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Liposome-based vascular endothelial growth factor-165 transfection with skeletal myoblast for treatment of ischaemic limb disease

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Abstract

The study aims to use cholesterol (Chol) + DOTAP liposome (CD liposome) based human vascular endothelial growth factor-165 ($VEGF_{165}$) gene transfer into skeletal myoblasts (SkMs) for treatment of acute hind limb ischaemia in a rabbit model. The feasibility and efficacy of CD liposome mediated gene transfer with rabbit SkMs were characterized using plasmid carrying enhanced green fluorescent protein (pEGFP) and assessed by flow cytometry. After optimization, SkMs were transfected with CD lipoplexes carrying plasmid-$VEGF_{165}$ (CD-p$VEGF_{165}$) and transplanted into rabbit ischaemic limb. Animals were randomized to receive intramuscular injection of Medium199 (M199; group 1), non-transfected SkM (group 2) or CD-p$VEGF_{165}$ transfected SkM (group 3). Flow cytometry revealed that up to 16% rabbit SkMs were successfully transfected with pEGFP. Based on the optimized transfection condition, transfected rabbit SkM expressed $VEGF_{165}$ up to day 18 with peak at day 2. SkMs were observed in all cell-transplanted groups, as visualized with 6-diamidino-2-phenylindole and bromodeoxyuridine. Angiographic blood vessel score revealed increased collateral vessel development in group 3 (39.7 $\pm$ 2.0) compared with group 2 (21.6 $\pm$ 1.1%, $P < 0.001$) and group 1 (16.9 $\pm$ 1.1%, $P < 0.001$). Immunostaining for CD31 showed significantly increased capillary density in group 3 (14.88 $\pm$ 0.9) compared with group 2 (8.5 $\pm$ 0.49, $P < 0.001$) and group 1 (5.69 $\pm$ 0.3, $P < 0.001$). Improved blood flow (ml/min./g) was achieved in animal group 3 (0.173 $\pm$ 0.04) as compared with animal group 2 (0.122 $\pm$ 0.016; $P = 0.047$) and group 1 (0.062 $\pm$ 0.012; $P < 0.001$). In conclusion, CD liposome mediated $VEGF_{165}$ gene transfer with SkMs effectively induced neovascularization in the ischaemic hind limb and may serve as a safe and new therapeutic modality for the repair of acute ischaemic limb disease.

Keywords: liposome • $VEGF_{165}$ • skeletal myoblast • ischaemic limb disease

Introduction

Ischaemic limb disease develops in about 500 to 1000 people per million population per year [1]. The common treatments of ischaemic limb disease include pharmacotherapy, percutaneous transluminal angioplasty and vascular surgery and are chosen depending on the severity of the symptoms and the arteries involved [2]. However, as many as 50% of patients with limb ischaemia will undergo limb amputation within 1 year because of an insufficient response to the treatments [2, 3].

New therapeutic strategies involving molecular and cellular approaches have been put into practice with the intent to restore peripheral blood flow by stimulating growth and development of

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collateral vessels [4–7]. The delivery of various angiogenic growth factors has been focussed to produce therapeutic vascularization and achieve biological bypass to the occluded blood vessel [4, 5, 8, 9]. The most commonly used approach is direct injection of naked DNA, non-viral vector coupled DNA or viral vector transgene delivery encoding for the angiogenic growth factor of interest [4, 8–13].

The use of naked DNA is limited by low-level transfection as well as expression efficiencies. Although viral vectors demonstrate higher transduction efficiency of therapeutic genes [4, 8–9], their clinical application has been delimited by multiple undesired effects such as immunogenicity and oncogenic [14]. Moreover, viral vectors cause inflammatory reactions, formation of anti-adenoviral antibodies, transient fever and increase of liver enzymes [15–17].

The use of non-viral vectors is safer and improve cellular uptake of transgene without viral vector related concerns [12, 13, 18]. We have already reported the efficacy of polyethylenimine-25 mediated vascular endothelial growth factor-165 ($VEGF_{165}$) gene transfer into human skeletal myoblasts (SkMs), which were subsequently used for the treatment of myocardial infarction [18]. Here, we report the efficacy of CD liposome mediated $VEGF_{165}$ for treatment of ischaemic limb disease using rabbit model. The study has been carried out to synthesize CD liposomes and optimise the transfection of $VEGF_{165}$ gene into rabbit SkM with minimum cytotoxic effects. The genetically modified cells were used for treatment of rabbit ischaemic limb. We anticipate that non-viral vector-based cell-mediated $VEGF_{165}$ delivery approach will concomitantly achieve angiogenesis and myogenesis.

Materials and methods

Preparation of liposome and lipoplexes with plasmid carrying enhanced green fluorescent protein (pEGFP)

The CD liposomes were prepared by mixing Chol (Sigma, St. Louis, MO, USA) and DOTAP (Roche, Mannheim, Germany) with ratio at 1: 4 (weight/weight: W/W) [12]. The CD liposomes complex with DNA (lipoplex) was prepared as described earlier [12]. Briefly, CD liposomes and plasmid DNA were diluted in 30 and 70 μl sample buffer (20 mM HEPES in 150 mM NaCl, pH7.4) separately. The CD lipoplexes were developed based on the ratio (W/W) of CD liposome: pEGFP (4.7 Kb, Clontech Lab, Inc., Mountain View, CA, USA) by mixing the respective solutions containing CD liposome and plasmid DNA. After mixing, the mixture was incubated for 30 min. at 37°C. The CD lipoplex mixture was added into cell culture medium (10% M199) for transfection with SkMs for 24 hrs at 37°C in incubator.

Characterization of CD lipoplexes

The lipoplexes were characterized *in vitro* [12]. The particle size and the residual surface charge (ζ-potential) of CD lipoplexes were determined with a Zetasizer Nano-ZS (Malvern Instruments, Malvern, Worcestershire, UK) equipped with a 4 mW, 633 nm Ne–He laser at 25°C and a fixed scattering angle of 90°. The physical shape was imaged by scanning electron microscope. The lipoplex resistance to nuclease digestion was determined.

Rabbit SkM isolation and culture

Rabbit SkMs were isolated from the hind limbs (gracilis and semi-membranosus muscles) of adult New Zealand White female rabbits [4]. Briefly, the site of skeletal muscle biopsy was stimulated with intramuscular injection of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml) to stimulate the proliferation of SkMs with the animal under anaesthesia. Three days later, 2 g muscle biopsy specimen was excised and processed immediately under sterile tissue culture conditions to isolate SkM. The biopsy site was sutured and the animal was allowed to recover. The biopsy sample was minced into a coarse slurry followed by enzymic digestion using 0.2% collagenase, type XI (C-9407; Sigma), for 90 min. at 37°C, 2.4 units/ml of dispase (Gibco-BRL, Invitrogen, Carlsbad, CA, USA) for 45 min. at 37°C and finally, 0.1% trypsin (Sigma) for 15 min. at 37°C. The muscle cell extract was pre-plated four times at regular time intervals to remove the debris and the contaminating cell population. Cells were re-suspended in M199 supplemented with 20% foetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 0.1 mg/ml of L-glutamine. The cells were repeatedly passaged every 2–3 days to prevent their differentiation into myotubes *in vitro*. The purity of the isolated cells was determined by immunostaining for desmin expression and further confirmed by FACS analysis using Coulter flow cytometer (Epics Elite Esp, Brea, CA, USA) as described [4]. Non-stained SkMs were used for baseline setting of auto-fluorescence. Data were analysed using WinMDI version 2.8 with gating at 1%.

