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Enhancement of cell adhesion, retention, and survival of HUVEC/cbMSC aggregates that are transplanted in ischemic tissues by concurrent delivery of an antioxidant for therapeutic angiogenesis

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ABSTRACT

A recurring obstacle in cell-base strategies for treating ischemic diseases is the significant loss of viable cells that is caused by the elevated levels of regional reactive oxygen species (ROS), which ultimately limits therapeutic capacity. In this study, aggregates of human umbilical vein endothelial cells (HUVECs) and cord-blood mesenchymal stem cells (cbMSCs), which are capable of inducing therapeutic angiogenesis, are prepared. We hypothesize that the concurrent delivery of an antioxidant *N*-acetylcysteine (NAC) may significantly increase cell retention following the transplantation of HUVEC/cbMSC aggregates in a mouse model with hindlimb ischemia. Our *in vitro* results demonstrate that the antioxidant NAC can restore ROS-impaired cell adhesion and recover the reduced angiogenic potential of HUVEC/cbMSC aggregates under oxidative stress. In the animal study, we found that by scavenging the ROS generated in ischemic tissues, NAC is likely to be able to establish a receptive cell environment in the early stage of cell transplantation, promoting the adhesion, retention, and survival of cells of engrafted aggregates. Therapeutic angiogenesis is therefore enhanced and blood flow recovery and limb salvage are ultimately achieved. The combinatory strategy that uses an antioxidant and HUVEC/cbMSC aggregates may provide a new means of boosting the therapeutic efficacy of cell aggregates for the treatment of ischemic diseases.

1. Introduction

The efficacy of clinically available interventions for treating patients with critical limb ischemia is limited, resulting in a high incidence of limb amputation [1,2]. Cell-based approaches to increase the blood flow in the affected limb hold great promise in inducing therapeutic angiogenesis [1,3,4]. However, most current strategies of cell delivery result in very low engraftment efficiency and cell viability in ischemic tissues, limiting their therapeutic benefits [5–8].

In cell therapy, suspensions of dissociated cells that are obtained from trypsinization are typically administered into the target tissue *via* local injection. Owing to their inadequate physical size and lack of extracellular matrices (ECMs), significant fractions of the administered cells are mechanically washed out during delivery [9]. To improve cell retention, we previously developed a thermo-responsive methylcellulose (MC) hydrogel-based platform that

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can be utilized to prepare aggregates of human umbilical vein endothelial cells (HUVECs) and cord-blood mesenchymal stem cells (cbMSCs) [10–12]. Because of their bulkiness and well-preserved ECMs, the as-prepared cell aggregates were effectively entrapped at the sites of cell transplantation [8,13,14], stimulating therapeutic angiogenesis and restoring blood perfusion in the ischemic limb that was experimentally created in mice [10,11].

Beyond the acute cell loss during injection, the hostile inflammatory environment in ischemic tissues, which are characterized by the extracellular production of very large amounts of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($\cdot OH$), and superoxide anion radicals ($O_2\cdot^-$), also accounts for the limited cell retention [7,15,16]. Extracellular ROS have been reported to hinder the attachment of transplanted cells to the target tissue [17,18]. Moreover, the oxidative stress that is caused by high levels of ROS may inhibit the proliferation and spreading of transplanted cells, ultimately reducing their therapeutic capacity [19,20]. Therefore, establishing a receptive cell environment with reduced oxidative stress is highly favored to increase the efficiency of cell-based therapy. The oxidative stress in ischemic tissues may be eliminated upon treatment with an antioxidant, on which cell adhesion in the initial stage of cell transplantation depends, causing the transplanted cells to be retained in treated tissues.

N-acetylcysteine (NAC) is a powerful thiol-containing antioxidant that can directly scavenge free-radical oxidants by H-atom donation and electron transfer from its sulfur atom [16,21]. We hypothesize that the concurrent delivery of NAC may significantly augment cell retention following the intramuscular injection of HUVEC/cbMSC aggregates, improving their therapeutic benefits (Fig. 1). To test this hypothesis, whether NAC treatment preserved the adhesion of cells in HUVEC/cbMSC aggregates and their viability when those aggregates were under oxidative stress *in vitro* was determined; their angiogenic potency was also investigated using the Matrigel tube-formation assay. A mouse model with hindlimb ischemia was employed to evaluate the retention of the cells in concurrently transplanted HUVEC/cbMSC aggregates and NAC, and their efficacy in stimulating subsequent therapeutic angiogenesis. Animals that received saline, NAC, or cell aggregates alone served as controls.

2. Materials and methods

2.1. Cytotoxicity of NAC and its ability to scavenge ROS

Before NAC can be used in cell transplantation, its optimal dose that does not affect cell viability must be determined. The cytotoxicity of NAC was assessed *in vitro* by separately incubating HUVECs and cbMSCs with various concentrations of NAC (0, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 mM). After 24 h, the cells were treated with 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich, St. Louis, MO, USA) for 4 h at 37 °C. The metabolized MTT was dissolved in dimethyl sulfoxide (Sigma–Aldrich), and its absorbance was measured at 570 nm with a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

To determine the capacity of NAC to scavenge ROS, aqueous solutions of H_2O_2, which is a prevalent ROS [17], in different concentrations (0.125, 0.25, 0.5, and 1.0 mM) were reacted with a NAC solution for 1 h; their counterparts were treated with deionized water to provide a control. Following treatments, the amount of residual H_2O_2 in each test solution was assessed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.2. Restoration of adhesion and spreading of cell aggregates under oxidative stress by NAC

In the inflammatory environment of ischemic tissues, a variety of ROS that affect cell adhesion are produced [17,18]. The effects of NAC on the restoration of adhesion and spreading of HUVEC/cbMSC

