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ST266 inhibits neointimal hyperplasia after arterial balloon injury in rats

Objective To examine the effect of Human Amnion-Derived Multipotent Progenitor (AMP) cells and their novel ST266 secretome on neointimal hyperplasia after arterial balloon injury in rats.

Material and Methods Sprague-Dawley male rats were randomly divided into four groups (n=7): Control (PBS) group, systemic ST266 group, systemic AMP group and local AMP implant group. Neointimal hyperplasia was induced in the iliac using a 2F Fogarty embolectomy catheter. After surgery, the rats in the ST266 group were treated with 0.1, 0.5, or 1ml ST266 iv daily. In the systemic AMP groups, a single dose (SD) of 0.5×10^6 or 1×10^6 AMP cells was injected via the inferior vena cava after arterial balloon injury. In local AMP implant groups, 1×10^6, 5×10^6, or 20×10^6 AMP cells were implanted in 300 µl Matrigel (Mtgl) around the iliac artery after balloon injury. The iliac arteries were removed for histologic analysis at 28 days after the surgery. Re-endothelialization index was measured at 10 days after balloon injury.

Results ST266 (1 ml) group had a lower level of the Neointima/Neointima+Media ratio (N/N+M) 0.3±0.1 vs 0.5±0.1, p=0.004) and luminal stenosis (LS) percentage (18.2±1.9% vs 39.2±5.8%, p=0.008) compared with the control group. Single-dose AMP (1×10^6) decreased LS compared to the control group (19.5±5.4% vs 39.2±5.8%, p=0.033). Significant reduction in N/N+M were found between implanted AMPs (20×10^6) and the control group (0.4±0.1 vs 0.5±0.1, p=0.003) and the Mtgl-only group (0.5±0.1, p=0.007). Implanted AMPs (20×10^6) decreased the LS compared with both the control (39.2±5.8%, p=0.001) and Mtgl-only group (37.5±8.6%, p=0.016). ST266 (1 ml) significantly increased the re-endothelialization index compared to the control (0.4±0.1 vs 0.1±0.1, p=0.002).

Conclusion ST266 and AMP cells reduce neointimal formation and increase the re-endothelialization index after arterial balloon injury. ST266 is potentially a novel, therapeutic agent to prevent vascular restenosis in human.

Keywords Restenosis; neointima; balloon angioplasty; amnion-derived multipotent; progenitor cells

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Introduction

Cardiovascular diseases remain the number one cause of death and disability worldwide. Vascular restenosis due to neointimal hyperplasia is the limiting entity following balloon angioplasty. It is associated with significant morbidity, mortality, and extensive cost, and, thus, vascular restenosis represents a major clinical and economical problem. Despite significant advances in revascularization techniques, restenosis after conventional balloon angioplasty occurs in 30–60% of cases. It is estimated that in 2010, ~200,000 patients experience repeated revascularization in the USA alone, due to post-angioplasty arterial restenosis [1]. Proliferation of endothelial and smooth muscular cells (SMC) and accumulation of extracellular matrix have been shown to be involved in intimal hyperplasia after vascular injury [2]. Thus, reduction of SMC proliferation and migration with pharmacological intervention should be as an effective approach for prevention of intimal hyperplasia.

Amnion-derived multipotent progenitor (AMP) cells, isolated from the full-term human placenta, are a subpopulation of amnion epithelial cells that are grown in serum-free media [3]. AMP cells display many favorable characteristics of stem cells, including the ability to differentiate into various cell types. Wound healing therapy with AMP cells has been shown to reduce the incidence of laparotomy wound failure [4]. Treatment with AMP cells was shown to significantly attenuate axonal degeneration and improve motor impairment in a model of traumatic brain injury [5].

AMP cells also have been shown to secrete many cytokines and growth factors involved in tissue regeneration and wound repair, inhibition of macrophage migration, anti-apoptotic of SMC, and tissue inhibition of metalloproteinases [6]. ST266 (Noveome Biotherapeutics, Inc, Pittsburgh, PA, USA), is a secretome derived from AMP cells. It contains physiologic levels of multiple growth factors and cytokines, and it has been shown to enhance wound healing [7], promote macrophage activity [8], and exhibit both anti-inflammatory and neuroprotective properties in the treatment of a model of penetrating ballistic brain injury [6, 9, 10]. ST266 also stimulates Schwann cell proliferation and protects neural cells from staurosporine-induced apoptosis in vitro. Individual proteins in ST266 are found at concentrations in the pg/ml to ng/ml range [6], with the total concentration of secretome proteins approximating 100 μg/ml. Many of these cytokines and growth factors are involved in the mechanisms proposed to explain scarless fetal wound healing. However, there are no reports of the potential of APM cells and their secretome, ST266, to reduced neointimal hyperplasia after arterial balloon injury. Therefore, we aimed to explore the potential ability of the AMP cells and the AMP cell-derived secretome. ST266, to inhibit neointimal hyperplasia and arterial lumen stenosis in a post-balloon injury model in rats. In addition, and for the first time, we applied ST266 systemically by intravenous (iv) injection.

