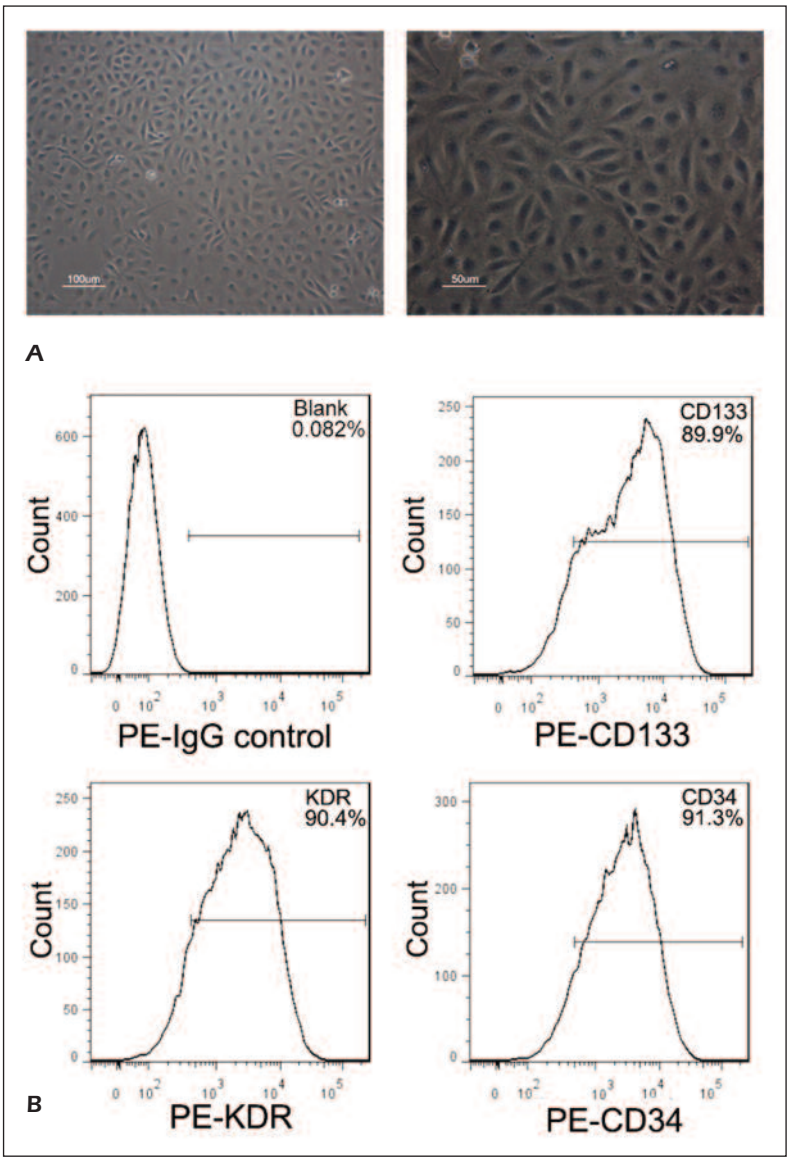


**Figure 1.** Morphology and characteristics of endothelial progenitor cells (EPCs). **A,** Representative microscopic image of EPCs. **B,** Flow cytometric analysis of the three EPC surface makers: CD34, KDR and CD133.

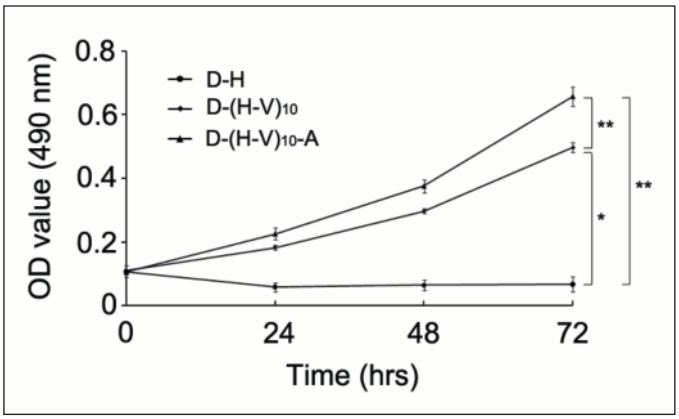


**Figure 2.** *In vitro* evaluation of cytotoxicity of covered steel sheets. The cytotoxicity of the D-H group, D-(H-V)10 group and D-(H-V)10-A group was evaluated by MTT assay. Triplicates for each sample were analyzed and each datum point represents the mean ± SD (n=3). Statistically significant differences are indicated: *$p$<0.05, **$p$<0.01, Student's t-test. *OD value*, optical density value.