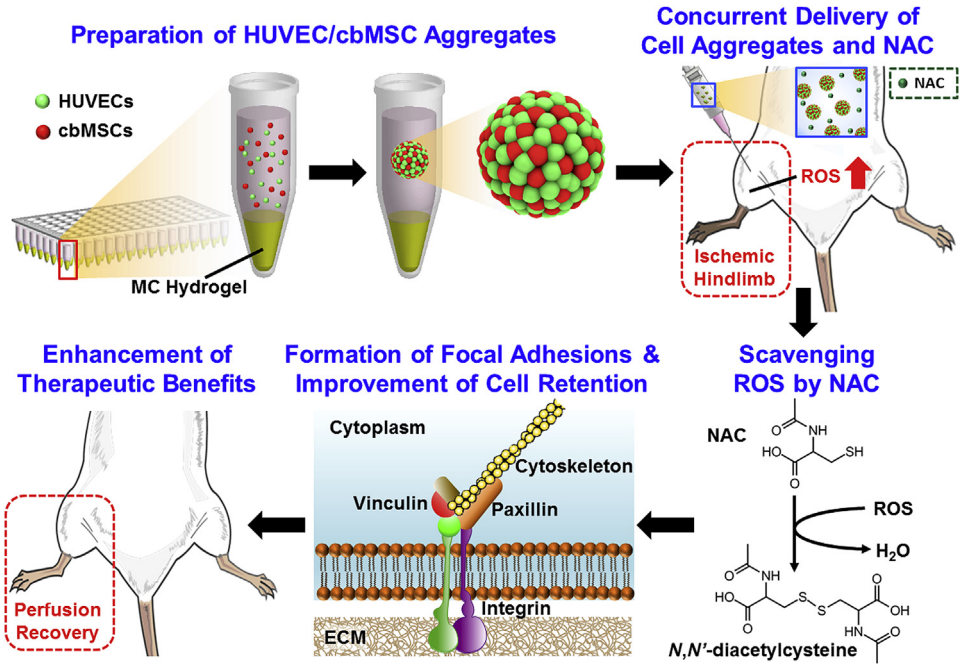

Fig. 1. Schematic illustrations showing preparation of HUVEC/cbMSC aggregates using an MC hydrogel-based system and concurrent delivery of cell aggregates and antioxidant NAC into a mouse model of hindlimb ischemia. Consequent reduction of oxidative stress in target tissue promotes retention of engrafted cells, which subsequently induces therapeutic angiogenesis and contributes to recovery of blood perfusion and limb salvage. HUVEC: human umbilical vein endothelial cell; cbMSC: cord-blood mesenchymal stem cell; MC: methylcellulose; NAC: *N*-acetylcysteine; ROS: reactive oxygen species.

aggregates that were under oxidative stress were investigated. The human cbMSCs that were used in this study were genetically engineered to express red fluorescent protein (RFP) [22]. HUVECs and cbMSCs were cultured and formed HUVEC/cbMSC aggregates in a 1:1 ratio and a density of 10,000 cells in wells that contained the MC hydrogel system, as described elsewhere [10–12]. The as-prepared HUVEC/cbMSC aggregates were plated into μ-Dishes (ibidi, Munich, Germany) that had been coated with fibronectin (5 $\mu g/cm^2$) to mimic the ECMs in native muscular tissues [23], before being treated with NAC, H_2O_2, or NAC + H_2O_2. At pre-determined times, the adhesion and spreading of cells in each studied group were monitored under an inverted phase-contrast microscope (Eclipse TE200, Nikon, Tokyo, Japan).

Paxillin and vinculin are multi-domain adaptor proteins that are key regulators of focal-adhesion formation and cell adhesion [24,25]. To elucidate in greater detail the mechanism by which NAC restores cell adhesion upon exposure to oxidative stress, distributions of the focal-adhesion proteins, paxillin and vinculin, in the cell aggregates were examined. Four hours following treatment, the test cell aggregates were fixed in 4% para-formaldehyde (Sigma–Aldrich), stained with primary antibodies against paxillin or vinculin (Abcam, Cambridge, MA, USA), and visualized using Alexa Fluor 488 or 633-conjugated secondary antibodies (Life Technologies). The cells were then counter-stained with 4,6-diamidino-2-phenylindole (DAPI; Life Technologies) and observed using a confocal laser scanning microscope (CLSM; LSM 780, Carl Zeiss, Jena, Germany). The areas that were covered by focal-adhesion proteins were quantified (n = 10) using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA).

Cell viability was qualitatively evaluated using a live/dead assay based on calcein-AM and ethidium homodimer and quantitatively determined by the MTT assay. In the live/dead assay (LIVE/DEAD Viability/Cytotoxicity Kit; Life Technologies), calcein-AM is enzymatically converted into green fluorescent calcein in live cells, while ethidium homodimer stains the nuclei of dead cells with red fluorescence. The test samples were then examined by a CLSM. Images of the optical sections that corresponded to the plane of the adhered cells were acquired, and the areas over which the cells had spread were determined (n = 15) using Image-Pro Plus software.

2.3. Tube formation assay

Cell adhesion reportedly plays a fundamental role in angiogenesis [26]. Accordingly, the ROS-mediated impairment of the adhesion of cells of HUVEC/cbMSC aggregates may reduce their angiogenic potential. To evaluate the effects of NAC, which can scavenge ROS, on rescuing the angiogenic capacity of cell aggregates, test samples were seeded onto growth factor-reduced Matrigel (BD Biosciences, San Jose, CA, USA) to perform a tube formation assay, and H_2O_2 was added to culture medium to simulate oxidative stress in ischemic tissues. The tube formation assay is one of the most extensively used *in vitro* assays to model the reorganization of angiogenesis [27].

The grown tubular networks were photographed under a phase-contrast microscope at pre-determined times, and the branching points and area of which the tubes spread were quantified using Image-Pro Plus software. The secretions of vascular endothelial growth factor (VEGF)-A, VEGF-D, fibroblast growth factor (FGF)-2, placental growth factor (PlGF), hepatocyte growth factor (HGF), and epidermal growth factor (EGF) into the culture supernatants were quantified using ELISA kits (n = 6; R&D systems, Minneapolis, MN, USA), according to the manufacturer's directions.

2.4. Animal study

All animal experiments were carried out in compliance with the "Guide for the Care and Use of Laboratory Animals" (Department of Health and Human Services, National Institutes of Health, Publication No. 85-23, revised 1996). The Institutional Animal Care and Use Committee of Veterans General Hospital (Taichung, Taiwan) approved all of the protocols that were utilized in this study.

Unilateral hindlimb ischemia was induced in a mouse model (BALB/c, male, eight weeks old; National Laboratory Animal Center, Taipei, Taiwan). Both the proximal and the distal points of the left femoral artery in each test mouse were ligated with sutures, and the segment between the ligatures was excised [28,29]. Immediately following arterial dissection, the HUVEC/cbMSC aggregates and NAC were concurrently delivered by suspending cell aggregates (1×10^6 cells in total) in 150 μL saline that was supplemented with NAC and then intramuscularly injecting the same into the quadriceps muscle that was adjacent to the proximal ligation site. To realize immunosuppression, cyclosporine A was administered to the test animals (10 mg/kg/day; Novartis, Rueil-Malmaison, France) intramuscularly one day before surgery and then daily until euthanasia. The animals that were treated with saline, NAC, or cell aggregates alone served as controls.

2.5. Evaluation of oxidative stress in ischemic limbs

In cell therapy in ischemic tissues, engrafted cells face increased oxidative stress in the recipient microenvironment immediately following delivery [18,30]. To evaluate the oxidative stress in tissues, test animals were euthanized on the first day following treatment, and their quadriceps muscles were harvested and processed for immunostaining. Tissue sections were separately incubated with primary antibodies against oxidative-damage markers 8-hydroxy-2′-deoxyguanosine (8-OHdG; JaICA, Shizuoka, Japan) and 4-hydroxynonenal (4-HNE) [31,32], visualized by fluorophore-conjugated secondary antibodies (Life Technologies), counterstained with DAPI, and then observed using a CLSM.

