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Brief Article

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Novel TEMPO-PEG-RGDs Conjugates Remediate Tissue Damage Induced by Acute Limb Ischemia/Reperfusion

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Supporting Information

ABSTRACT: We have recently developed new Tempo-PEG-RGDs conjugates and have quantitatively examined their antithrombotic and antioxidant capabilities. These compounds were therapeutically beneficial when characterized in both in vitro platelet aggregation assays and a rat model of arterial thrombosis. Moreover, these compounds demonstrated significant protection from organ damage in a rat model of ischemia/reperfusion. Our data indicate that Tempo-PEG-RGDs represent a new class of adjuvants with therapeutic efficacy in acute and transient ischemic damage.

INTRODUCTION

Affecting more than 200000 patients annually, acute ischemia of the lower limbs is frequently encountered in multiple disease states and surgical procedures, including acute atherosclerotic thrombosis, traumatic arterial injuries, and thromboembolic events.[1] Acute limb ischemia is a two-stage process, in which the early stage manifests as continued growth of the arterial thrombus, resulting in collateral vessel occlusion. The later stage manifests as narrowing and obstruction of the microvasculature due to progressive edema.[2,3] Therapeutic intervention for acute limb ischemia generally entails intraarterial application of thrombolytic agents such as streptokinase, urokinase, recombinant tissue plasminogen activator, and/or reteplase because prompt reperfusion of the ischemic limb is mandatory for the restoration of baseline organ function. Nonetheless, accumulating evidence indicates that the process associates with additional cellular injury, primarily in the form of muscle necrosis and edema that may even progress to systemic complications such as multiple organ dysfunction.[4] Currently, there remain no universally effective treatments for the remote organ injury associated with ischemic reperfusion damage.

Production of the reactive oxygen species (ROS), which are damaging to intracellular biomacromolecules, is significantly enhanced with the onset of reperfusion. Predominantly generated in the mitochondria, ROS result in expansion of a leukocyte-mediated inflammatory reaction that exacerbates reperfusion-induced tissue injury.[1−4] Accordingly, antioxidant supplementation represents a rationale therapeutic approach to mitigate ROS-associated damage.[5] Of the numerous compounds employed therapeutically in this setting, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-*N*-oxyl (Tempo) has gained increasing attention.[6] It has been suggested that the antioxidant capacity of Tempo is structurally related because it is a stable nitroxide radical compound. Additionally, Tempo's antioxidant capacity is likely enhanced because it possesses superoxide dismutase (SOD)-mimicking activity.[6]

Thrombosis, a consequence of capillary occlusion by platelets and neutrophils, is also extensively observed in tissue damaged by I/R events.[4] Thrombotic activity leads to reduced perfusion and exacerbation of tissue injury during reperfusion stage, and a large component of the complications associated with reperfusion damage is thrombosis-related.[4] Accordingly, investigative efforts have been directed toward antithrombotic therapeutic interventions, including the use of the Arg-Gly-Asp (RGD) peptide, a well-known motif contained in integrin ligands. This motif recognizes the platelet membrane integrin-glycoprotein IIb/IIIa (GPIIb/IIIa) receptor, and it has been demonstrated that binding of the latter to fibrinogen mediates platelet aggregation. Because the RGD motif can prevent fibrinogen binding to GPIIb/IIIa on the surface of activated platelets, the use of this motif may provide an antithrombotic therapeutic strategy via inhibition of platelet aggregation.[7] Additionally, polyethylene glycol (PEG; FDA-approved for several human applications) also manifests beneficial effects in I/R injury by blocking the coagulation system and subsequent reduction of platelet adhesion both in vitro and in vivo.[8] Through formation of a molecular barrier on the glycocalyx, PEG prevents acute platelet deposition on damaged arteries; moreover, grafting of pericardium with PEG inhibits calcium deposits and reduces platelet and leukocyte adhesion.[9] Finally, evidence suggests that PEG repairs cell membranes and inhibits free radical production both in in vitro and in vivo models.[9] On the basis of the above observations, we hypothesized that conjoining RGD sequences with Tempo and PEG would result

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in compounds with enhanced capacity to attenuate oxidative stress and prevent platelet aggregation, thereby leading to reduced thrombotic activity. The current report summarizes the synthesis of these Tempo-PEG-RGDs conjugates, and our ensuing evaluations of antiplatelet aggregation and antithrombotic activities, as well as the antioxidant capabilities, of these new compounds.