Optimization of SkMs transfection with CD lipoplexes

The highest transfection efficiency was achieved when the ratio between Chol and DOTAP (W/W) was at 1:4 [12]. The ratios (W/W) between CD liposome and pEGFP (2 μg) was first increased from 6:1 to 15:1. Next, the amount of pEGFP DNA was increased from 2 to 5 μg using the optimal ratio between CD liposome and pEGFP. The CD lipoplexes were developed as described above and added into culture medium for transfection with trypsinized SkM for 24 hrs at 37°C in incubator.

Transfection efficiency and cell viability

Transfection efficiency and cell viability of SkMs were analysed by flow cytometry using Coulter flow cytometer (Epics Elite Esp). The cells with an adequate size and granularity were accounted for in the statistical analysis [12, 18]. Non-transfected and CD liposome transfected SkMs were used as baseline settings of auto-fluorescence limit. Data were analysed using WinMDI version 2.8 (Scripps Research Institute, La Jolla, CA, USA) with gating at 1%.

Characterization of SkMs with CD-p$VEGF_{165}$

A plasmid (4.75 Kb) containing human $VEGF_{165}$ (p$VEGF_{165}$) was used [12, 18]. Transfection of SkMs was carried out with CD lipoplexes carrying

$pVEGF_{165}$ using the optimized transfection conditions based on flow cytometry results. The $VEGF_{165}$ gene expression efficiency was analysed by immunostaining, quantitative RT-PCR (QRT-PCR) and ELISA as described [12, 18].

Cell labelling

Before cell transplantation, SkMs were incubated in 10% M199 containing 2 μg/10 ml 4, 6-diamidino-2-phenylindole (DAPI) and 1:500 diluted bromodeoxyuridine (BrdU) labelling reagent from BrdU Labeling and Detection Kit (Roche) for 24 hrs at 37°C in 5% CO_2 incubator. After thorough washing with PBS, cells were resuspended in M199 and prepared for cell transplantation. The detection of BrdU was performed with the same kit as per instruction.

Experimental animals

All animals were maintained by Animal Holding Unit of National University of Singapore (NUS). All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC), NUS.

Hind limb ischaemia was produced in 47 New Zealand White rabbits (2.5–3 kg body weight) by permanent ligation of femoral artery [4]. Briefly, the superficial and deep femoral arteries and internal iliac artery of the right hind limb were exposed and ligated from the inguinal ligament through a longitudinal incision to a point just proximal to the patella with the animal under anaesthesia. Multiple ligatures of the artery beginning from the inguinal ligament down to the point where it bifurcates into the popliteal and saphenous arteries were closed with 4–0 polypropylene (Prolene) sutures [4]. Wounds were closed, and the animals were allowed to recover. Complete occlusion of the ligated blood vessel was confirmed by angiography.

The animals were randomized into three groups: basal M199 without cells injected group 1: $n = 17$ (six animals died between 2 and 6 weeks after M199 injection and one animal died during second angiogram, mortality = 41.2%); non-transfected SkM transplanted group 2: $n = 13$ (two died at 4 and 6 weeks after SkM transplantation and one died during second angiogram, mortality = 23.1%); CD-$pVEGF_{165}$ transfected SkM transplanted group 3: $n = 11$ (one died at 4 weeks after CD-VEGF-SkM transplantation, the rest survived and used in the study, mortality = 9%). Another six rabbits ($n = 2$ each group) were harvested at 1 week after cell transplantation and used for immunohistochemical studies.

SkMs transplantation

Ten days after development of the animal model, 1.5 ml of basal M199 containing 3×10^7 allogenic SkMs were injected into rabbit ischaemic hind limb: non-transfected SkMs in group 2, and CD-$pVEGF_{165}$ transfected SkMs in group 3. A total of 20 sites were injected intramuscularly into the centre and periphery of femoral artery ligation points. The injected site of the muscle was marked with 4–0 polypropylene sutures. All animals were maintained with 5 mg/kg cyclosporine from 3 days before and until 6 weeks after treatment. Animals also received 40 mg/kg Cefazolin for 5 days after operation to prevent any possible infection.

Histochemical and immunohistochemical studies

The explanted tissue from cell injection sites was sectioned into 5-mm cross-sectional pieces. Cryo-sections of 6–8-μm thickness were cut and observed for DAPI fluorescence with a fluorescent microscope (Olympus, Tokyo, Japan) and confirmed by BrdU. The $DAPI^+$ tissue sections from the cell-transplanted tissues were used for staining of skeletal myosin heavy chain as described [18]. Skeletal muscle samples at 1 week were used to detect VEGF expression from the transplanted cells [18].

Blood vessel density quantification

Dual fluorescent immunostaining for CD31 (goat anti-CD31, Santa Cruz Biotech, Santa Cruz, CA, USA) and smooth muscle actin (SMA) (mouse anti-SMA, Sigma) was carried out for blood vessel quantification at the site of the graft at 6 weeks after treatment [18]. For each tissue section, the number of blood vessels was counted from 4 randomly selected microscopic fields at high power magnification (400×). A total of eight randomly selected tissue sections from six animals per group were used to measure the final average blood vessel density.

Regional blood flow study

Rabbit harvested at 6 weeks ($n = 6$ animal each group) after treatment received fluorescent microsphere injection to assess regional blood flow in the ischaemic limb [18, 19]. The animals were anaesthetized and maintained using isoflurane (0.2%) and 100% oxygen (2 l/min.). Rabbit heart was exposed by a limited left side thoracotomy between the fourth and fifth intercostal spaces. At the same time, left femoral artery was isolated and prepared for blood sampling. One-millilitre solution containing 1×10^5 Fluospheres® yellow-green polystyrene microspheres (Molecular Probes, Invitrogen, Carlsbad, CA, USA) was directly injected into left ventricle while arterial reference blood samples were obtained from at a constant rate of 5 ml/min. The animals were killed and tissue samples from sites of injection and reference blood samples were processed to extract the fluorescent dye. The fluorescent intensity in each sample was measured using a Perkin Elmer LS-50B spectrophotometer at excitation and emission wavelengths according to the manufacturer's instructions. Regional blood flow (Q) was calculated as $Q = (fl/fl_{ref}) \times R$, where fl and fl_{ref} are the fluorescence of the tissue sample and the reference blood sample, respectively, and R is the withdrawal speed of the arterial reference sample (ml/min.).

Angiographic assessment

The animals underwent angiography at 10 days and 6 weeks after treatment ($n = 7$ each group). After anaesthesia, the left femoral artery was isolated. A 22-gauge intravenous catheter was placed into the artery. The catheter was used to infuse 10 ml of iodinated contrast medium as a bolus (Iomeprol, IOMERON 350; Bracco, Leatherhead, Surrey, UK). Serial filming of the right hind limb was performed with an Advantex angiography machine (GE Pacific Ltd., Singapore). After this the catheter was removed, the punctured artery was closed, and the animal was allowed to recover. Angiographic score of the right thigh at femoral artery ligation was quantified at 4-sec. post-injection film with direct counting of the number of arteries crossing 15 lines drawn vertically across the mid-thigh [4, 20].