To further evaluate the capacity of NAC to scavenge regional ROS, the retrieved hindlimb muscles were homogenized (TissueLyser II, Qiagen, Valencia, CA, USA), and the levels of malondialdehyde (MDA) that they contained were determined using a colorimetric Thiobarbituric Acid Reactive Substances Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), following the manufacturer's guidelines. MDA, which is an end product of lipid peroxidation, which is caused by oxidative free radicals, is a biomarker of oxidative damage [30]. The MDA values herein are expressed as nmol/mg protein.

2.6. Assessment of retention of cells of transplanted HUVEC/cbMSC aggregates

To assess the retention of the cells of the transplanted HUVEC/cbMSC aggregates with or without the concurrent delivery of NAC in the ischemic limbs, the RFP that was constitutively expressed by cbMSCs was tracked. On days one, seven, and 14, ischemic limbs of the test animals were retrieved, cryosectioned, counterstained with DAPI, and examined under a CLSM to detect RFP fluorescence.

The retention of the transplanted cells was further determined by quantifying the non-coding human Alu sequences by a real-time quantitative polymerase chain reaction (PCR) system (7500 Real-time PCR System, Applied Biosystems, Carlsbad, CA, USA) [8,33]. The genomic DNA of the harvested tissues was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). The

sequences of the primers and the TaqMan probe that was used to detect human Alu sequences were as follows: forward, 5′-GAG ATC GAG ACC ATC CCG GCT AAA-3′ and reverse, 5′-CTC AGC CTC CCA AGT AGC TG-3′; TaqMan probe, 5′-GGG CGT AGT GGC GGG-3′ (Applied Biosystems). A calibration curve was generated by serially diluting the human genomic DNA that was extracted from equal amounts of HUVECs and cbMSCs into DNA that had been prepared from mouse C2C12 myoblasts [34].

2.7. Blood flow recovery

Blood flow recovery in the ischemic hindlimb was investigated noninvasively using single-photon emission computed tomography (SPECT; NanoSPECT/CT scanner, Bioscan, Washington DC, USA) on days one (as the baseline) and 14 after treatment (n = 5 for each test group). The animals were intravenously injected with ^{99m}Tc-diethylenetriaminepentaacetic acid (17.5 MBq/0.1 mL) and underwent dynamic planar imaging or SPECT imaging using a single-pinhole collimator or four multi-pinhole collimators, respectively, followed by X-ray acquisition for anatomy correlations. The obtained emission images were quantified as the perfusion ratio between the ischemic and the nonischemic contralateral limbs or were reconstructed three-dimensionally using the PMOD software workstation (version 3.2, PMOD Technologies Ltd., Zurich, Switzerland).

2.8. Histological analysis

On day 14 following treatment, the test animals in each group were photographed and their gross appearance examined. Subsequently, all animals were sacrificed, and their quadriceps muscles were harvested, fixed in 10% phosphate-buffered formalin, and then processed for histological analyses. The muscle degeneration and tissue fibrosis were evaluated by hematoxylin and eosin (H&E) and Masson's trichrome staining, respectively. For transmission electron microscopy (TEM) analysis, the fixed muscle samples were dissected into 3 mm^3 pieces, post-fixed in 2% aqueous osmium tetroxide, dehydrated in ascending concentrations of ethanol, and embedded in Araldite 502 (Electron Microscopy Sciences, Hatfield, PA, USA). One-micron thick sections were made, stained with toluidine blue for observation under a light microscope and screening. Selected blocks in which the muscle had a longitudinal orientation were cut into 800 Å-thick sections, mounted on 200-mesh copper grids, doubly stained with uranyl acetate and lead citrate, and then examined using a Phillips CM-10 electron microscope with a LaB 6 cathode (Phillips Electronic Instruments Co., Eindhoven, The Netherlands), operated at 80 kV.

To explore the therapeutic angiogenesis in each studied group, tissue sections were immunostained with primary antibodies against von Willebrand factor (vWF) and smooth muscle actin (SMA; Abcam), and visualized using a horseradish peroxidase-based detection kit (EnVision, Dako, Glostrup, Denmark). To quantify the capillary and arteriole densities, the vWF- and SMA-stained vascular structures were counted, respectively, in a blinded fashion.

2.9. Statistical analysis

All data are expressed as mean ± standard deviation. The one-tailed Student *t* test was performed to assess differences between pairs of groups, whereas one-way analysis of variation (ANOVA) was followed by the Bonferroni *post hoc* test to assess variations among three or more groups. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

One of the main challenges that are encountered in cell-based strategies for therapeutic angiogenesis is to ensure the transplanted cells to reside in ischemic tissues. In this study, we hypothesize that a receptive cell environment in the target tissue can be realized by reducing its regional oxidative stress in the initial stage of cell transplantation using an antioxidant. Based a mouse model of hindlimb ischemia, this study demonstrates that the concurrent delivery of antioxidant NAC with HUVEC/cbMSC aggregates can significantly enhance the retention of engrafted cells and their ability to induce therapeutic angiogenesis when compared with their counterparts without NAC, improving the salvaging of the limb.

3.1. Cytotoxicity and antioxidant capacity of NAC

To determine the maximum concentration of NAC that can be used for cell transplantation, the cytotoxicity of NAC toward HUVECs and, separately, cbMSCs was assessed using the MTT assay. According to Fig. 2a, the NAC-induced cytotoxicity was undetectable in both types of cells up to a concentration of 5 mM, which was therefore utilized for the subsequent experiments.

The capacity of NAC (5 mM) to scavenge ROS was evaluated: H_2O_2 was employed to mimic the ROS that were generated during tissue ischemia [16,17]. Owing to the lack of any other ROS in the *in vitro* environment, the concentrations of H_2O_2 that were used herein were significantly exceeded that in ischemic tissues (*ca.* 0.05 mM) [16,35]. As displayed in Fig. 2b, treatment with NAC considerably reduced the amount of H_2O_2 *in vitro* ($P < 0.01$), indicating the capacity of NAC to scavenge H_2O_2. These experimental results suggest that NAC may reduce the environmental oxidative stress following delivery into ischemic tissues.