Material and methods

Animals

Male Sprague – Dawley rats (10–12 wks old) were purchased from Shanxi Medical University Laboratory Animal Center. Filtered tap water and a standard rat pellet diet were available ad libitum. The rats were housed in cages kept at 25±2^{0}C and at 50±5% relative humidity, i.e., a controlled environment. A 12-hr light/dark cycle (light period, 6 am to 6 pm) was maintained throughout the experiment. All procedures were approved by the Institutional Animal Care and Use Committee of Shanxi Medical University.

Balloon injury model

All surgeries were performed under general isoflurane anesthesia. Vascular balloon injury was inflicted in the right iliac artery as previously described [11]. Briefly, a longitudinal aortotomy in the abdominal aorta was made to insert a 2F French Fogarty embolectomy catheter into the right iliac artery. The balloon was inflated to 1.5–1.6 atm and retracted to the aortotomy site three times to assure a well-established endoluminal injury. The aortic incision was repaired with 7–0 prolene sutures (0.4 metric, Ethicon, San Lorenzo, PR, USA). The rats were killed at 28 days after surgery by an overdose of inhaled isoflurane. The iliac arteries were harvested and fixed with formalin for histomorphometric analysis.

Re-endothelialization-index assessment

Ten days after balloon angioplasty, 0.5 ml of 0.5% Evans blue solution was injected through a tail vein, and the rats were euthanized 15 min later. The injured iliac artery was then harvested and dissected longitudinally. The arteries were photographed with 10× scope and the images were analyzed for Re-endothelialization index. Re-endothelialization was evaluated based on blue staining (no endothelium coverage) and no-staining (with endothelium coverage) area using the US National Institutes of Health (NIH) ImageJ software (version 1.43).

AMP cell culture and activation

Proprietary serum-free culture media and cryopreserved AMP cells were provided by Noveome Biotherapeutics, Inc. (PA, USA) at a concentration of 1×10^6/ml and stored in the vapor phase of liquid nitrogen. The cells were thawed and cultured in proprietary serum-free culture media at 37°C, 5% carbon dioxide, and 95% air humidity and were grown to 70% confluency in 75 ml cell culture plates. The AMP cells were activated with 10 ng of interferon gamma (Cell Signaling Technology, USA) 48 hrs before using them in-vivo.

AMP cell implantation

Activated AMP cells were trypsinized, rinsed twice in cold phosphate buffered saline (PBS), and resuspended in 1 ml of Matrigel Matrix (Corning, NY, USA). Matrigel scaffolds facilitated the perivascular implantation of the cells around small-caliber blood vessels, since it rapidly solidifies at physiological temperature. Then, 300 μl of the cell suspension was implanted perivascularly around the right iliac artery after balloon angioplasty. Initially, the bottom part of the artery was covered with 150 μl Matrigel, and then the upper surface of the artery was covered by slowly dropping another 150 μl of the cell suspension. The artery was completely encircled by Matrigel.

Histology and histomorphometric measurements

Morphometric analysis was performed on hematoxylin and eosin stained slides. For imaging, the arteries were observed with an EVOS FL Cell Imaging System (Life Technologies, CA, USA) using a 4× or 10× objective in the bright field mode. Morphometric measurements were performed using NIH ImageJ software (version 1.43).

The area of each vascular layer was measured. Media was defined as the area between external elastic lamina (EEL) and the internal elastic lamina (IEL). The neointima was defined as the area enclosed between the IEL and the lumen,