Chemistry. Tempo-RGDs and Tempo-PEG-RGDs conjugates were prepared using the benzotriazole-1-yloxy-tris (dimethyl-amino)-phosphoniumhexafluorophosphate (BOP):*N*-hydroxy-benzotriazole (HOBt):diisopropylethylamine (DIEA) (1:1:1) method at room temperature. Peptide segments were synthesized on a Wang resin using standard Fmoc solid-phase peptide synthesis (Figure S1, Supporting Information (SI)). All target species were purified by semipreparative RP-HPLC and structure confirmed by FAB MS. These compounds were further analyzed by EPR spectroscopy to confirm the presence of the intact nitroxide label. The chemical structures of these newly synthesized compounds are shown in Figure S2, SI.

RESULTS AND DISCUSSION

To evaluate platelet aggregation, we employed an antiplatelet aggregation assay, which characterized the activities of the Tempo-RGDs and Tempo-PEG-RGDs conjugates. This assay utilized an in vitro rabbit platelet aggregation system, which was induced either by adenosine 5-diphosphate (ADP) or the platelet activating factor (PAF).[10] Previous studies indicate that the formation of platelet aggregates is associated with free radical generation, and that these free radicals then trigger oxidative stress in platelets.[9] These studies also indicated that the interaction of ADP with platelets stimulates reducing activity in the platelet membrane as well as a significant increase in the number of sulfhydryl groups on the platelet surface. The presence of such highly reactive sulfhydryl groups is noteworthy, as the sulfhydryl groups are capable of reducing nitroxide to hydroxyamines.[9] In the present study, we observed that all of the tested compounds (except Tempo) possess some level of antiplatelet aggregation capacity. In particular, Tempo-PEG-RGDs (final concentration, 100 nM; incubation time 120 min) in vitro with ADP or PAF in the presence of platelets significantly altered the induction of platelet aggregation (Tables S1 and S2, SI). Compared to Tempo-PEG-RGDs, Tempo-RGDs exhibit antiplatelet aggregation activity to a lesser extent. In addition to the antiaggregation contributions from RGDs fragments, N−O$^{\bullet}$ group exerted SOD mimetic action[6] and was also responsible for the antiplatelet aggregation activity. Thus, we propose that the inhibition of nitroxide-RGDs conjugates on the platelet aggregation may originate from a synergistic effect. In addition, the different reactivity between Tempo-RGDs and Tempo-PEG-RGDs is likely affected by the alterations in the microenvironment. We hypothesize that these alterations are due to conformational changes, which result from the binding of ADP.

Following the previous work, we evaluated the antithrombotic effects of Tempo-RGDs and Tempo-PEG-RGDs using an in vivo rat model as previously described,[10] in which the weights of thrombi were determined as outcome measures (Table S3, SI). Thrombi weight following treatment with Tempo-RGD (S, V, F) was 25.9 ± 2.6, 25.1 ± 1.9, and 22.3 ± 2.2 mg (NS: 40.1 ± 2.2 mg), respectively. It is noteworthy that the antithrombotic activities of Tempo-PEG-RGD (S, V, F) (wet thrombi wt: 23.6 ± 2.8, 24.7 ± 2.2 and 20.7 ± 2.5 mg, respectively) were higher than aspirin (27.6 ± 2.2 mg) at equivalent amounts (5 μmol/kg). The accurate mechanism(s) of the antithrombotic activities of our newly synthesized Tempo-peptide conjugates remains to be determined, although their antiaggregation capacities, which were stimulated by various agents, suggests a common signaling pathway in the cascade of platelet aggregation.