3.2. NAC restores ROS-impaired cell adhesion

The HUVEC/cbMSC aggregates that were prepared using the MC hydrogel-based system were spherical with a diameter of around 300 μm. Our previous results revealed that most cells within these

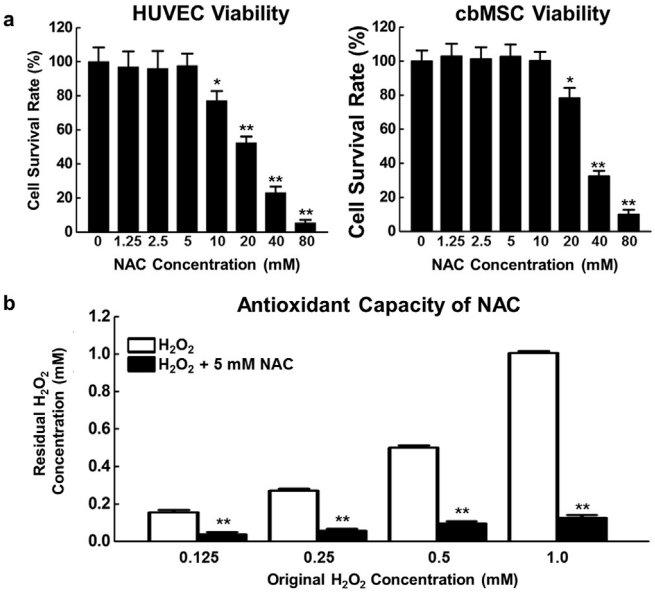

Fig. 2. (a) Cytotoxicity of NAC toward HUVECs and cbMSCs (n = 6). $^*P < 0.05$, $^{**}P < 0.01$ *vs.* untreated cells. (b) Antioxidant capacity of NAC in scavenging H_2O_2 (n = 5). $^{**}P < 0.01$ *vs.* H_2O_2-treated group.

cell aggregates were viable [10,11]. Since cell adhesion is a prerequisite for subsequent cell retention and survival following their being transplanted *in vivo*, the restoration of ROS-impaired cell adhesion by NAC was investigated. In this investigation, the prepared HUVEC/cbMSC aggregates were transferred into fibronectin-coated culture plates and supplied with H_2O_2 and NAC. Fibronectin is an important ECM protein that is present in all skeletal muscles [23]. Untreated cells and cells that were treated with NAC or H_2O_2 alone were used as controls.

Like the untreated cells (Control), the cells that received NAC migrated out of the aggregate and attached on the plate surface, proliferating over time, suggesting that NAC treatment did not disturb the normal cellular functions of the seeded cells (Fig. 3a). Conversely, the cell aggregate that was treated with H_2O_2 could not spread over the culture plate, indicating that the exogenous ROS inhibited the adhesion and growth of the cells. Notably, upon treatment with NAC, the H_2O_2-inhibited cell adhesion and proliferation could be effectively restored.

One of the best-characterized structures in adherent cells is focal adhesion, which is an integrin-based structure that mediates cell–ECM adhesion and bidirectionally transmits information [18,36,37]. During cell adhesion, the focal adhesion-associated adaptor proteins, paxillin and vinculin, recruit other cytoplasmic proteins, causing the assembly of focal adhesions that connect cells to ECM [24,25]. To elucidate the mechanism that underlies the ROS-mediated impairment of cell adhesion, the cytoplasmic patterns of paxillin and vinculin in the early stage of cell adhesion were investigated.

Following immunofluorescence staining, multiple stained spots of paxillin (Fig. 3b) and vinculin (Fig. 3c) were observed in the optical sections that corresponded to the plane of cell adhesion in the untreated control group and the group that received NAC, indicating the success of the adherent cells in establishing focal adhesions. In the H_2O_2-treated group, however, diffuse patterns of paxillin and vinculin were detected, suggesting that the extracellular ROS hindered the cell adhesion *via* disrupting the formation of focal adhesions. In contrast, in the presence of NAC, the H_2O_2-mediated disruption of focal-adhesion formation was recovered (Fig. 3d), probably because NAC effectively eliminated the extracellular oxidative stress (Fig. 2b).