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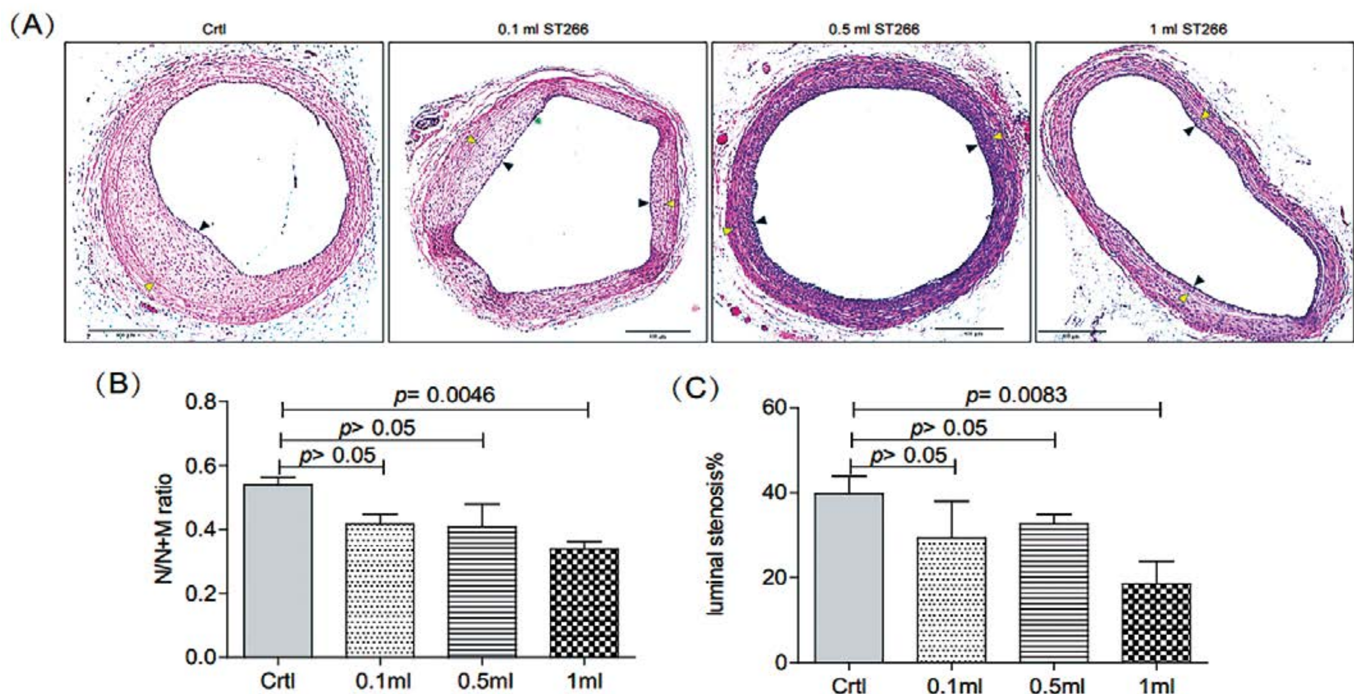
Figure 1. ST266 reduces neointima development



A: Representative photomicrograph of neointima hyperplasia 28 days post angioplasty. Yellow arrowhead = internal elastic lamina. Black arrowhead = endothelial layer. **B:** ST266 decreases the N/N+M ratio compared to control group (n=7 per group). **C:** ST266 decreased the luminal stenosis compared to control group (n=7 per group).

and the neointima/media+neointima ratio was calculated from these measurements. We also estimated the luminal stenosis percentage using the formula: (neointimal/IEL area) ×100.

Smooth muscle cell culture, scratch wound migration assay and Western blot analysis

Rat SMCs were plated in 6-well plates using rat SMC growth medium (Cell Application, Inc.) containing10% FBS and cultured until cell monolayers formed. The monolayers were wounded by manual scraping with a 10 μl micropipette tip and then washed. The cells were then incubated with rat SMC growth medium containing 1% FBS alone or combined with the indicated concentrations of ST266 and PBS for 48 hrs. The cells were photographed using an inverted microscope at times zero, 24 hrs, and 48 hrs. Thecells that migrated past the wound edge were quantified in three high-power fields (left field, middle field, and right field) [12]. 24 hrs after treating with 40% ST266 or with 40% PBS (diluted with rat SMC growth medium) or rat SMC growth medium alone (control), protein extracted from cultured rat SMCs were used to measure Sirtuin1 (SIRT1) expression with Western blotting.

Western blot analyses were performed using antibody raised against SIRT1 (Abcam, 1:1000). GAPDH (Santa Cruz Biotechnology, Inc., 1:1000) was used as control to normalize protein concentrations. Protein separation was performed with 10% SDS polyacrylamide gel electrophoresis (Invitrogen, Thermo Fisher Scientific), with 15 μg of protein per each lane. Subsequently, the separated proteins were transferred to nitrocellulose high bound ECL membranes (Invitrogen, Thermo Fisher Scientific). Membranes were blocked with 3% non-fat milk (Quality Biological, Inc.) for 1 hr at room temperature and probed with primary antibody in blocking buffer overnight at 4^0C. After being washed three times with PBST (PBS-Tween20), the membranes were incubated with corresponding secondary antibodies (Life technologies.) 1:4000 for 1 hr at room temperature. After three washes, immunocomplexes were visualized with a Chemilumine Science detection kit (Denville Scientific Inc.).