Vessel numbers from different animals within each group were summed and averaged to obtain the angiographic score for that group.

Statistical analysis

All data are expressed as mean ± S.E.M. All statistical analyses were performed with SPSS (version 10.0). The difference between groups was analysed by the method of ANOVA using Bonferroni test. The difference of mortality among three groups was determined by Mantel–Haenszel chi-square test. All tests were performed with a significance level of 5%.

Results

Characterization of lipoplexes

$VEGF_{165}$ DNA lipoplexes were micro-spheres with average diameter between 50 and 120 nm when the ratio of CD: DNA at 9:1 (Fig. 1A). The average particle size and ζ-potential of lipoplexes showed concentration dependence: larger particle size and higher ζ-potential at higher CD:DNA ratio (Fig. 1B and C). CD liposome protected the encapsulated DNA from degradation for more than 120 min., while the naked DNA was fully degraded after 30 min. by DNase-I (Fig. 1D).

Optimization of CD-pEGFP transfection with SkMs

SkMs were about 80% pure for desmin expression (Fig. 2A–C). This was further confirmed by FACS for desmin expression (Fig. 2D and E). The Chol: DOTAP ratio was fixed at 1:4 and ratio of CD: pEGFP (W/W) was adjusted from 6:1 to 15:1 on trypsinized SkMs (Fig. 2F). It was at 9:1 of CD: pEGFP that the highest transfection efficiency was achieved (efficiency = 13.83 ± 3.68%; viability = 87.23 ± 8.86%) when 2 μg pEGFP was used. Furthermore, pEGFP was increased from 2 to 5 μg on trypsinized hSkM with CD: pEGFP at 9:1 which further increased transfection efficiency to 16.0 ± 3.83% with cell viability at 84.5 ± 1.46%) (Fig. 2G). Thus, the optimal transfection conditions using CD liposome were CD: DNA ratio of 9:1 with 3 μg plasmid DNA/1×10^5 on trypsinized SkMs and were used throughout the study.

Characterization of CD-$pVEGF_{165}$ transfected SkM

Immunostaining revealed $VEGF_{165}$ expressing SkM *in vitro* (Fig. 3A). QRT-PCR results showed that the gene expression of $VEGF_{165}$ from CD-$hVEFG_{165}$ transfected SkM increased 8.24 ± 0.13 times at day 2, 6.31 ± 0.58 times at day 4, 4.03 ± 0.66 times at day 8 and 1.54 ± 0.2 times at day 18 (Fig. 3B). ELISA showed that the CD-$hVEFG_{165}$ transfected SkMs secreted $VEGF_{165}$ up to 18 days of observation (0.91 ± 0.09 ng/ml) with peak level expression at day 2 after transfection (14.5 ± 0.25 ng/ml) (Fig. 3C).

Fate of the transplanted SkMs

The labelling efficiency for DAPI was almost 100%, while it was 50% for BrdU (Fig. 4A and B). After transplantation into rabbit ischaemic hind limb, extensive survival of allogenic rabbit SkMs shown as $DAPI^+$ or $BrdU^+$ nuclei were observed at the site of the graft (Fig. 4C–F) at 6 weeks after cell transplantation. Fluorescence immunostaining for skeletal myosin heavy chain expression demonstrated that some of the DAPI+ donor SkMs nuclei integrated into host muscle fibres to form hydrid muscle fibres at 6 weeks (Fig. 4G–I). The transplanted CD-$pVEGF_{165}$ transfected SkMs expressed $VEGF_{165}$ for at least 1 week *in vivo* (Fig. 4J–L).

Evidence for angiogenesis

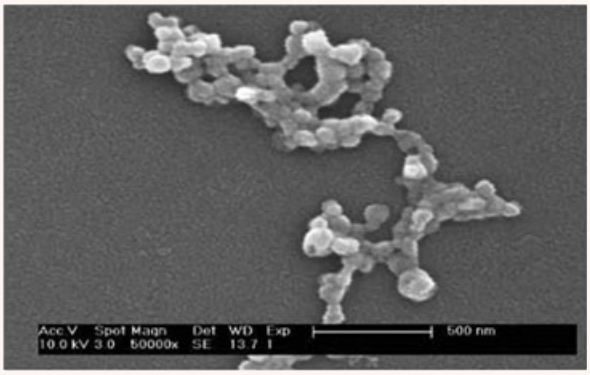
Blood vessel density based on PECAM-1 immunostaining (at 400× magnification) was highest in group 3 (15.04 ± 0.77) as compared with group 1 (5.71 ± 0.15; $P < 0.001$) and group 2 (8.5 ± 0.18; $P < 0.001$) at 6 weeks after treatment (Fig. 5A, D, G and J). The blood vessel density based on SMA immunostaining was also highest in group 3 (10.92 ± 0.37) as compared with group 1 (3.96 ± 0.12; $P < 0.001$) and group 2 (6.04 ± 0.2; $P < 0.001$) (Fig. 5B, E, H and K). Dual immunostaining for PECAM-1 and SMA showed that percentage of the mature blood vessels in group 3 was 72.93 ± 1.5%, which was similar to those of group 1 (69.2 ± 2.2; $P = 0.58$) and group 2 (71.13 ± 2.1; $P = 1$) (Fig. 5C, F, I and L).

Regional blood flow

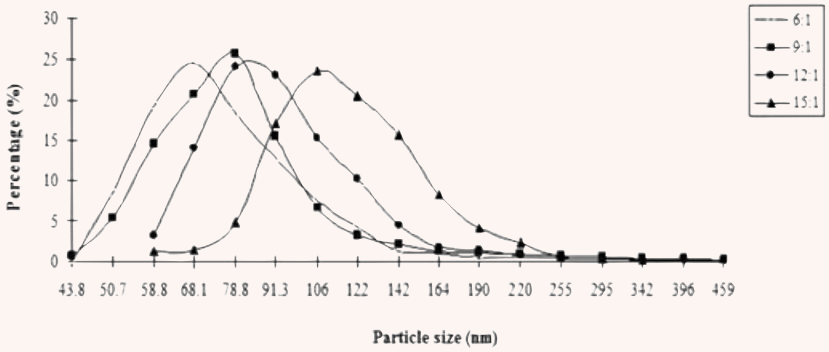
The blood flow (ml/min./g) of rabbit ischaemic limb was significantly reduced in group 1 (0.06 ± 0.012) as compared with group 2 (0.12 ± 0.016; $P = 0.016$) and group 3 (0.17 ± 0.011; $P < 0.001$) (Fig. 5M). Group 3 receiving CD-pVEGF-SkMs transplantation had better-improved perfusion than that of group 2 ($P < 0.001$).

Angiographic assessment

Ten days after femoral artery ligation, angiographic assessment revealed occlusion of the ligated blood vessels (Fig. 6A–F). By 6 weeks after treatment, significantly increased angiographic score was observed in group 3 (39.7 ± 2.0) compared with group 2 (21.6 ± 1.1; $P < 0.001$) and group 1 (16.9 ± 1.1; $P < 0.001$) (Fig. 6G).