In the present study, we have demonstrated that Tempo-RGDs and Tempo-PEG-RGDs conjugates have potential free radical scavenging activities in an acetylcholine-induced relaxation of the rat thoracic aorta assay. Ach-induced vasodilatation may result from an increased production of $ONOO^-$ (formed from the interaction between ROS and NO$^{\bullet}$).[9] In vessels, NO$^{\bullet}$ is released mainly from the endothelium and spreads to the smooth muscle, which leads to vasorelaxation. Although myocytes and neutrophils also produce NO$^{\bullet}$, the vascular endothelium is an important site for NO$^{\bullet}$ synthesis and is responsible for the regulation of NOS activity.[9] Endothelial cells also generate O_2^- when stimulated by cytokines, hypoxia-reoxygenation, and ischemia-reperfusion. O_2^- overproduction in the vascular endothelium contributes to vasodilation.[9] It is known that O_2^- overproduction coupled with NO$^{\bullet}$ formation can lead to $ONOO^-$ generation. $ONOO^-$ is a physiologically active toxic metabolite of NO$^{\bullet}$ that can lead to vascular and myocardial dysfunction.[9] As shown in Table S4, SI, we observed that Tempo is a weak inhibitor of acetylcholine-induced vasorelaxation. In contrast, acetylcholine-induced relaxation could be significantly reversed by Tempo-RGDs or Tempo-PEG-RGDs conjugates, and their inhibition effects are dose-dependent. We also observed that Tempo-PEG-RGDs are more effective inhibitors of acetylcholine-induced relaxation than Tempo-RGDs. However, there were no statistically significant differences between these groups.

Because increased superoxide and NO$^{\bullet}$ formation are vital for the initiation of ischemia/reperfusion (I/R) injury, blockade or scavenging of either NO$^{\bullet}$ or superoxide most likely would prevent reperfusion injury and enhance the recovery of organs. Although Tempo can scavenge superoxide anion to give hydroxylamines, the half-life of Tempo in blood is only 15 s.[6] The rapid clearance of Tempo from the bloodstream is most likely due to fast renal clearance and the rapid reduction of the Tempo radical form to the corresponding hydroxylamine form in the blood. In the present study, we found that PEGylation seems to be able to slow down the reduction of the Tempo radical. From the predicted lowest energy conformations of Tempo-PEG-RGDs (Figure S2, SI), we can visualize that the Tempo radical sits in the center of the Tempo-PEG-RGDs conjugate. We therefore propose that when the Tempo radical is surrounded by a long PEG chain, an increase in reduction resistance occurs, which may lead to prolonged blood circulation time and enhanced beneficial effects of Tempo-PEG-RGDs conjugates.

Nitroxides undergo one electron reduction and oxidation, which is the key feature of their antioxidant characteristics. The free radical scavenging effect of nitroxide derivatives is correlated with selected molecular and biochemical parameters such as the highest occupied molecular orbital (HOMO) energy, the net charge, and the difference in heat of formation between hydroxylamine and its radical. Along these lines, the ionization energy of the HOMO can be employed as a measure of an antioxidant's capacity to participate in radical scavenging. For example, the HOMO energy of melatonin (a natural antioxidant) is −10.425 eV. In comparison, the HOMO energy of Tempo-PEG-RGDs is as follows: $E_{\text{HOMO (Tempo-PEG-RGDS)}}$ = −5.804 eV, $E_{\text{HOMO (Tempo-PEG-RGDV)}}$ = −5.908 eV, and

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$E_{HOMO\ (Tempo\text{-}PEG\text{-}RGDF)}$ = −5.596 eV. As a general rule, the higher the HOMO energy, the more active the compound is as an antioxidant. Theoretically, Tempo-PEG-RGDs should possess higher radical trapping potential than melatonin. Among these Tempo-PEG-RGDs conjugates, Tempo-PEG-RGDF is generally viewed as the most active free radical scavenger. However, our research indicates Tempo-PEG-RGDF exhibits better antiaggregation, antithrombotic, and free radical scavenging activities than Tempo-PEG-RGD (S, V). However, there were no statistically significant differences between these groups.