Statistical analyses

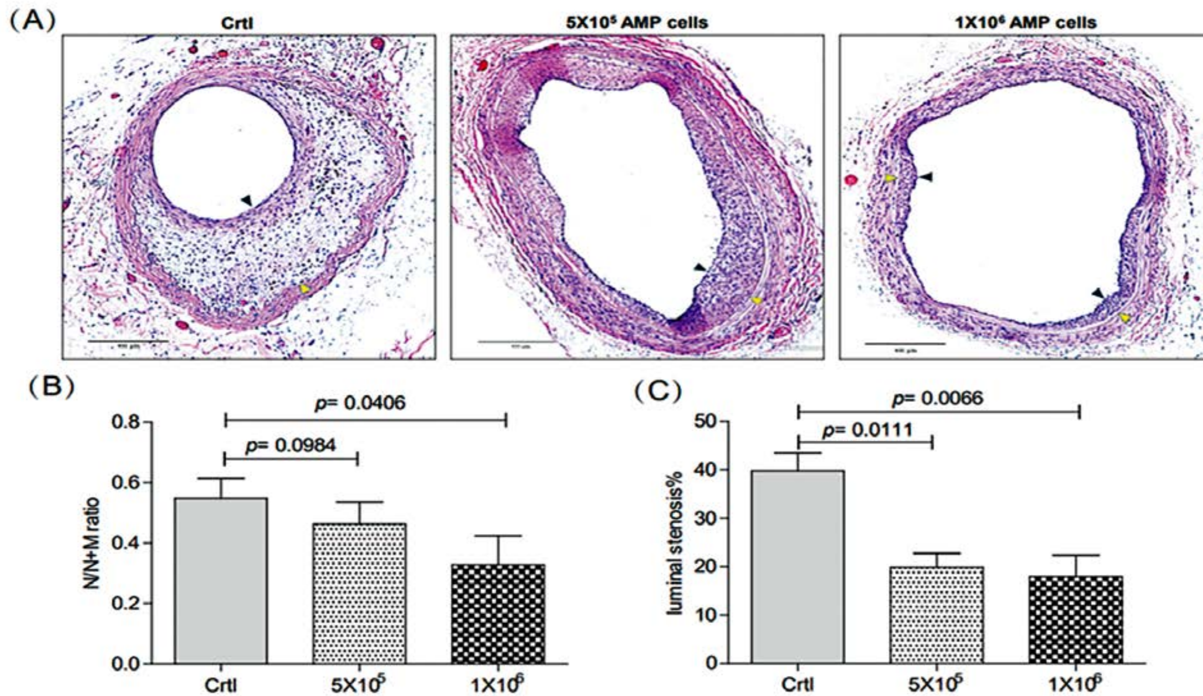
Each individual experiment was repeated 3 times. Data were analyzed with GraphPad Prism 5 (Graph Pad Software, La Jolla, CA, USA), data were expressed as the mean±SEM. Statistical significances were assessed by one-way ANOVA and student's t test. Statistically significant was defined when $P < 0.05$.

Results

Systemic ST266 decreases neointimal hyperplasia and luminal stenosis

Since ST266 is a secretome derived from AMP cells, containing physiologic concentrations of multiple growth factors and cytokines, we evaluated the capacity of different

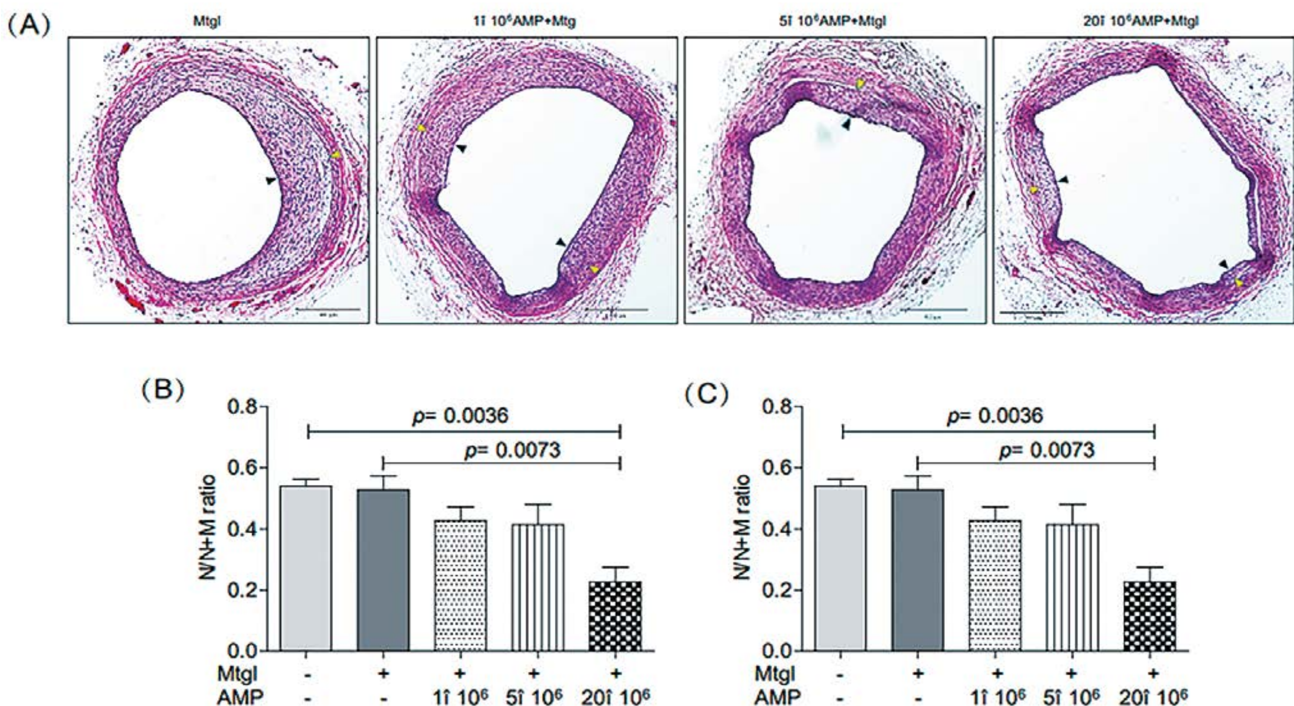
Figure 2. Systemic injection of AMP cells reduces neointima development