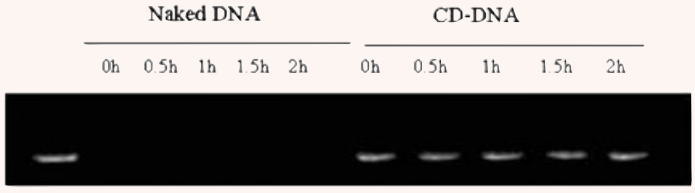
A



B

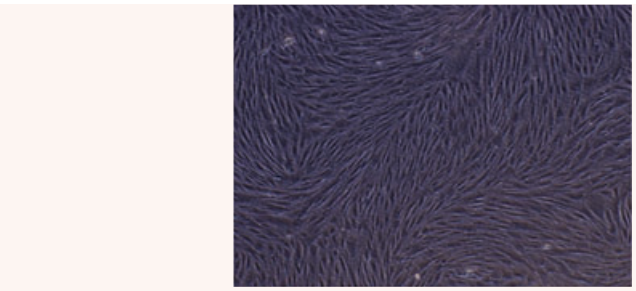
CD: DNA ratio	6:1	9:1	12:1	15:1
Particle size (nm)	82.79 ± 0.26	87.46 ± 7.74	98.21 ± 0.4	125.63 ± 6.0
Zeta potential (mV)	41.5 ± 0.45	44.28 ± 1.93	46.05 ± 1.33	49.15 ± 1.95

C



D

Fig 1. (**A**) Scanning electron micrographs showing CD-DNA lipoplex particle size between 50 and 120 nm when lipoplexes were formed in HEPES buffer with CD:DNA ratio at 9:1 using 3 μg DNA (bar = 500 nm). (**B**) Size distribution of CD-DNA lipoplexes at various CD:DNA ratios: 6:1, 9:1, 12:1 and 15:1 using 3 μg DNA. (**C**) Average size and ζ-potential of CD-DNA lipoplex increased from 6:1 to 15:1 using 3 μg DNA. (**D**) CD liposome encapsulated DNA showed stability against DNase-I for up to 2 hrs as compared with the non-complexed DNA.



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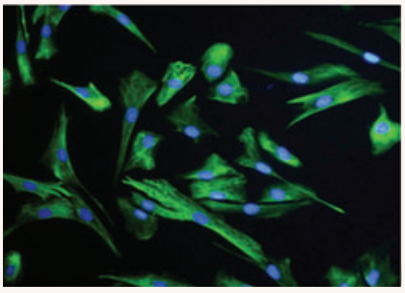

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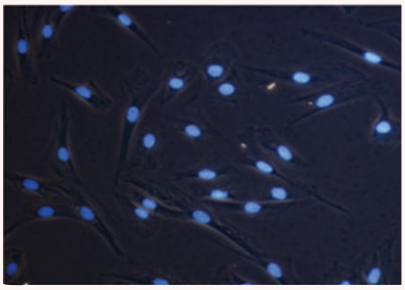

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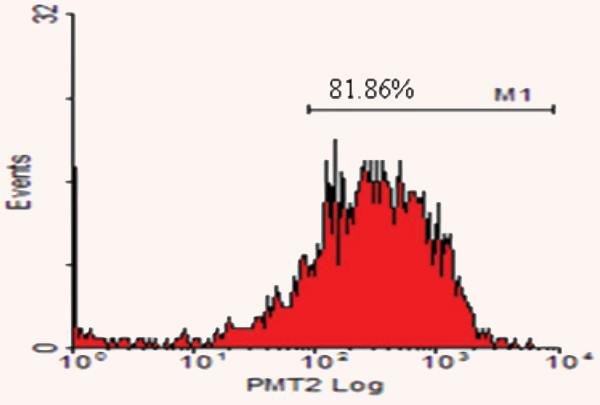

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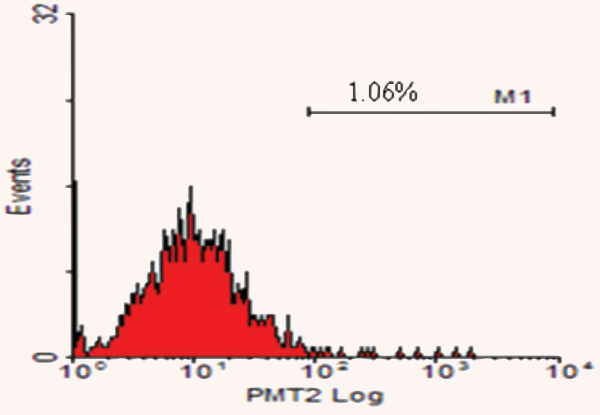

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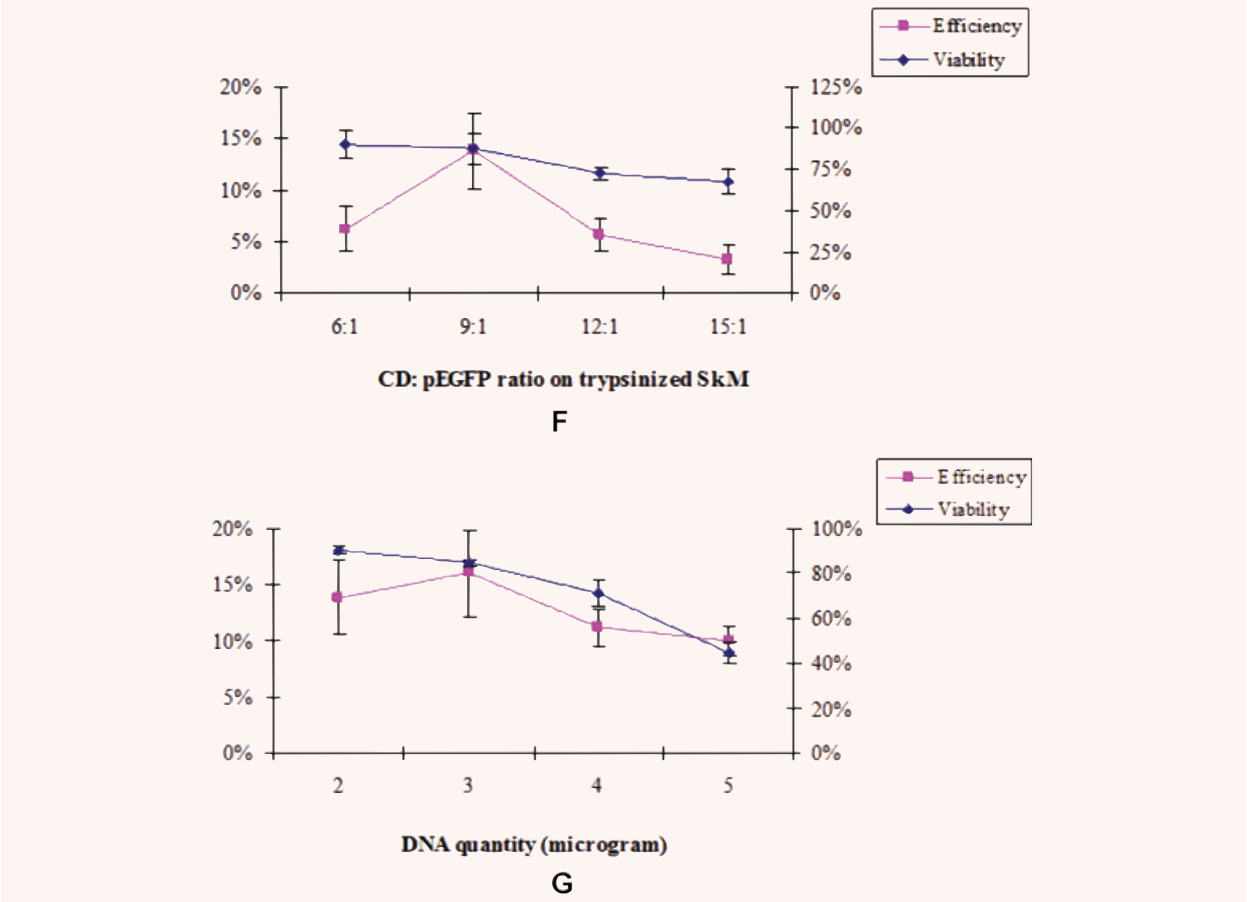