90.4% for KDR Figure 1B demonstrated that EPCs were successfully isolated. The isolated EPCs were used for the following *in vitro* evaluation of coated steel sheets.

### *In vitro evaluation of cytotoxicity of covered steel sheets*

The cytotoxicity of the steel sheets, from the D-H, D-(H-V)$_{10}$ and D-(H-V)$_{10}$-A groups, was evaluated and compared by MTT assay. Our results revealed that there was bare no EPC cells growth in the D-H group. In the D-(H-V)$_{10}$ group, EPCs had an obvious growth compared with the D-H group. The EPCs grew significantly faster in the D-(H-V)$_{10}$-A group even than in the D-(H-V)$_{10}$ group, suggesting simultaneously coating with VEGF and CD34 antibody could highly promote the growth of EPCs on the steel sheets (Figure 2). Our results suggested that steel sheets simultaneously coated with VEGF and anti-CD34 antibody had a less toxic effect on the seeded EPCs than solely coated with VEGF or bare metal.

### *Steel sheets coated with VEGF and anti-CD34 antibody promoted the differentiation of EPCs*

The effects on differentiation from EPCs to endothelial cells have been evaluated for the D-H, D-(H-V)$_{10}$ and D-(H-V)$_{10}$-A group using flow cytometry assay. The results showed that the percentage of CD31 positive cells was significantly increased in the D-(H-V)$_{10}$-A group than that of the D-H group (Figure 3A). Meanwhile, the percentage of VE-cadherin positive cells was also significantly increased in the in the D-(H-V)$_{10}$-A group than the D-H group (Figure 3B). However, the percentage of endothelial precursor cell marker KDR was significantly reduced in the D-(H-V)$_{10}$-A group than the D-H group (Figure 3C). Our results suggested that simultaneously coated stainless steel with VEGF and anti-CD34 antibody could significantly promote the differentiation of EPCs.

## Discussion

Restenosis after intracoronary stenting is one of the major limitations for long-term recovery from coronary artery surgery. Drug-eluting stents (DESs), such as rapamycin drug-eluting stents and paclitaxel-eluting stents, have been reported to greatly reduce the rate of in-stent restenosis through significantly reducing migration and proliferation of the medial SMCs[7]. However, the anti-proliferative effects of these drugs also delay the formation of a functional endothelial lining over the stent, which may increase the risk of thrombosis in the long-term recovery[16-18]. Long-term follow-up studies showed that DESs implantation increased the long-term risk of thrombosis by 15%-35% compared with bare-metal stents implantation[8].

Novel DES with biocompatible and biodegradable polymers has been developed to minimize the risk of stent thrombosis. Recent studies[19-21] have reported that vascular endothelial damage may provoke a cascade of cellular and biochemical events which initiates the restenosis after coronary stenting. Thus, accelerating endothelial repair gains more importance than inhibiting the proliferation of SMCs, which could reduce the exposure time of stents in blood, decrease the rate of long-term thrombosis and shorten the time of antiplatelet therapy for patients.

Circulating endothelial progenitor cells specifically are CD34$^+$ and VEGFR-2$^+$/CD31$^+$. EPC capture stent coated with anti-CD34 antibody is a novel technology that has opened up a new horizon in coronary stent implantation, of which the safety and feasibility have been demonstrated in registry

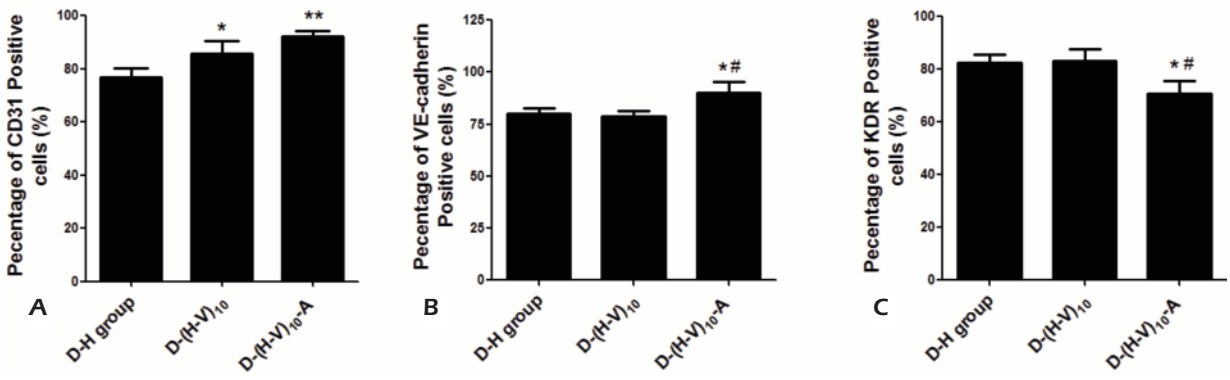


**Figure 3.** Double coating the steel with VEGF and anti-CD34 antibody promoted the differentiation of EPCs. Flow cytometric detection of the CD31 (**A**), VE-cadherin (**B**) and KDR (**C**) in the D-H group, D-$(H\text{-}V)_{10}$ group and D-$(H\text{-}V)_{10}$-A group.