A: Representative photomicrograph of neointima hyperplasia 28 days post-angioplasty. Yellow arrowhead = Internal Elastic Lamina; Black arrowhead = endothelial layer. **B:** Single IV injection of 1×106 AMP cells significantly reduced the N/N+M ratio compared to the control group (n=7 per group). **C:** Single IV injection of 1×106 AMP cells significantly reduced luminal stenosis compared to control group (n=7 per group).

doses of systemic ST266 on the results of the arterial histomorphometric analysis after balloon injury. Treatment with 1 ml of ST266 significantly decreased the N/N+M ratio compared to the control (PBS) group (0.4±0.1 vs. 0.5±0.1, p=0.005; n=7; Figure 1 A&B). In addition, administration of 1 ml ST266 significantly decreased the luminal stenosis

Figure 3. Perivascular Implantation of AMP cells reducesneointima development



A: Representative photomicrograph of neointima formation 28 days after angioplasty. Yellow arrowhead = internal elastic lamina. Black arrowhead = endothelial layer. **B:** Perivascular implantation of 20×106AMP AMPs cells reduced the N/N+M compared to the both control and Mtgl-only groups (n=7 per group). **C:** Perivascular implantation of 20×10^6 AMP AMPs cells reduced luminal stenosis compared to the both control and Mtgl-only groups (n=7 per group).

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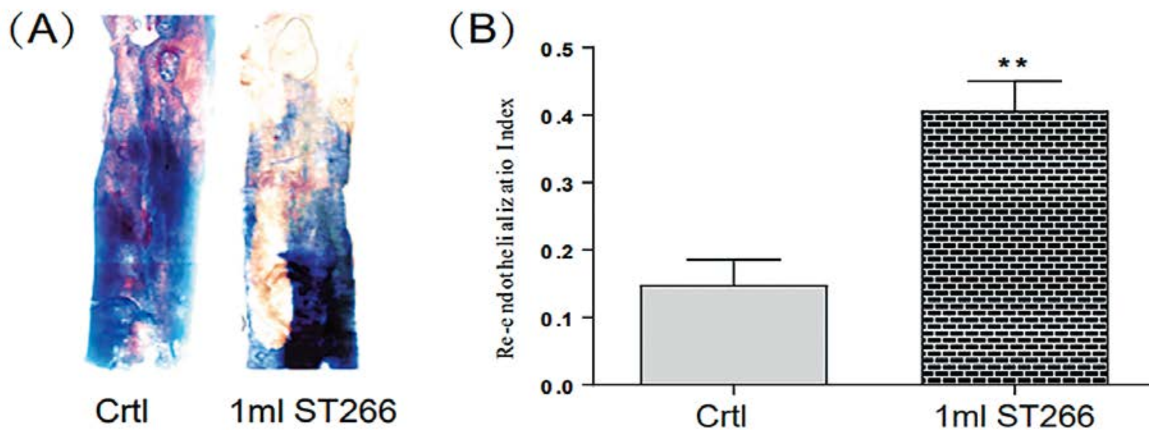
Figure 4. ST266 improve Re-endothelialization



A: Representative Evans-Blue photomicrograph of iliac arteries shows the level of re-endothelialization (non-blue color) 10 days post-angioplasty.
B: 1ml ST266 iv significantly increased the re-endothelialization index compared to control (PBS) group (0.4±0.1 vs 0.1±0.1% resp., p=0.002).

percentage compared with the control group (18.2±1.9% vs. 39.2±5.6%, p=0.008, n=7; Figure 1 A&C). Although a decreasing trend was seen in both the N/N+M ratio and the luminal stenosis in both the 0.1 ml and 0.5 ml ST266 groups, they did not show significant differences compared with the control group.