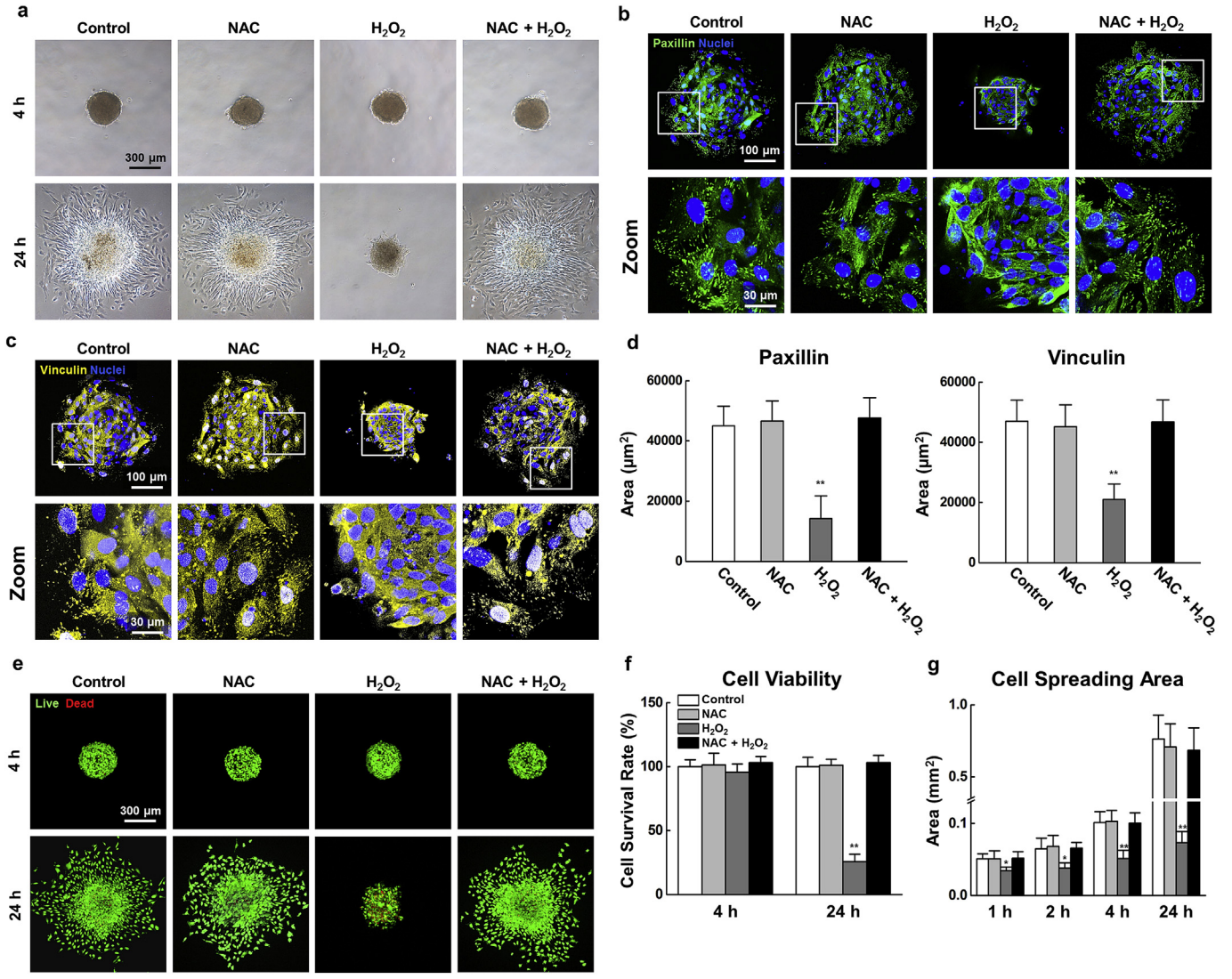

Fig. 3. Cell adhesion and spreading of HUVEC/cbMSC aggregates that were seeded into fibronectin-coated culture plates under various treatment conditions. (a) Representative photomicrographs of test cell aggregates. (b & c) Cytoplasmic patterns of focal adhesion-associated adaptor proteins paxillin and vinculin and (d) their corresponding quantitative results in early stage of cell adhesion. Lower panels magnify white box in corresponding upper panel. (e) Representative CLSM images of live/dead staining of seeded cell aggregates; corresponding (f) cell viability, evaluated using MTT assay (n = 6) and (g) cell spreading area (n = 15). $^*P < 0.05$, $^{**}P < 0.01$ *vs.* untreated control group.

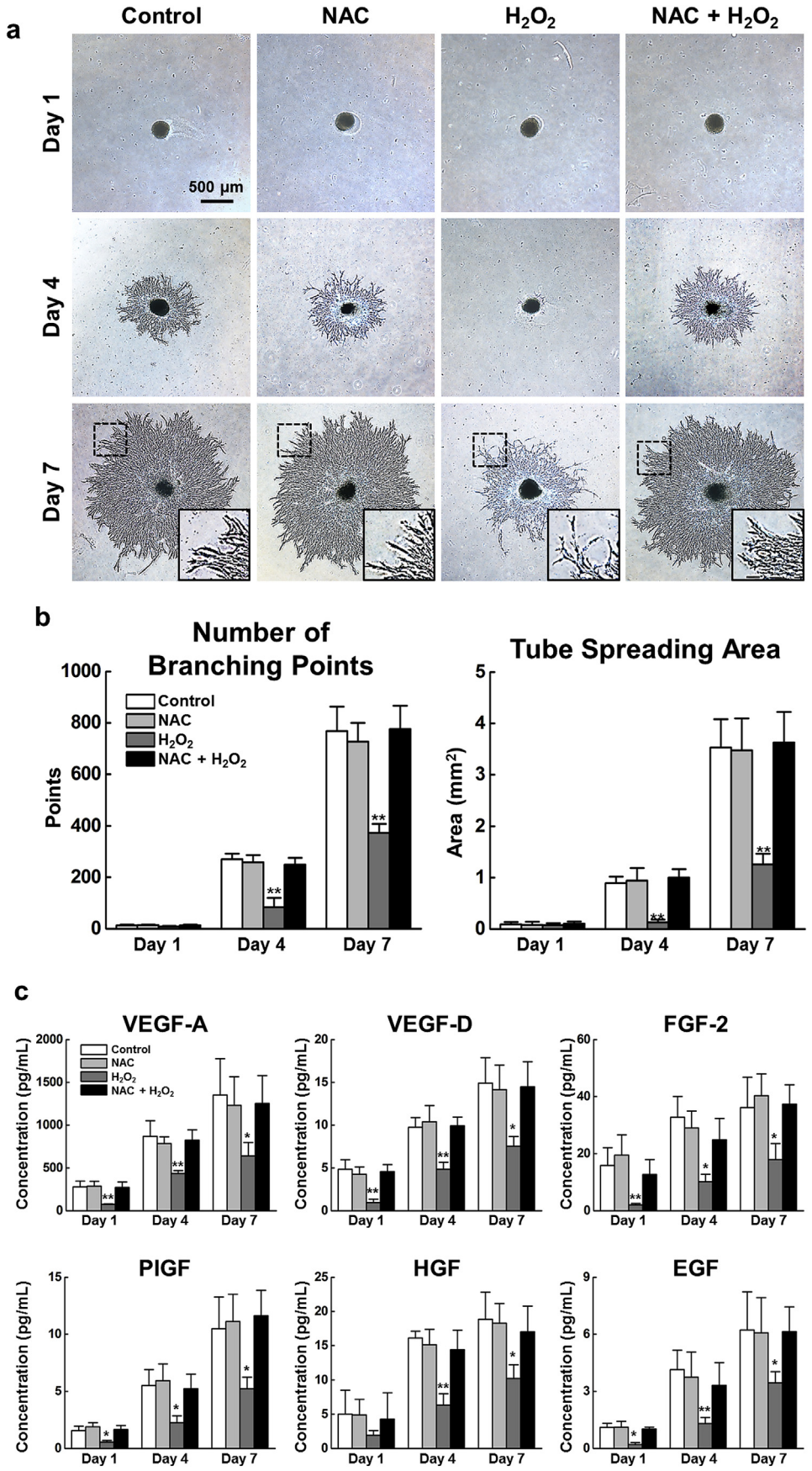

Fig. 4. Angiogenic capacity of HUVEC/cbMSC aggregates, evaluated using Matrigel tube formation assay. (a) Representative photomicrographs of tubular networks that were formed by cell aggregates on Matrigel, taken at predetermined times and corresponding (b) number of branching points and area of tube spreading (n = 6) and (c) levels of pro-angiogenic and pro-survival factors that were secreted into culture media (n = 6). Insets show magnified views. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* untreated control group.

The survival and spreading of anchorage-dependent cells that have been seeded in a new environment require favorable cell–ECM interactions *via* focal adhesions [7]. The formation of too few focal adhesions can result in suboptimal cell–ECM contacts, ultimately leading to cell apoptosis, which is known as anoikis [17]. The cell viability in each studied group was evaluated using a live/dead staining method and the MTT assay, and the area over which viable cells spread was estimated using an image analysis system.

The results of live/dead staining four hours following seeding of the cell aggregate showed that the majority of the cells in the H_2O_2-treated group were viable, suggesting that H_2O_2 treatment did not induce massive acute cell death (Fig. 3e). At 24 h, however, the H_2O_2-treated cells exhibited significantly reduced cell viability (Fig. 3e and f) and they were spread over a smaller area (Fig. 3g) than the other test groups ($P < 0.01$). The decreases in cell viability and spreading area following H_2O_2 treatment could be efficiently reversed to the control levels by treatment with NAC ($P > 0.05$). The above experimental data clearly demonstrate that the antioxidant NAC can restore the ROS-impaired cell adhesion and spreading of HUVEC/cbMSC aggregates, indicating its potential for augmenting the retention and survival of cells following their transplantation in ischemic tissues.