Fig. 2 (**A**) Phase contrast photomicrograph of rabbit SkM. (**B**) Immunostaining of rabbit SkM for desmin expression. (**C**) Phase contrast photomicrograph of the picture B. (Magnification: A = 40×, B and C = 200×). FACS analysis of isolated rabbit cells for desmin expression. (**D**) 80% of these cells were positive for desmin expression using (**E**) non-stained rabbit cells as a control for auto-fluorescence. Optimization of CD-pEGFP transfection with rabbit SkM. (**F**) Gene transfection efficiency and viability of SkM when CD:pEGFP increased from 6:1 to 15:1 with Chol: DOTAP ratio at 1:4 using 2 μg pEGFP. (**G**) Gene transfection efficiency and cell viability on trypsinized SkMs when pEGFP was increased from 2 to 5 μg with CD:pEGFP ratio at 9:1.

Clinical Score and rabbit mortality

Four rabbits in group 1 had limb shrinkage and died between 2 and 6 weeks, while two rabbits in group 2 had limb shrinkage and died at 4–6 weeks after treatment. However, none of the rabbit in group 3 had limb shrinkage. Though no significant difference of mortality after treatment ($P = 0.0602$) among three groups after treatment, SkMs and CD-pVEGF-SkMs transplantation have a tendency to reduce mortality in this rabbit model of acute ischaemic limb disease.

Discussion

This study demonstrates the feasibility of SkM transfected with CD-pVEGF$_{165}$ lipoplexes as an alternative treatment option for acute ischaemic limb disease. Using nanosized lipoplex technology, gene transfection efficiency was reached up to 16%, which is significant keeping in mind the notoriety of non-viral vectors for poor transfection efficiency. Transplantation of CD-pVEGF$_{165}$ transfected SkMs increased neovascularization, improved regional blood flow and angiographic score in rabbit model of ischaemic limb disease.

One of the obstacles to *ex vivo* non-viral vector-mediated gene transfer of primary SkM is that the transfection efficiency is typically very low for non-viral vectors. One of the main impediments to the efficiency of liposome-mediated gene transfection is liposome composition [21, 22]. In our previous study, we found that combination of Chol with DOTAP provided better transfection efficiency as compared with DOTAP as alone [12]. Thus, we chose to combine Chol with DOTAP as composition of the liposome and optimized the transfection procedure.

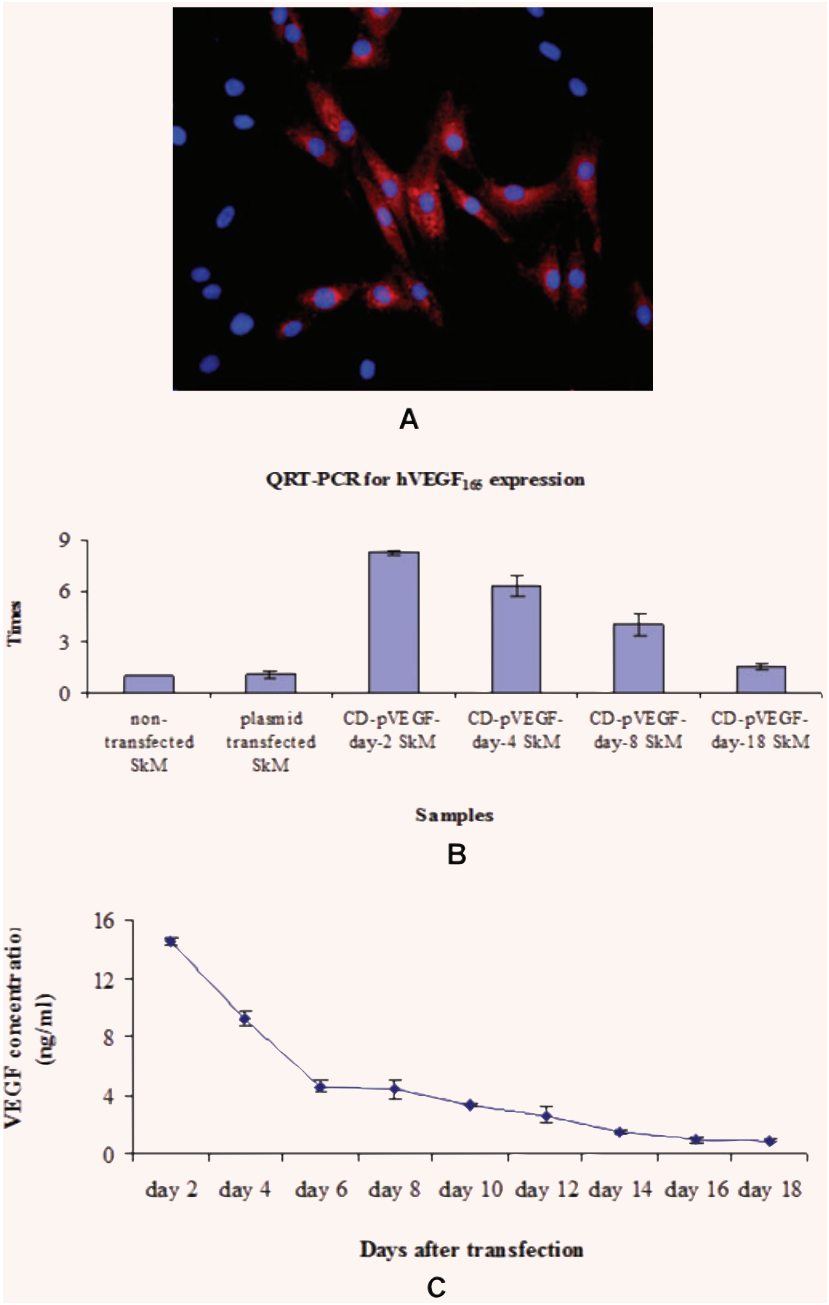
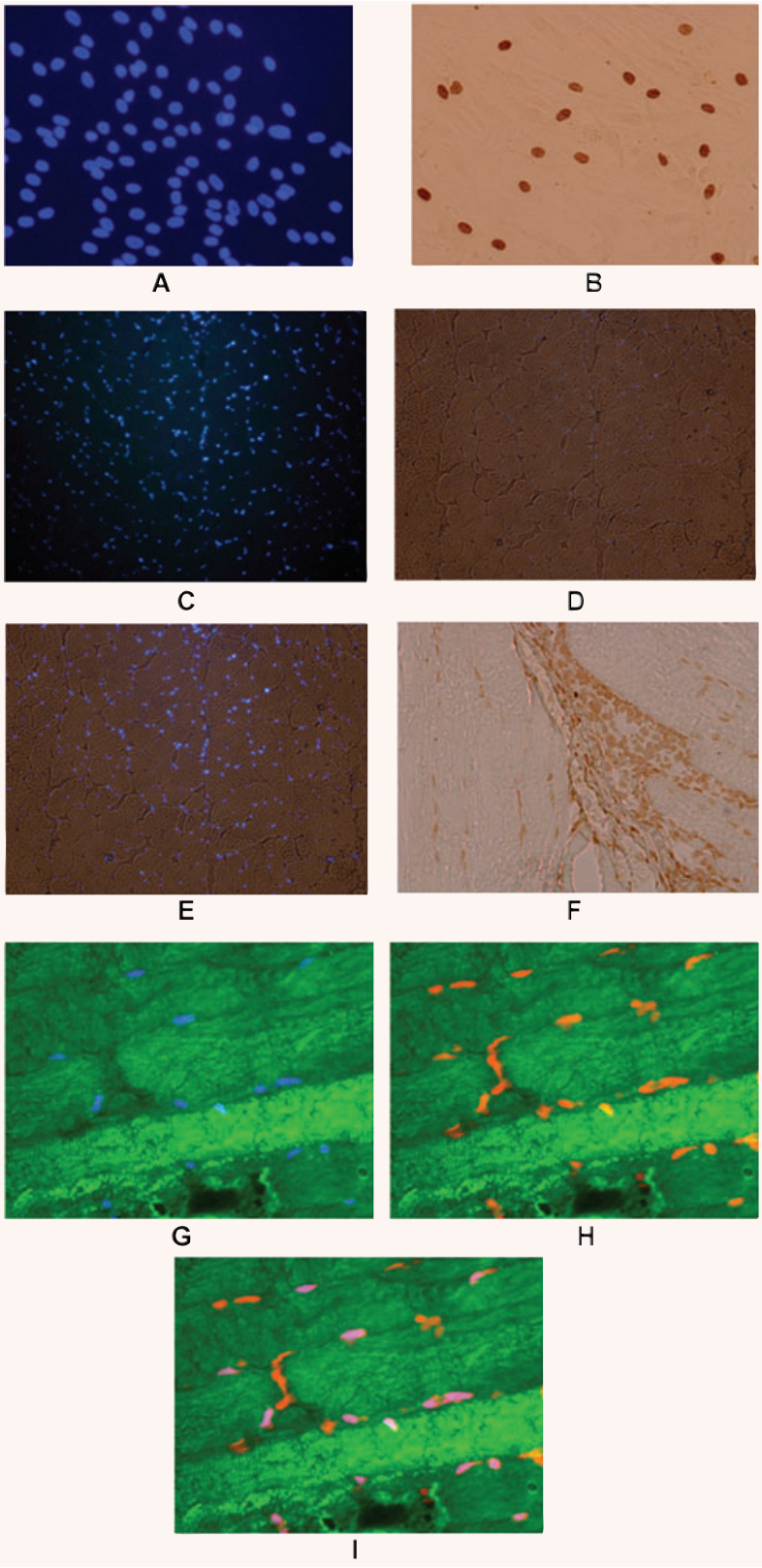