Treatment of AMP cells decreases neointima formation and luminal stenosis

To investigate the therapeutic effects of AMP cells on neointima formation and luminal stenosis in the balloon-injured artery, we injected different doses (0.5×10^6 and 1×10^6) of AMP cells into the inferior vena cava immediately after aortotomy. Strikingly, treatment with 1×10^6 AMP cells significantly reduced the N/N+M ratio compared with the control group (0.3±0.1 vs. 0.5±0.1; p=0.041; n=10 Figure 2 A&B). Moreover, the 1×10^6 AMP cell treatment showed less luminal stenosis compared with the control, 18.6±2.5 vs. 39.2±5.8, p=0.007, n=10; Figure 2 B), Importantly, treatment with 0.5×10^6 AMP cells did not significantly affect the N/N+M ratio (0.5±0.1 vs. 0.5±0.1; p=0.0984, n=10; Figure 2 A&C), but it did significantly inhibit luminal stenosis compared with the control group (19.4±2.2 vs. 39.2±5.8, p=0.011; Figure 2 A&C).

Perivascular AMP cell implantation decreased the neointima formation and luminal stenosis

To further detect the inhibitory role of the AMP cells on neointima hyperplasia, we seeded different doses of activated AMP cells around the balloon-injured arteries, and we examined neointima formation at 28 days after angioplasty. The result showed that 20×10^6 implanted AMP significantly decreased the N/NM ratio compare to the control group (0.4±0.1 vs. 0.5±0.1, p=0.0036, n=7) and Matrigel-only groups (0.4±0.1 vs. 0.5±0.1, p=0.0073, n=7; Figure 3 A&B) Activated AMP also decreased the luminal stenosis (16.8±2.5%, n=7) compare to the both control (39.2±5.8%, p=0.001, n=7) and Matrigel-only groups (37.5±8.6%, p=0.0161, n=7; Figure 3 C).

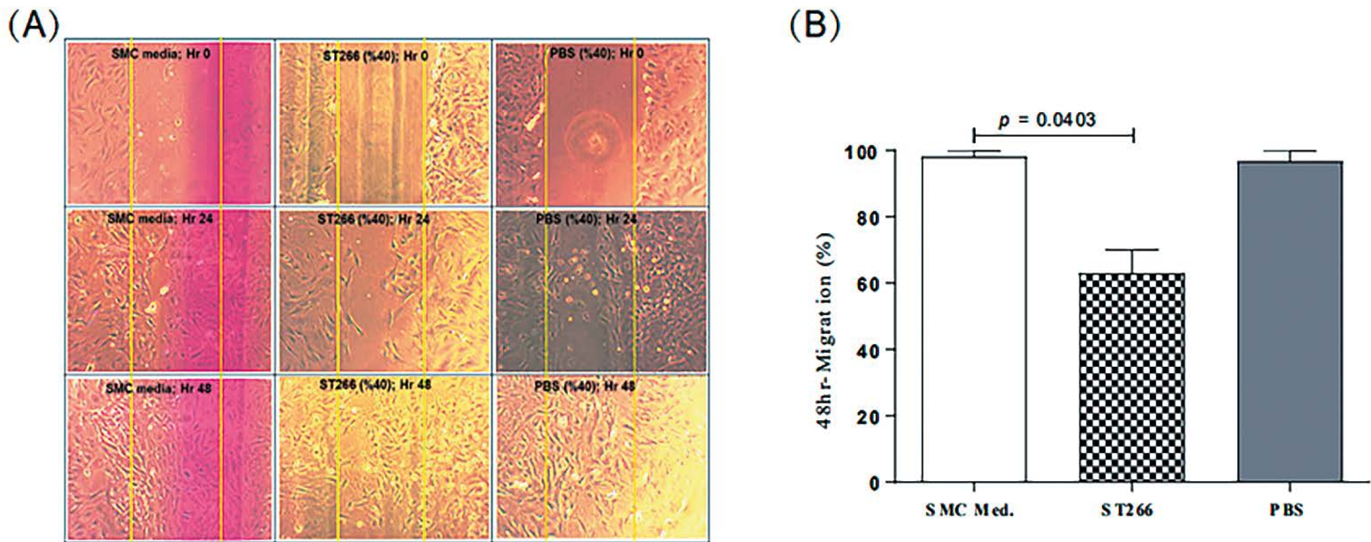
Re-endothelialization index can be improved by treatment of ST266

To test the effect of ST266 on re-endothelialization of injured arteries, we injected intravenously 0.5 ml of 5% Evans blue into the rats' arteries at 10 days after balloon angioplasty 1 ml ST266 injection, and then the injured iliac artery was harvested and dissected longitudinally, and the re-endothelialization index was analyzed. We found that ST266 significantly increased the re-endothelialization index at 10 days after balloon injury compared with the control group (0.4±0.1 vs. 0.1±0.1, p=0.0021, n=7; Figure 4 A&B). These results confirmed that ST266 is a potent wound healer and has therapeutic and preventive potential for treatment of post-balloon injury restenosis.

ST266 decreases migration of Smooth Muscle Cell

The results of the in vitro migration assay showed that 48 hrs after inducing a scratch wound in the rat SMC culture, 40% ST266, diluted with the rat SMC growth medium, markedly decreased vascular SMC migration compared to the growth medium (46.4%±11.8% vs. 98.2±1.8, p=0.0403) and to PBS plus the same growth medium at 40% dilution (46.4%±11.6% vs. 96.8±3.2, p=0.0490; Figure 5 A&B). These results are in agreement with the in vivo findings, which showed a lower N/N+M ratio with a higher dose of systemic ST266.