3.3. Tube formation assay

As demonstrated in our earlier investigations, HUVEC/cbMSC aggregates can induce substantial therapeutic angiogenesis *via* both the direct formation of new blood vessels and the indirect paracrine secretion of growth factors following intramuscular transplantation [10,12]. Since angiogenesis is strongly associated with cell adhesion [38], the oxidative stress that is generated in ischemic tissues may be expected to impair the pro-angiogenic activity of the engrafted HUVEC/cbMSC aggregates. The *in vitro* Matrigel tube-formation assay was conducted to evaluate whether the angiogenic potential of HUVEC/cbMSC aggregates would be suppressed by H_2O_2 and be restored by NAC treatment.

The network that was formed from the cell aggregate that was treated with H_2O_2 had significantly fewer branching points and a smaller spreading area ($P < 0.01$) than the long-lasting capillary-like networks that were formed by the untreated cell aggregate or that received NAC, indicating the ROS-mediated impairment of its angiogenic capability (Fig. 4a and b). Conversely, co-treatment with NAC recovered the ability of the H_2O_2-treated cell aggregate to form tubes.

The levels of growth factors that were released from test cell aggregates during their formation of tubes were determined to evaluate the effects of ROS on the indirect paracrine secretions. According to Fig. 4c, treating HUVEC/cbMSC aggregates with H_2O_2 significantly reduced the secretions of the pro-angiogenic factors VEGF-A, VEGF-D, FGF-2, and PlGF and pro-survival factors HGF and EGF ($P < 0.05$). The reduced secretions of these paracrine factors are attributed to the decreases in cell adhesion and tube formation of test cell aggregates under oxidative stress. In contrast, the concentrations of the paracrine growth factors that were secreted into the culture media in the presence of both H_2O_2 and NAC were comparable to those of the untreated control or of the cell aggregates that had received only NAC. These experimental results reveal that the reduced effectiveness of HUVEC/cbMSC aggregates in stimulating angiogenesis in an oxidative environment can be sufficiently restored by treatment with the antioxidant NAC.

When transplanted into ischemic tissues, HUVEC/cbMSC aggregates under oxidative stress may not be able to adhere properly to be retained in the therapeutic target and exert their pro-angiogenic effects [9]. By scavenging the regional ROS, NAC can reestablish a receptive cell environment, promoting the adhesion, retention and survival of cells of the engrafted HUVEC/cbMSC aggregates, which may therefore subsequently induce therapeutic angiogenesis, ultimately enhancing blood flow recovery.

3.4. In vivo reduction of oxidative stress by NAC

In cell-based strategies, initial cell retention is crucial to ensure the subsequent outcome of treatment, as only retained cells can perform their therapeutic functions on the target tissue [7,9]. The potential of NAC to reduce regional oxidative stress in ischemic tissues and promote the retention of cells of the engrafted HUVEC/cbMSC aggregates was investigated in a mouse model with experimentally generated hindlimb ischemia. The cell aggregates and NAC were concurrently injected into the ischemic limb in mice; animals that received only saline (untreated control), NAC or cell aggregates alone served as controls.

Tissue samples were harvested at 24 h after cell administration and then immunostained to identify DNA damage (8-OHdG) and lipid peroxidation (4-HNE) in the cells that had been injured by oxidative stress. The numbers of 8-OHdG- and 4-HNE-positive cells in the group to which the cell aggregates and NAC had been concurrently delivered was much lower than those in the group that had received HUVEC/cbMSC aggregates alone (Fig. 5a). A similar result was obtained for levels of tissue MDA, which is another oxidative stress marker of lipid peroxidation, as determined by a colorimetric assay ($P < 0.05$; Fig. 5b). These experimental results suggest that NAC effectively scavenges ROS and reduces the oxidative stress in ischemic tissues, potentially enhancing the retention of transplanted cells.

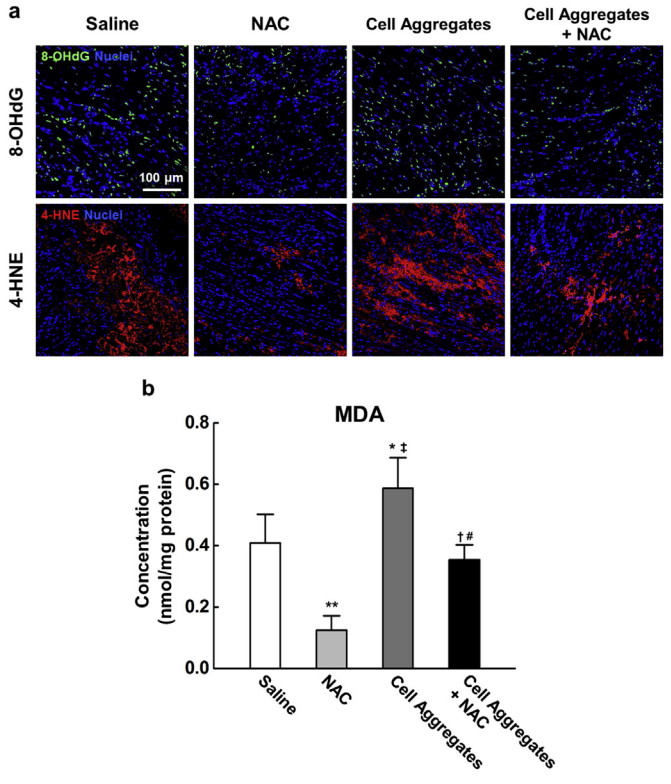

Fig. 5. Oxidative stresses in ischemic limbs following various treatments. (a) Representative immunofluorescence images of oxidative stress markers 8-OHdG and 4-HNE and (b) levels of tissue MDA (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* saline-treated group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ *vs.* NAC-treated group; $^{\#}P < 0.05$ vs. cell-aggregate-treated group. 8-OHdG: 8-hydroxy-2′-deoxyguanosine; 4-HNE: 4-hydroxynonenal; MDA: malondial dehyde.