To further investigate above findings, we employed a rubber band tourniquet model to induce limb I/R damage in rats, an animal model widely employed to mimic the clinical setting of acute I/R damage. This model has clinical relevance because tourniquet application is broadly employed in a variety of surgical protocols in order to ensure a bloodless surgical field. The literature also indicates that free oxygen radicals are produced in the ischemic tissue, and upon tourniquet release, these radicals facilitate endothelial injury, increased micro-activated permeability, and tissue edema in addition to activated adhesion molecules and cytokines that may underpin a systemic inflammatory response.[1] In the current protocol, 3 h of unilateral lower limb ischemia was followed by 4 h of reperfusion, after which time the tourniquet was released. This study design was employed to examine a representative compound, Tempo-PEG-RGDF, as a mitigator of local and remote organ injury. We employed a laser Doppler imager to assess limb I/R. Figure 1

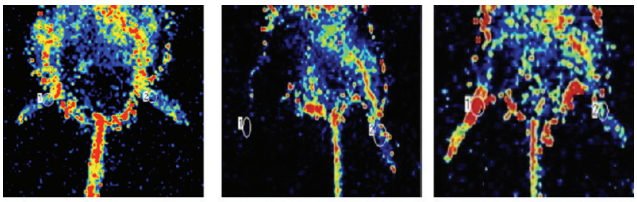

Figure 1. Representative baseline (A), ischemia (B), and reperfusion (C) laser images. The orange outline of the major lower extremity vessels and limbs is clearly visible in the laser Doppler images.

shows the interval of ischemia associated with an absence of visible blood vessels in the lower extremity of the ischemic limb. After 3 h of ischemia, however, blood flow of this limb recovered to near baseline flow within minutes of reperfusion, followed by a rapid decrease of blood flow. Doppler imaging further revealed a decrease in blood flow in the nonischemic contralateral limb during this period, and reperfusion following the ischemic interval (3 h) led to a persistent hypoperfusion of the contralateral limb.

Tissue edema, linked to microvascular injury, venous outflow congestion, and a concomitant of I/R damage, was evaluated by the wet/dry (W/D) tissue ratio (Table S5, SI). We observed substantial tissue edema in reperfused limbs (5.60 ± 0.37; sham limbs, 3.18 ± 0.25). Tempo-PEG-RGDF intervention significantly mitigated this muscle edema (3.45 ± 0.21), although the mechanism of edema remains unclear. It is possible that Tempo treatment could alleviate muscle edema to a lesser extent (4.49 ± 0.45).

We also quantified oxidant stress via quantitation of malondialdehyde (MDA; a metabolite of the free radical-mediated lipid peroxidation) and superoxide dismutase (SOD),[11] based upon the hypothesis that tissue I/R would associate with lipid peroxidation and free radical damage. Both MDA and SOD are representative of the tissue levels of free radical and inflammatory response of tissues during I/R damage. In plasma samples from the above animals (Table S6, SI), we found that MDA levels significantly increased following I/R damage as compared to sham animals, and these increases were significantly attenuated with Tempo-PEG-RGDF administration. Hence, we conclude that Tempo treatment could reverse the elevated MDA levels to a lesser extent. SOD inhibits the expression of free radicals and adhesion molecules and thereby exerts a protective effect on muscle I/R injury. We found plasma SOD activity significantly reduced in I/R treated rats (Table S6, SI). SOD represents the primary enzyme antioxidant defense system, and its reduction during I/R likely triggers lipid peroxidation in membranes, an observation consistent with elevated MDA levels. The intervention with Tempo-PEG-RGDF significantly mitigated the reduced SOD activity. Tempo treatment reversed this decrease in activity to a lesser extent.