Figure 5. ST266 decreases Smooth Muscle Cell migration



A: Representative photograph of effect of rat SMC medium, ST266 and PBS on SMC migration in a wound-healing scratch assay. **B:** Quantitative analysis of migration distances demonstrated that compared to SMC medium and PBS. ST266 significantly decreased SMC migration after 48 hrs (p=0.0403).

ST266 increases the expression of SIRT1 protein in the cultured SMCs

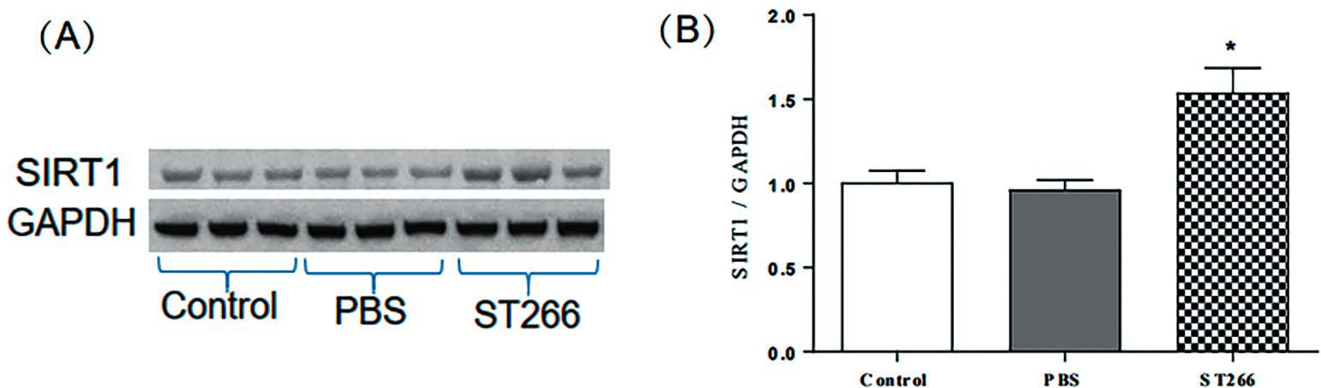
The preventive role of SIRT1 protein on neointimal formation was formerly shown by Li et al. [13]. Also, Khan et al. had shown in an optic neuritis model that ST266 increased the SIRT1 expression in the retina and optic nerve of the rats [14]. Therefore, we examined whether ST266 had any effect on SIRT1 expression on stressed cultured rat SMCs. Interestingly, the Western blot analysis showed that treatment of ST266 significantly increased the expression of SIRT1 in injured arteries compared with the control and with treatment with PBS (Figure 6 A&B).

Discussion

In the present study, we tested the anti-restenotic potential of systemic and perivascular implanted human AMP cells and their secretome «ST266» on vascular histomorphology in a rat model of arterial restenosis induced by classically applied balloon injury. Our findings indicated that effective doses of the AMP cells and ST266 decreased the neointima thickness and luminal stenosis. Our results also showed that ST266 significantly increased the re-endothelialization index in vivo and inhibited SMC migration in vitro.

The core sequential milestones in the process of arterial restenosis after balloon injury are very comparable with the wound-healing pathophysiology [15]. The characteristic features of the AMP cells and ST266 in wound healing acceleration have been clarified in preclinical and clinical studies [5, 6, 9, 16]. Steed et al. demonstrated that ST266 contained physiologic concentrations of cytokines relevant to wound healing, including platelet-derived growth factor

Figure 6. SIRT1 protein expression in the rat SMCs treated with control (rat SMC medium), PBS or ST266



A: ST266 significantly increased SIRT1 expression in the SMCs after 24 hrs (p=0.0403 vs PBS and control).
B: Representative Western blot analysis of the cultured rat SMCs extracts using anti-SIRT1 IgG and anti-GAPDH IgG.

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Table 1. Selected known proteins in the ST266 solution and their biological function

Protein	Biological Function
TIMP 1 and 2	MMP inhibitor, wound healing
PDGF-BB, CTGF	Cell growth promotion, wound healing
Angiogenin, VEGF	Blood Vessel Formation, wound healing
MMP-3 and 9	Proteases, wound healing
Decorin, Epiphycan, Biglycan, Keratocan	Collagen alignment, wound healing
Osteonectin (SPARC)	Collagen mineralization, wound healing
Destrin	Regulates actin, cell motility
MIC-1	Immune modulation
Hyaluronan	Matrix protein, wound healing
Mucin 1, 2, 4, 5AC, 13, 15, 16, 21	Lubrication of the eyes
Annexin 2 and 5, Osteopontin	Anti-apoptotic

TIMP, tissue inhibitor metalloproteinase; PDGF, platelet-derived growth factor; CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; MIC-1, macrophage-inhibitory cytokine 1.