Fig. 3 (**A**) $VEGF_{165}$ expression from CD-$pVEGF_{165}$ transfected SkMs. (VEGF = red fluorescence, DAPI = blue fluorescence) (magnification A = 200×). (**B**) QRT-PCR of CD-pVEGF transfected SkMs at 2, 4, 8 and 18 days after transfection. (**C**) $VEGF_{165}$ protein secretion from CD-pVEGF transfected SkMs as a function of time.

The lipid size is influenced by lipid DNA ratio, and the amount of plasmid DNA [22–24]. Smaller lipoplexes resulted in poorer transfection efficiency as compared with larger particle. Previous studies have shown size-dependent internalization of particles during transfection procedure [23]. Microspheres with a diameter of <200 nm are taken up predominantly *via* clathrin-mediated endocytosis and are processed along this pathway to the lysosomal compartment. In contrast, larger particles (500 nm) are internalized almost exclusively *via* the caveolae-dependent route and are never seen in the lysosomal compartment. The average lipoplex size was between 82.79 and 125.63 nm in the present study. Thus, most of the CD lipoplexes entered into cytoplasm of SkM through clathrin-mediated endocytosis with subsequent destruction of an endosome within SkM [23]. Compared with 22 kD polyethylenimine (PEI22) and 25 kD polyethylenimine (PEI25), CD liposome however, showed lower transfection efficiency in