studies and long-term follow-up studies[10,22-24]. Coating stent with VEGF is also a novel strategy that has been reported to accelerate re-endothelialization[12,25]. Previously, with the hope of facilitating rapid restoration of a functional endothelium, we have successfully developed a novel coating strategy for simultaneously coating VEGF and anti-CD34 antibody[13]. Our results indicated that both VEGF and antibodies against CD34 were successfully coated on the 316 stainless steel sheets. In this study, we further evaluated the cytotoxicity of the double-coated stainless steel sheets with MTT assay. Our results showed that, comparing with the bare stainless steel or VEGF-coated one, steel double coated with VEGF and anti-CD34 antibody has a less toxic effect on the seeded EPCs.

It has been reported that circulating progenitor cells in peripheral blood, such as the endothelial progenitor cells (EPCs), play an important role in re-endothelialization. Hibbert et al[19] found that 80% of the in-stent neointimal is derived from circulating progenitor cells in blood including EPCs not from the vascular wall. Other studies also proved that EPCs could induce the in-stent neointimal due to accelerating endothelial repair[26-29]. In our study, we found that simultaneously coating with VEGF and anti-CD34 antibody could significantly promote the differentiation of EPCs *in vitro* when compared with the bare stainless steel and VEGF-coated one.

## Conclusions

Our study provided preliminary results that double coated the steel sheets with VEGF and anti-CD34 antibody not only have less cytotoxicity effects but also promote the differentiation of EPCs *in vitro*. Future studies should be carried out to further evaluate the effects of this novel coating strategy on re-endothelialization in both *in vitro* and *in vivo*.

## Acknowledgements

The study was supported by the following funds: National Natural Science Foundation of China through the National Outstanding Youth Science Fund (Grant number: 51103059); National Natural Science Foundation of Jilin Province (Grant numbers: 201115071, 20140101054JC); Science and Technology Development Planning Project of Jilin Province (Grant number: 20150519025JH); Guiding Fund for Strategic Adjustment of Economic Structure Special Projects (Grant number: 2015Y030-3); Science and Technology Research Projects of the 12th Five-Year Plan for Jilin Province (Grant numbers: [2014] B016 and [2015] 524).

## Conflict of Interests

The Authors declare that they have no conflict of interests.

## References

1. Deaton C, Froelicher ES, Wu LH, Ho C, Shishani K, Jaarsma T. The global burden of cardiovascular disease. J Cardiovasc Nurs 2011; 26: S5-14.
2. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, Detre K, Veltri L, Ricci D, Nobuyoshi M. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. N Engl J Med 1994; 331: 496-501.

3. Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G, Emanuelsson H, Marco J, Legrand V, Materne P, et al. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. N Engl J Med 1994; 331: 489-495.
4. Ross R, Glomset JA. The pathogenesis of atherosclerosis (first of two parts). N Engl J Med 1976; 295: 369-377.
5. van der Hoeven BL, Pires NM, Warda HM, Oemrawsingh PV, van Vlijmen BJ, Quax PH, Schalij MJ, van der Wall EE, Jukema JW. Drug-eluting stents: results, promises and problems. Int J Cardiol 2005; 99: 9-17.
6. Yang SS, Tang L, Ge GH, Ma JW, Qiao ZY, Hou YM, Zhang L, Liu HJ, Cao H, Hao ZM, Cheng WB, Wang HW, Zhang RY. Meta-analysis of the long term effects of different interventions on chronic total coronary occlusions. Eur Rev Med Pharmacol Sci 2013; 17: 1583-1589.
7. Carter AJ, Aggarwal M, Kopia GA, Tio F, Tsao PS, Kolata R, Yeung AC, Llanos G, Dooley J, Falotico R. Long-term effects of polymer-based, slow-release, sirolimus-eluting stents in a porcine coronary model. Cardiovasc Res 2004; 63: 617-624.
8. Byrne RA, Sarafoff N, Kastrati A, Schomig A. Drug-eluting stents in percutaneous coronary intervention: a benefit-risk assessment. Drug Saf 2009; 32: 749-770.
9. Yang SS, Tang L, Ge GG, Li RG, Qu XK, Fang WY, Ma JG, Qiao ZY, Hou YM, Cao H, Liu HJ, Zhang L, Hao ZM, Weng H. Efficacy of drug-eluting stent for chronic total coronary occlusions at different follow-up duration: a systematic review and meta-analysis. Eur Rev Med Pharmacol Sci 2015; 19: 1101-1116.
10. Aoki J, Serruys PW, van Beusekom H, Ong AT, McFadden EP, Sianos G, van der Giessen WJ, Regar E, de Feyter PJ, Davis HR, Rowland S, Kutryk MJ. Endothelial progenitor cell capture by stents coated with antibody against CD34: the HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First In Man) Registry. J Am Coll Cardiol 2005; 45: 1574-1579.
11. Lahtinen M, Blomberg P, Baliulis G, Carlsson F, Khamis H, Zemgulis V. In vivo h-VEGF165 gene transfer improves early endothelialisation and patency in synthetic vascular grafts. Eur J Cardiothorac Surg 2007; 31: 383-390.
12. Swanson N, Hogrefe K, Javed Q, Malik N, Gershlick AH. Vascular endothelial growth factor (VEGF)-eluting stents: in vivo effects on thrombosis, endothelialization and intimal hyperplasia. J Invasive Cardiol 2003; 15: 688-692.
13. Song CL, Li Q, Yu YP, Wang G, Wang JP, Lu Y, Zhang JC, Diao HY, Liu JG, Liu YH, Liu J, Li Y, Cai D, Liu B. Study of novel coating strategy for coronary stents: simutaneous coating of VEGF and anti-CD34 antibody. Rev Bras Cir Cardiovasc 2015; 30: 159-163.
14. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000; 95: 3106-3112.
15. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000; 95: 952-958.
16. Valgimigli M, Airoldi F, Zimarino M. Stent choice in primary percutaneous coronary intervention: drug-eluting stents or bare metal stents? J Cardiovasc Med (Hagerstown) 2009; 10 Suppl 1: S17-26.
17. Krylov AL. [Thrombosis of stents with an antiproliferative coating. Review of the literature]. Angiol Sosud Khir 2009; 15: 59-66.
18. Stefanini GG, Holmes DR, Jr. Drug-eluting coronary-artery stents. N Engl J Med 2013; 368: 254-265.
19. Hibbert B, Ma X, Pourdjabbar A, Holm E, Rayner K, Chen YX, Sun J, Filion L, O'Brien ER. Inhibition of endothelial progenitor cell glycogen synthase kinase-3beta results in attenuated neointima formation and enhanced re-endothelialization after arterial injury. Cardiovasc Res 2009; 83: 16-23.
20. Gupta S, Gupta MM. Stent thrombosis. J Assoc Physicians India 2008; 56: 969-979.
21. Inoue T, Node K. Molecular basis of restenosis and novel issues of drug-eluting stents. Circ J 2009; 73: 615-621.
22. Pereira-da-Silva T, Bernardes L, Cacela D, Fiarresga A, Sousa L, Patricio L, Cruz Ferreira R. Safety and effectiveness of the genous endothelial progenitor cell-capture stent: follow-up to 5 years. J Invasive Cardiol 2013; 25: 666-669.
23. Pereira-da-Silva T, Bernardes L, Cacela D, Fiarresga A, Sousa L, Patricio L, Ferreira RC. Safety and effectiveness of the Genous endothelial progenitor cell-capture stent in the first year following ST-elevation acute myocardial infarction: A single center experience and review of the literature. Cardiovasc Revasc Med 2013; 14: 338-342.
24. Sethi R, Lee CH. Endothelial progenitor cell capture stent: safety and effectiveness. J Interv Cardiol 2012; 25: 493-500.
25. Swanson N, Hogrefe K, Javed Q, Gershlick AH. In vitro evaluation of vascular endothelial growth factor (VEGF)-eluting stents. Int J Cardiol 2003; 92: 247-251.
26. Ribeiro F, Ribeiro IP, Alves AJ, do Ceu Monteiro M, Oliveira NL, Oliveira J, Amado F, Remiao F, Duarte JA. Effects of exercise training on endothelial progenitor cells in cardiovascular disease: a systematic review. Am J Phys Med Rehabil 2013; 92: 1020-1030.
27. Van der Heiden K, Gijsen FJ, Narracott A, Hsiao S, Halliday I, Gunn J, Wentzel JJ, Evans PC. The effects of stenting on shear stress: relevance to endothelial injury and repair. Cardiovasc Res 2013; 99: 269-275.
28. Werner N, Junk S, Laufs U, Link A, Walenta K, Bohm M, Nickenig G. Intravenous transfusion of endothelial progenitor cells reduces neointima formation after vascular injury. Circ Res 2003; 93: e17-24.
29. Kong D, Melo LG, Mangi AA, Zhang L, Lopez-Ilasaca M, Perrella MA, Liew CC, Pratt RE, Dzau VJ. Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells. Circulation 2004; 109: 1769-1775.