We speculate that the protective effect of Tempo-PEG-RGDF against lipid peroxidation follows a mechanism similar to other nitroxides. These protective effects include blockade of primary initiation, prevention of peroxide-dependent initiation, and scavenging of membrane-associated radicals.[6] Because nitroxide can maintain iron in the oxidized state and prevent initiation of iron-dependent lipid peroxidation, iron-dependent initiation is blocked. In addition, a mechanism of slowing peroxidation before access to membrane lipids is gained. Moreover, during this process, nitroxide is reduced to a corresponding hydroxylamine, which also represents a radical scavenger that can deter lipid peroxidation. Overall, our results (MDA, SOD, etc) indicate that Tempo-PEG-RGDF is an effective in vivo ROS scavenger.

Cross-sections of skeletal muscle from sham rats (sham cohort) displayed normal architecture (images not shown; H/E: hematoxylin-eosin staining), with a typical perimysium-enclosed fascicle and myofibers of uniform size arranged in a mosaic pattern. Conversely, muscle edema, neutrophil accumulation, and cell necrosis were observed following I/R (Figure 2A–C).

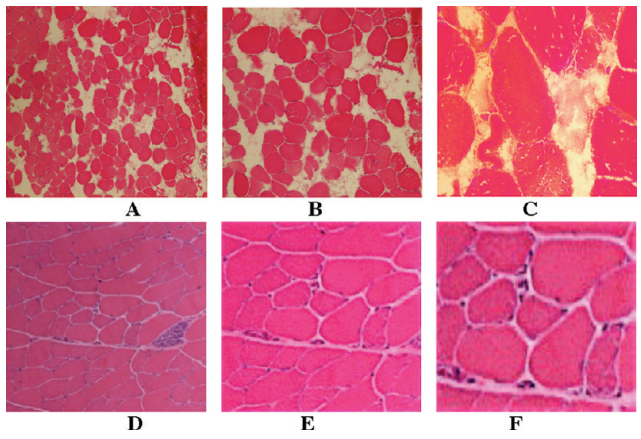

Figure 2. Histology of cross-section of skeletal muscle stained with hematoxylin-eosin. (A–C) Representative muscle tissue sections were obtained from rats exposed to limb I/R.; (D–F) Representative muscle tissue sections were obtained from I/R+ Tempo-PEG-RGDF treated subjects. Magnification: (AD) ×100; (B,E) ×200; (C,F) ×400.

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Modified Gomori trichrome staining revealed interstitial edema, membrane ruptures with dissolution of myofibrils, necrotic and swollen cells, and scattered "ragged red fibers", indicative of widespread cellular damage (Figure 3A–C). In addition, irregular

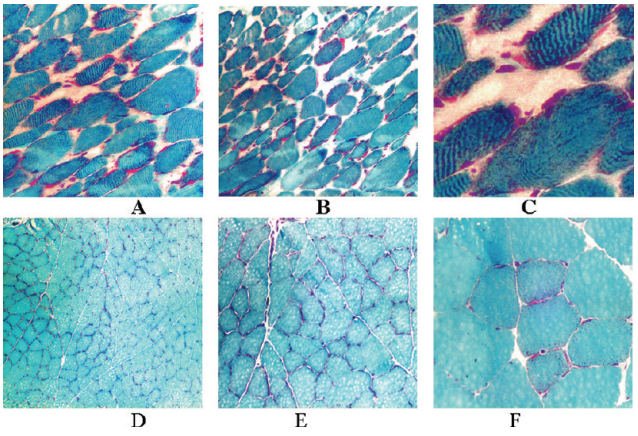

Figure 3. Histology of cross-section of skeletal muscle stained with modified Gomori trichrome. (A–C) Representative muscle tissue sections were obtained from the rats exposed to limb I/R. (D–F) Representative muscle tissue sections were taken from I/R+ Tempo-PEG-RGDF treated subjects. Magnification: (A,D) ×100; (B,E) ×200; (C,F) ×400.

distribution of oxidative metabolism enzymes was obvious in the NADH (nicotinamide adenine dinucleotide)-stained skeletal muscle tissues from I/R group rats (Figure 4A–C). Typically,