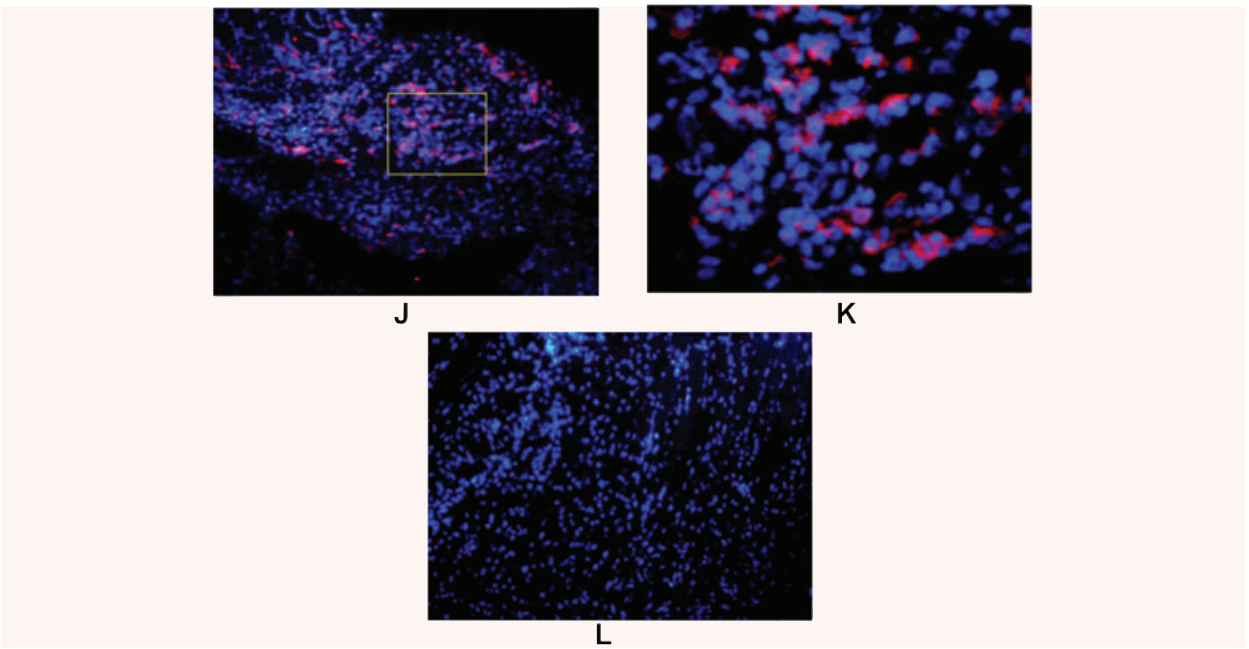


Fig. 4 (**A**) DAPI labelled rabbit SkMs (blue fluorescence). (**B**) BrdU-labelled rabbit SkMs as shown brown colour after immunochemical staining. (**C**) DAPI-labelled rabbit SkMs survived in rabbit skeletal muscle until 6 weeks after transplantation. (**D**) Picture C under light microscope to show the rabbit skeletal muscle. (**E**) Overlap of picture C and D. (**F**) BrdU labelled rabbit SkM in rabbit skeletal muscle at 6 weeks after transplantation. (**G**) $DAPI^+$ rabbit muscle tissue was immunostained for expression of skeletal myosin heavy chain expression *as green fluorescence*. (**H**) The same tissue was counter-stained with propidium iodine to show all the nuclei. (**I**) Overlap of picture G and H to simultaneously show the co-localization of donor SkM nuclei with host skeletal muscle nuclei. (**J**) $DAPI^+$ tissue was immunostained for $VEGF_{165}$ expression at 1 week after CD-p$VEGF_{165}$ transfected SkM transplantation in rabbit skeletal muscle. (**K**) The selected area of picture I was magnified to show co-localization of DAPI nuclei (blue) and $VEGF_{165}$ protein (red). (**L**) Non-transfected SkM transplanted rabbit tissue was used as negative control (magnification: A, B, J and L = 200×, C, D and E = 40×, F = 100×, G, H, I and K = 400×).

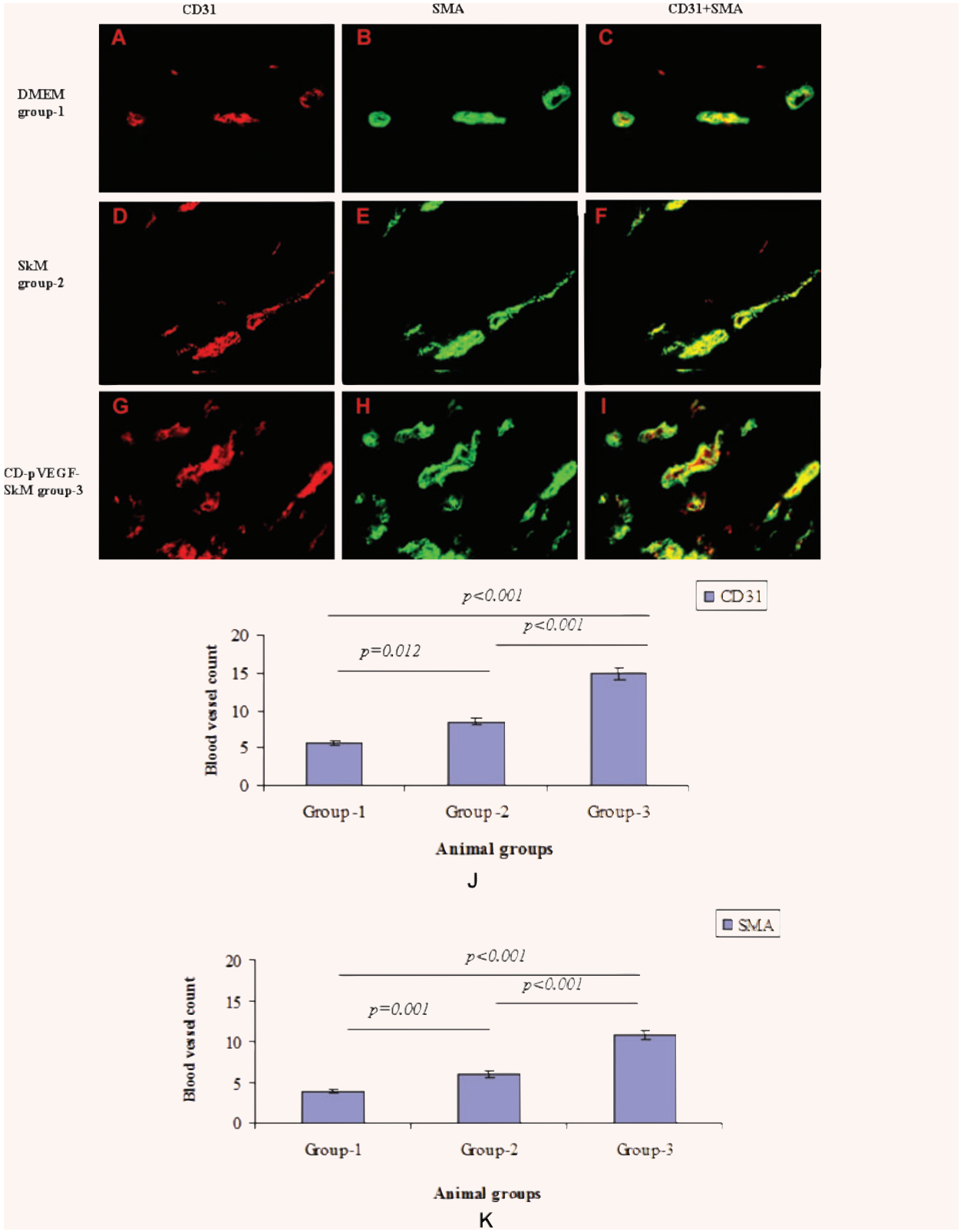
human SkMs [12]. This is due to the fact that CD lipoplexes can only translocate plasmid DNA into nuclei predominantly during the S/G2 phase of the cell cycle [25].

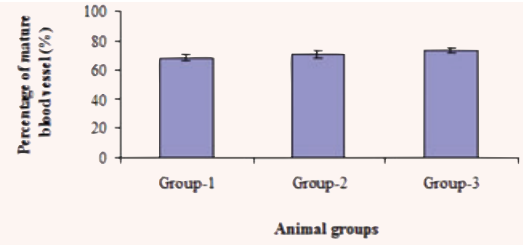
The ζ-potential, the residual charge on the particles, is preferred to be higher to achieve higher transfection efficiency. Our previous study showed that values as low as 26.17 mV are sufficient for binding complexes with the cell surface [12]. In current study, the liposomes used in the study have ζ-potential more than 41.5 mV. This suggested that ζ-potential is not a key factor in determine the gene transfection efficiency.

The level of secreted $hVEHGF_{165}$ (14.5 ng/ml) from the transfected SkMs was sufficient to initiate and maintain neovascularization. This level was lower that our previous study which used adenoviral vector for gene transduction and achieved a higher level of $VEGF_{165}$ protein expression (19.52 ± 4.12 ng/ml) [4]. In both the studies, however, efficient neovascularization was observed with improved regional blood flow and angiogenic score. Previous studies have shown that the presence of minimal VEGF is required for initiation as well as maintenance of newly formed blood vessels. A low 2.78 ± 0.2 ng/ml VEGF protein concentration has been reported to efficiently stimulate neovascularization [26].

A transient VEGF gene and protein expression mode was observed in transfected rabbit SkMs. The transfected CD-p$VEGF_{165}$ SkMs expressed $VEGF_{165}$ at least for up to 18 days *in vitro* and 1 week *in vivo*. Although adenoviral vector transduction is more efficient, use of adenoviral vector has severe problems of high immunogenicity, tumorigenic potential and induction of host inflammatory reaction limited their clinical application [14–16]. Besides, long-term expression of the VEGF may result in deleterious effects, such as haemangioma formation [27].