(PDGF), vascular endothelial growth factor (VEGF), angiogenin, transforming growth factor β2 (TGF- β2), and tissue inhibitor of metalloproteinase (TIMP) – 1 and 2[6] (Table 1). This distinctive aspect of ST266 makes it a potential, risk-free, and «natural» treatment after balloon to reduce the rate of in-stent restenosis.

More evidence supporting our findings include studies that examined the effect of the ST266 proteins on endothelial function or neointima formation, individually. For instance, the positive impacts of VEGF on the results of in-stent angioplasty were reported by Swanson et al. [17] and Tang et al. [18].

The other active components in ST266 that support our findings of less neointima formation are tissue inhibitor metalloproteinases (TIMP) and matrix metalloproteinases (MMP) 3 and 9. According to Linjen et al., TIMP-1 impairs arterial neointima formation after vascular injury [19]. They showed that following MMP activity, TIMP-1 impairs SMCs migration and neointima formation. It has also been shown that MMP-9 is necessary for the regulation of SMC cell replication and migration after arterial injury [19, 20].

The potent role of angiogenin, another secreted protein in the AMP cell secretome, in repairing the endothelial layer and inhibiting SMCs migration should also be cited. The regulatory role of angiogenin in the dynamic interactions between SMC and endothelial layer has been reported previously [21–23]. According to Hatzi et al., angiogenin is a protein with angiogenic activities which inhibits proliferation of SMCs [21]. On the other hand, SMCs and pericytes suppress endothelial cell proliferation [22]. Endothelial cell conditioned-media stimulates or inhibits SMC growth depending on endothelial cell density [23]. This modulatory action of angiogenin explains our findings of simultaneous inhibitory effect of ST266 on SMC migration while provoking re-endothelialization.

The results of our study also indicated that ST266 caused overexpression of Sirtuin 1 (SIRT1) in rat smooth muscle cells. Khan et al. previously showed that intranasal administration of ST266 significantly increased the SIRT1 expression in the retina of the rats in a model of optic neuritis [14].

In agreement with our findings, Li et al. discovered that SIRT1 inhibited vascular SMC proliferation by blocking cell-cycle entry into the S phase. Likewise, wound healing assays indicated that the overexpression of SIRT1 resulted in a significant inhibition of vascular SMC migration [13]. The role of SIRT1 protein in aging, metabolism, and tolerance to oxidative stress has been shown previously [24]. SIRT1 is also a key regulator in the endothelial homeostasis by controlling angiogenesis, vascular tone, and endothelial dysfunction as well as by decreasing atherosclerosis [25].

The immunomodulatory traits of the AMP cells and ST266 can also explain the promising outcomes of this study. The underlying inflammatory mechanisms in the processes of SMC proliferation and migration, neointima formation, and luminal stenosis have been called "central" [26, 27]. The number of vessel wall monocytes/macrophages positively correlates with the neointima area, which indicates the role for monocytes in restenosis. Conversely, blockade of monocytes recruitment results in attenuation of neointima hyperplasia [28]. The inhibitory effects of AMP cells on the peripheral blood monocytes have been verified by Banas et al. [3, 29].

The results of this study showed that systemic ST266 and both systemic and perivascular implantation of the AMP cells at their most effective dose significantly decreased the N/N+M ratio and luminal stenosis after balloon injury. Based on the findings of Banas et al., we propose that our results might be explained by the immunomodulatory effects of the AMP cells and their secretory product on the inflammatory component of the restenosis process.

Our findings with perivascular implantation of different doses of AMP cells also showed significantly decreased neointima thickness and luminal stenosis compared to the both control and Matrigel-only groups. The regulatory roles of adventitia and vasa vasorum in vascular response to the injury have been proven previously [30].

Therefore, it was hypothesized that enriching adventitia with protective factors provides control over the response the vascular injury. According to published clinical and preclinical data, surgically or radiologically implanted endothelial progenitor cells on biomaterials, such as gelatin

matrices, positively influenced the vessel response after a revascularization procedure [31–33]. The comparable results between AMP cell therapy and endothelial progenitor cells makes the AMP cells a potential candidate for cell-based therapy during endovascular interventions.

A limitation of this study was the use of healthy rats. Therefore, impact of the AMP cells or ST266 in a repeat balloon angioplasty on an existing lesion was not assessed. According to the outcomes of this research, compared to the control groups, systemic injections of the AMP cells or ST266 and perivascular implantation of the AMP cells concurrent with balloon angioplasty improved the long-term vascular histomorphology. These promising results would be helpful in bringing more novelty in the revascularization therapeutic approach, stent technology as well as stem/progenitor cell containing sheet coats to optimize the results of endovascular procedures.

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No conflict of interest is reported.

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