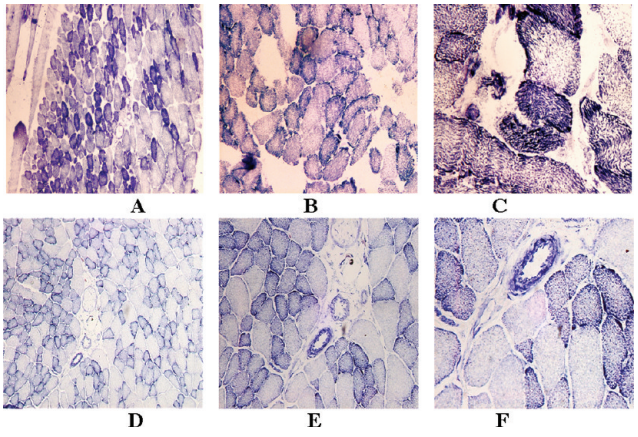

Figure 4. Histology of cross-section of skeletal muscle stained with NADH. (A–C) Representative muscle tissue sections were obtained from the rats exposed to limb I/R. (D–F) Representative muscle tissue sections were taken from I/R+ Tempo-PEG-RGDF treated subjects. Magnification: (A,D) ×100; (B,E) ×200; (C,F) ×400.

slow-twitch muscle fibers stain darkly in NADH stain because they are oxidative and mitochondria-rich. In contrast, fast-twitch fibers stain lighter because they are glycolytic and mitochondria-poor.

Tempo-PEG-RGDF intervention, however, mitigated the majority of these damages, although occasional "ragged red fibers" were still observed (Figure 3D–F) in addition to infrequent vacuolated fibers seen in H/E sections (Figure 2D–F). The majority of muscle fibers were intact, with well-defined edges, consistent texture, and homogeneous uniform morphology. Additionally, NADH reactions were weak in most fibers (Figure 4D–F), reflecting substantial tissue protection associated with Tempo-PEG-RGDF intervention following I/R treatment.

Finally, H/E–stained sections derived from untreated rats displayed normal myocardium architecture, including short myocytes with proportionally enlarged nuclei. In contrast, tissues derived from subjects exposed to I/R revealed edema between wavy muscle fibers and surrounding blood vessels (Figures 5–7A–C) as well as pyknotic nuclei and contraction

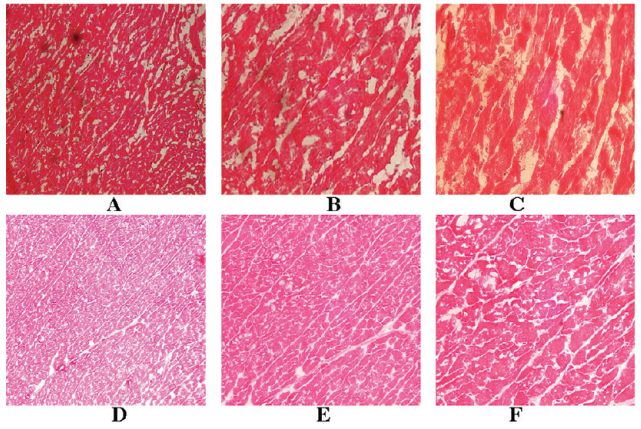

Figure 5. Histology of myocardial tissues stained with hematoxylin and eosin. (A–C) Representative myocardium tissues were obtained from the rats exposed to limb I/R. (D–F) Representative myocardium tissues sections derived from I/R+ Tempo-PEG-RGDF treated subjects. Magnification: (A,D) ×100; (B,E) ×200; (C,F) ×400.

bands. Gomori trichrome staining of these tissues also revealed relaxed collagen fibers surrounding individual myocardial tracts. These phenomenon are associated with disruption of the collagen fibers in the interstitial region and interstitial fluid separating the lateral myocyte arrangement (Figure 6A–C).