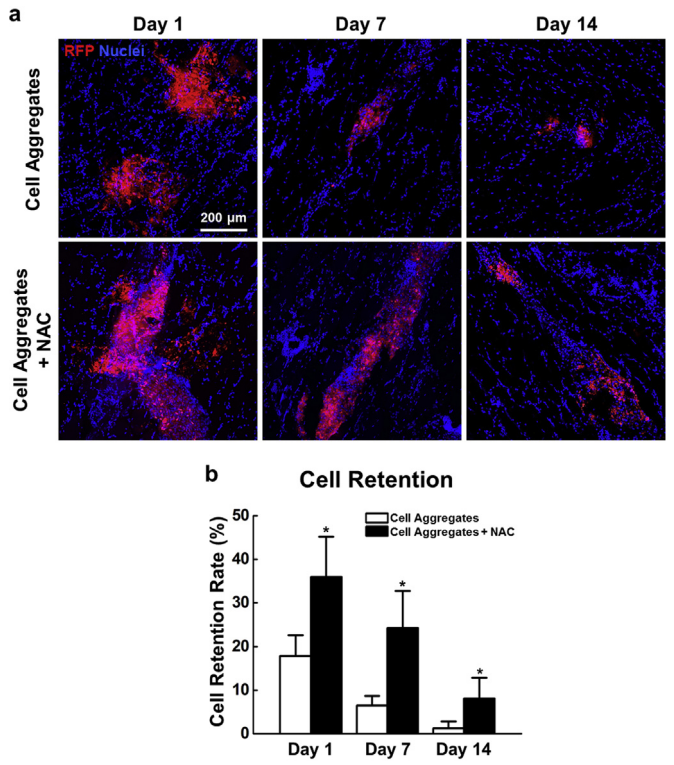

Fig. 6. Retention of cells of transplanted HUVEC/cbMSC aggregates in ischemic limbs. (a) Representative CLSM images of red fluorescent protein (RFP) that is expressed by engrafted cbMSCs. (b) Rate of retained human cells, as determined by quantitative polymerase chain reaction (n = 5). $^{*}P < 0.05$ *vs.* cell-aggregate-treated group.

3.5. Enhancement of cell retention by NAC

To evaluate the cell retention of HUVEC/cbMSC aggregates that were engrafted into ischemic limbs, the RFP that was expressed by cbMSCs was tracked using a CLSM. As displayed in Fig. 6a, more RFP-positive cells were presented in CLSM images following the concurrent delivery of cell aggregates and NAC than following treatment with cell aggregates alone. The PCR results concerning human Alu sequences, which represent engrafted human cells in the mouse tissue, supported the enhancement of cell retention by NAC ($P < 0.05$; Fig. 6b). NAC probably improves cell retention by its antioxidant activity (Fig. 2b).

Despite the encouraging improvement of cell retention rate after the concurrent delivery of NAC, the long-term survival of exogenously engrafted cells remains limited, as only 8.1% of the transplanted cells can be found on day 14 (Fig. 6b). Upon treatment with NAC, the oxidative stress in target ischemic tissues can be eliminated significantly, resulting in improved cell retention in the initial stage of transplantation. However, after the depletion of NAC, the oxidative stress caused by the inflammatory environment in ischemic tissues may raise again, thereby adversely influencing the survival of the engrafted cells.

3.6. Therapeutic effects

To explore the effects of the concurrent delivery of HUVEC/cbMSC aggregates and NAC on therapeutic angiogenesis in ischemic limbs, the blood flows of test animals were assessed noninvasively using SPECT on day one (baseline) and day 14 following treatment. At the baseline, ligation of the femoral artery drastically reduced blood perfusion in the affected limb (Fig. 7a) in all surgically treated mice, as verified by a mean perfusion ratio of 0.20 ± 0.01 (Fig. 7b). The animals that received only saline

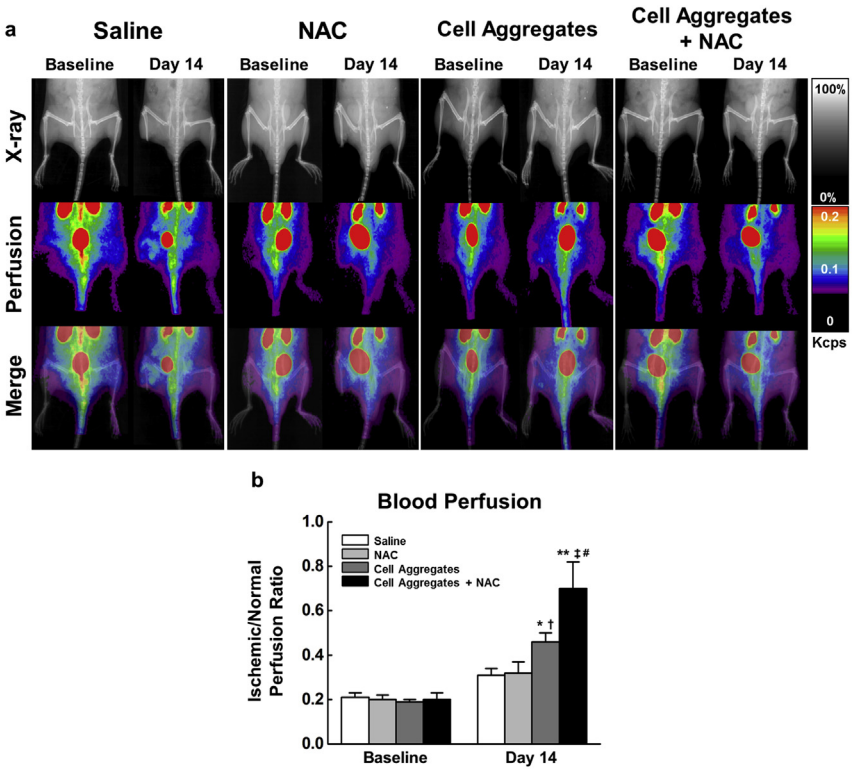

Fig. 7. Blood flow recovery, assessed by SPECT. (a) X-ray and perfusion images and (b) perfusion ratio of test animals on day one (baseline) and day 14 after various treatments (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* saline-treated group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ *vs.* NAC-treated group; $^{\#}P < 0.05$ vs. cell-aggregate-treated group.

(untreated control) or NAC exhibited limited recovery of blood perfusion 14 days after treatment, suggesting that NAC did not contribute to the restoration of blood flow. In contrast, the perfusion ratio that was observed in the ischemic limb that received HUVEC/cbMSC aggregates was significantly increased. Notably, the concurrent delivery of cell aggregates and NAC further accelerated blood flow recovery (Fig. 7a, b and Supplementary Video 1). These analytical results indicate that the extent of restoration of perfusion in the ischemic limb was enhanced by treatment with the cell-aggregate alone and improved even more by the combined therapeutic approach that involved the cell-aggregate and NAC.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.09.043.