It has been shown that VEGF has cytoprotective effects on the ischaemic muscle [18]. VEGF expressed from CD-p$VEGF_{165}$ transfected SkMs protected ischaemic skeletal muscle in the early stage, and subsequently stimulated neovascularization to increase regional blood flow at a later stage. Thus, the lowest mortality was observed in group-3 animals as compared with the control groups. It was interesting that only SkM transplantation maintained blood vessel density in group 2. This effect may be related with the paracrine factors released from SkMs, including VEGF, hepatocyte growth factor and platelet-derived growth factor [19, 28, 29]. Compared with transplantation of $CD34^+$ mononuclear cells [30] or hydrogels containing growth factors [31], transplantation of





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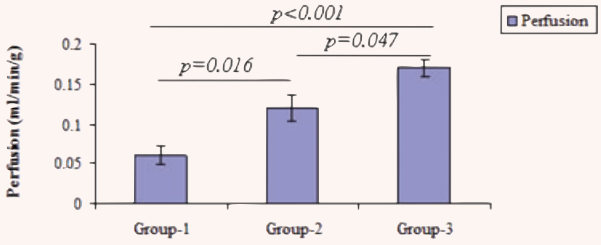

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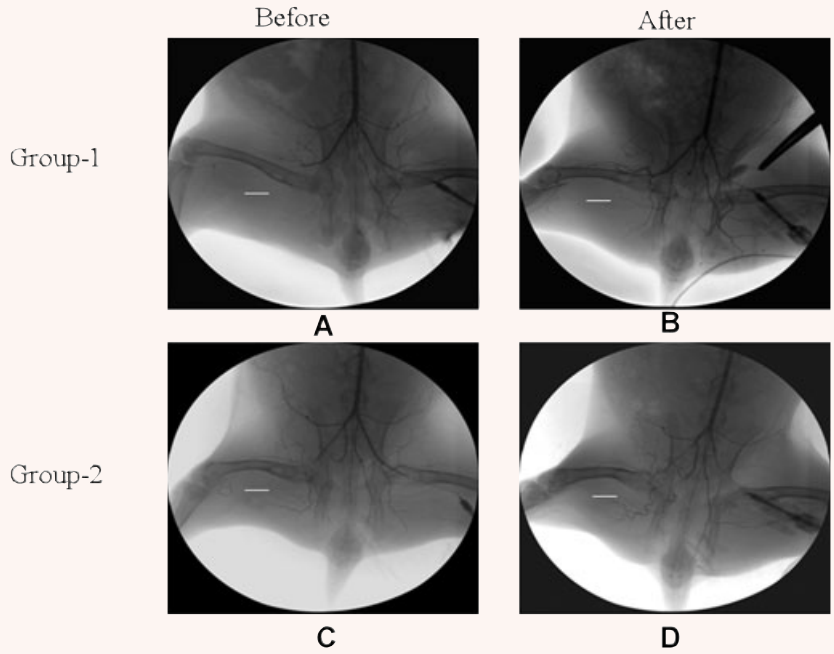
Fig. 5 Dual fluorescence immunostaining for CD31 (**A**, **D**, **G**) and SMA (**B**, **E**, **H**) at 6 weeks after treatment. Merged images of CD31 and SMA from each group **(C, F, I)** were used to assess blood vessel maturation index. (Red = CD31; green = SMA) (magnification A–I = 400×). Significantly increased blood vessel density count based on CD31 (**J**) and SMA (**K**) was observed in group 3 as compared with groups 1 and 2. (**L**) Blood vessel maturation index varied insignificantly in all animal groups. (**M**) Regional blood flow in group 3 was significantly improved as compared with groups 1 and 2 at 6 weeks after cell transplantation.



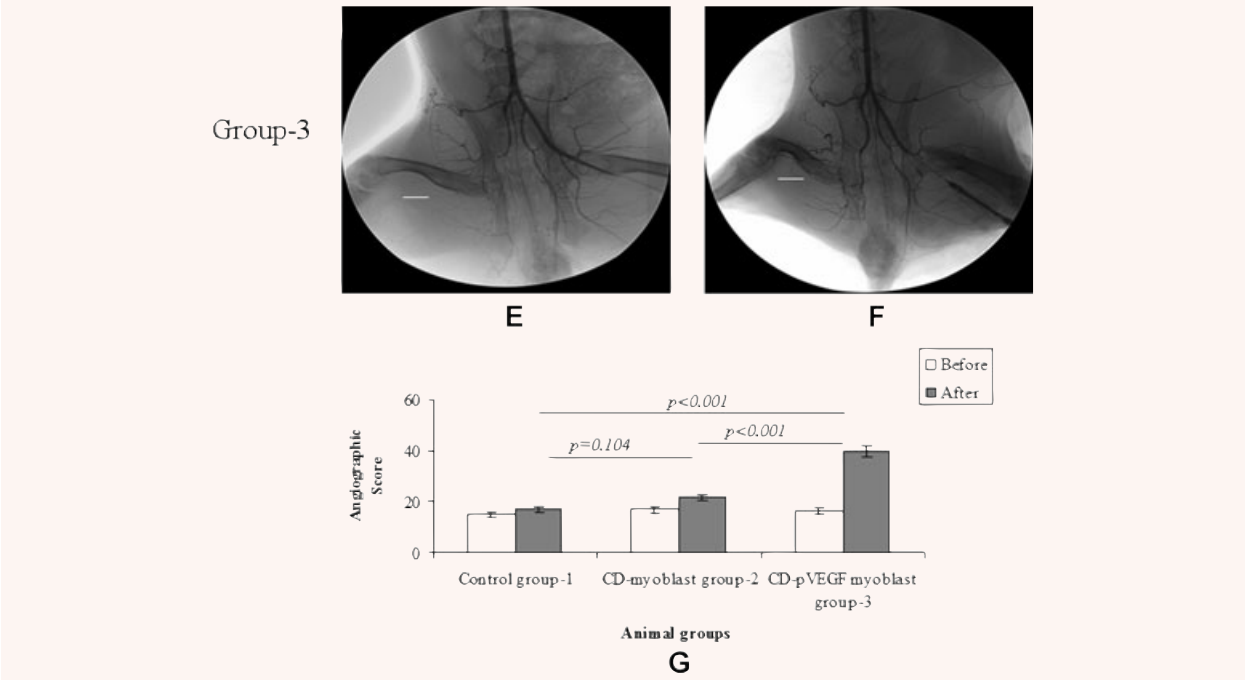


Fig. 6 Typical angiographs of rabbit hind limbs at 10 days (baseline) after ligation (**A**, **C**, **E**) and 6 weeks after respective treatment (**B**, **D**, **F**). Higher number of collateral blood vessels were seen in group-3 animals. (**G**) Angiographic score at 10 days after ligation and 6 weeks after treatment. (The white bar represents the area where M199 or rabbit SkMs injected.)

SkMs carrying angiogenic gene has one significant advantage that it can impart simultaneous angiogeneis and myogenesis. In summary, our study highlights the feasibility and efficacy of CD liposome-mediated $VEGF_{165}$ transfection with rabbit SkMs for angiomyogenesis in treatment of *acute* ischaemic limb disease.

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Conflict of interest: None.

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