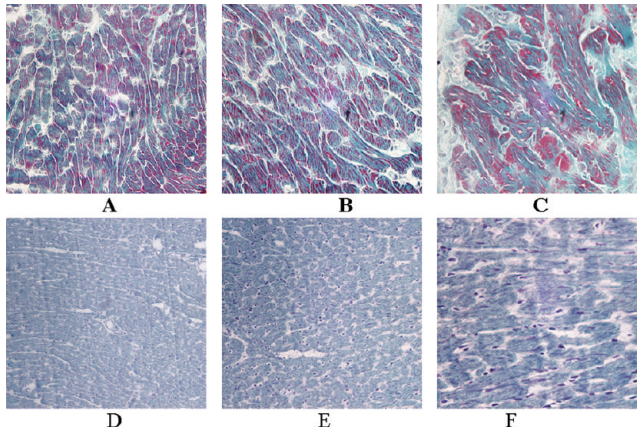

Figure 6. Myocardial tissues stained with modified Gomori trichrome. (A–C) Representative myocardium tissues derived from rats exposed to limb I/R. (D–F) Representative myocardium tissues sections from I/R+ Tempo-PEG-RGDF treated subjects. Magnification: (A,D) ×100; (B,E) ×200; (C,F) ×400.

Tempo-PEG-RGDF intervention with I/R resulted in a tightly woven network of collagen and only mild edema between fibers, representing a considerable improvement over the nontreatment cohort (Figures 5–7D–F).

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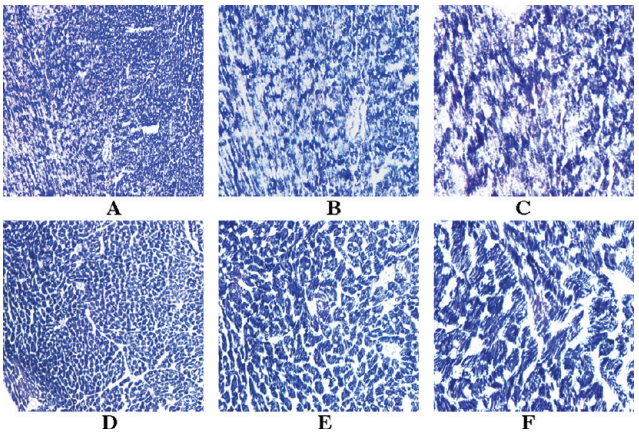

Figure 7. Histology of myocardial tissues stained with NADH. (A–C) Representative myocardium tissues were obtained from the rats exposed to limb I/R. (D–F) Representative myocardium tissues sections derived from I/R+ Tempo-PEG-RGDF treated subjects. Magnification: (A,D) ×100; (B,E) ×200; (C,F) ×400.

CONCLUSION

This research developed novel Tempo-PEG-RGDs conjugates that are active in ADP- or PAF-induced in vitro platelet aggregation assays and are efficacious in rat arterial thrombosis assays. The findings suggest that Tempo-PEG-RGDs conjugates possess potent antiplatelet aggregation and antithrombotic activities. In addition, the free radical scavenging activities of Tempo-PEG-RGDs conjugates were evaluated using a rat aortic strip assay. Tempo-PEG-RGDF (as a representative compound) also displayed a significant capacity to reduce lipid peroxidation as well as mitigation of local and remote organ injury induced by I/R injury. We hypothesize that Tempo-PEG-RGDs likely exert their antithrombotic effects through inhibition of tissue factor synthesis via stimulated human monocytes as well as via the ability of the nitroxide moiety to function as an intracellular scavenger of superoxide anions and hydroxyl radicals. PEGylation may lead to prolonged blood circulation time, thereby enhancing the beneficial effects of Tempo-PEG-RGDs conjugates. Our prediction is that our newly prepared Tempo conjugates will become an important agent for attenuation of I/R injury in the future.

ASSOCIATED CONTENT

Supporting Information

Details of synthetic procedures, characterization of target compounds, and detailed protocols for biological test. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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