The morphologies of the ischemic limbs in each studied group were grossly examined and photographed at the baseline (on day 1) and on day 14 following treatment. According to Fig. 8a and b, the mice that received saline or NAC alone underwent complete limb loss by autoamputation. Conversely, treatment with HUVEC/cbMSC aggregates increased limb salvaging, although foot necrosis and muscle atrophy were observed in the test animals. Notably, concurrent delivery of the cell aggregates and NAC resulted in substantial recovery of the ischemic hindlimb, such that tissue degeneration was limited to the toes. Immunohistological staining of the tissue sections to visualize vWF and SMA was conducted to identify the capillaries and arterioles, respectively. As shown in Fig. 8c and d, combined treatment with HUVEC/cbMSC aggregates and NAC increased capillary and arteriole densities in the affected

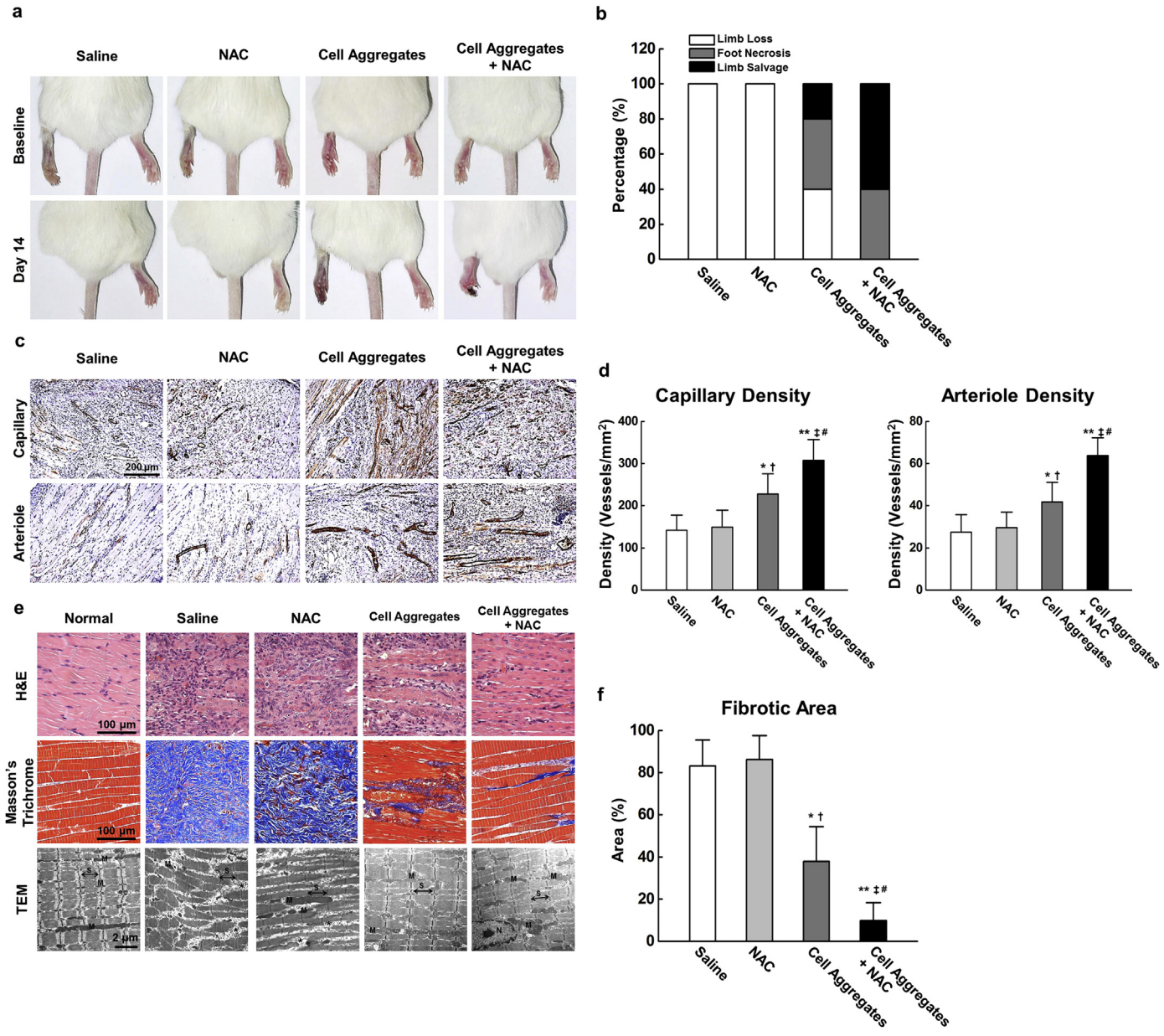

Fig. 8. Postmortem morphometric and histological analyses following various treatments. (a) Gross appearances of test mice at baseline and on day 14 after treatment. (b) Physiological status of ischemic limbs on day 14 after treatment. (c) Representative immunohistochemistry images of vWF (capillary) and SMA (arteriole) and (d) corresponding quantitative results (n = 5). (e) Representative H&E and Masson's trichrome staining images and TEM micrographs of retrieved limb tissues. S: sarcomere; N: nucleus; M: mitochondria. (f) Fibrotic areas quantified according to the results of Masson's trichrome staining (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* saline-treated group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ *vs.* NAC-treated group; $^{\#}P < 0.05$ vs. cell-aggregate-treated group.

limb more than did treatment with cell aggregates alone ($P < 0.05$).

Results of the H&E and Masson's trichrome staining revealed that the ischemic tissues in the control groups that received only saline or NAC suffered from considerable muscle degeneration and tissue fibrosis (Fig. 8e and f). However, both delivery of HUVEC/cbMSC aggregates alone and the concurrent transplantation of the cell aggregates and NAC effectively reduced tissue injury, maintaining muscle integrity. The ultrastructure of the myocytes in the affected limbs was also evaluated using TEM (Fig. 8e). Ischemic injuries in saline or NAC-treated animals contained many degenerating myocytes with swollen and degenerated mitochondria and poorly preserved sarcomeres. The transplantation of HUVEC/cbMSC aggregates supported the restoration of myofibrils although some continued to exhibit a degree of cytoplasmic edema. The most advantageous treatment regimen involved the co-administration of cell aggregates and NAC. Most of the myocytes contained parallel arrays of intact sarcomeres as well as interspersed mitochondria of various sizes. The above results clearly demonstrate that the intramuscular administering of a combination of HUVEC/cbMSC aggregates and NAC caused limb salvaging with attenuated tissue damage owing to the therapeutic angiogenesis and restoration of sufficient blood flow to maintain tissue viability.

4. Conclusions

This study demonstrates the feasibility of using the antioxidant NAC in the early stage of cell transplantation to scavenge regional ROS that are generated in ischemic tissues, creating an environment that is receptive to engrafted cells. The NAC-mediated reduction of oxidative stress significantly enhances the adhesion, retention, and survival of cells following the transplantation of HUVEC/cbMSC aggregates, promoting therapeutic angiogenesis and ultimately resulting in salvaging of the limb.

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