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The effect of a nitroxide antioxidant on ischemia-reperfusion injury in the rat *in vivo* hind limb model

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Abstract
Microsurgical procedures such as free tissue transfer or replantations of amputated digits involve an obligatory ischemic period leading to regional tissue oedema, rhabdomyolysis, systemic acidosis, hypercalcemia and multiple organ dysfunction syndrome reflecting ischemia-reperfusion (I/R) injury. Since nitroxide stable radicals act as antioxidants their potential protective effects were tested. Anaesthetized Sabra rats were subjected to regional ischemia of the hind limb for 2 h using a tourniquet. Upon reperfusion rats were injected with 4-OH-2,2,6,6-tetramethylpiperidine-1-oxyl (TPL). Systemic I/R-induced damage was assessed by sampling blood for differential count, lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) serum levels. Regional injury was evaluated by analysing excised muscle samples for oedema (tissue water content) and inflammatory infiltrate (number of cell nuclei in histomorphometric analysis). I/R-induced changes of biomarkers reflecting systemic damage peaked about 8 h following the start of reperfusion and fully disappeared as the biomarkers relaxed to their pre-ischemic values after 24 h. TPL facilitated the recovery of some of these parameters and partially affected release of cellular CPK and LDH. The parameters of I/R-induced regional tissue injury did not demonstrate any recovery and were not inhibited by TPL.

Keywords: *Ischemia/reperfusion, rat hind limb, oxidative stress, nitroxide.*

Background

Obligatory ischemia/reperfusion (I/R)

Microsurgical procedures, such as transfer of free tissues or replantations, involve an obligatory ischemic period which generally lasts 1.5–4 h followed by reperfusion. Ischemia is associated with post-revascularization syndrome, which can lead to both local and general complications such as compartment syndrome, rhabdomyolysis, acidosis, hypercalcemia, hypovolaemic shock, function failure of various organs and even amputation and death. To better restore function of replanted extremities and transplanted muscle, not only should the ischemic period be shorter, but also post-ischemic reperfusion injury of skeletal muscle should be minimized [1]. White cell inhibitors [2], enriched substrates and/or controlled rates of reperfusion have been shown to facilitate recovery of impaired muscles [2] and improve their viability. The utilization of O_2 critically needed following ischemia carries both a blessing and a potential curse since the metabolism of this life-sustaining molecule results in the production of potentially harmful radical and non-radical reactive species. In accordance with the model of site-specific damage, the physiological defense as well as common

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strategies for intervention are based upon dismutation of $O_2^{\cdot -}$, binding of redox-active metals, scavenging of $^{\cdot}OH$ and other radicals and removal of H_2O_2. The key role played by radical species [3,4] and metal ions [5] in the I/R injury is well documented [6]. Scavengers of radical species [7–13], antioxidative enzymes [7–9] and chelators of redox-active metals [14] have been tested using several experimental models in an attempt to attenuate I/R injury [15] after prolonged ischemia and variable (1–3 h) periods of reperfusion.

McCord and Fridovich [16] showed the protective effect of SOD against oxidative damage, whereas other studies [9], though not all [7,8], demonstrated partial protection by SOD and catalase also against damage triggered by tourniquet-induced ischemia in rat limb. Obviously the short half-life in the blood of exogenously added SOD and its inability to penetrate into the cell limited the potential of such treatment and led to a search for cell permeable SOD-mimics [17]. Like the catalytic dismutation of $O_2^{\cdot -}$ by SOD, which involves an alternating reduction and oxidation of the transition metal of the metalloenzyme in a 'ping-pong' like mechanism, various complexes of iron, manganese and copper, which readily undergo redox reactions, are reportedly effective treatment in various pathological conditions [18,19]. Yet, such metal complexes are prone to dissociation in the cell and consequently can be rendered inactive.

Nitroxide antioxidants

An alternative strategy of intervention involves the use of cyclic nitroxides, which are synthetic stable radicals ($RNO^{\cdot}$) that 'shuttle' among three oxidation states, by undergoing 1-electron transfer reactions. They are readily reduced to hydroxylamines (RNOH) or oxidized to oxoammonium cations ($RN^+=O$). Consequently, all three forms can be present in the tissue, as shown for TPL in Scheme 1.

In the presence of a 2-electron reductant, such as NADPH, $RN^+=O$ can be directly reduced to RNOH [20]. In the tissue, nitroxides are rapidly reduced, predominantly in the mitochondria through enzyme-associated mechanisms, yielding almost exclusively the respective RNOH [21–24]. The oxidation of RNOH to $RNO^{\cdot}$ can also occur at high rates [22–24], thus achieving distribution of $RNO^{\cdot}$/RNOH, irrespective of which of the two forms is administered.

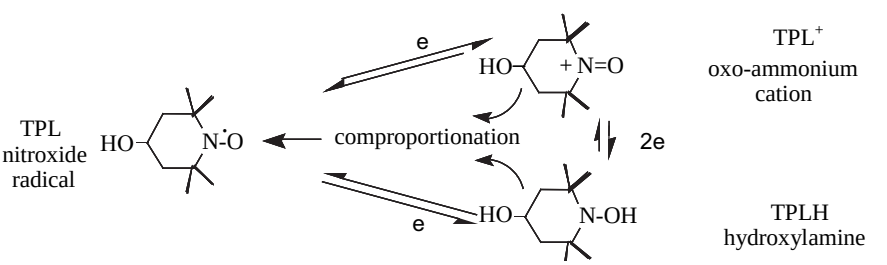
SOD-mimic activity of nitroxides

Nitroxides were found to catalyse removal of superoxide by flip-flopping between two oxidation states, both capable of reacting with $HO_2^{\cdot}/O_2^{\cdot -}$. Unlike most enzyme/substrate systems, where the substrate concentration far exceeds that of the enzyme, the cellular level of SOD exceeds about 10^6-fold that of its substrate $HO_2^{\cdot}/O_2^{\cdot -}$. Similarly, $RNO^{\cdot}$ catalyses dismutation of $HO_2^{\cdot}/O_2^{\cdot -}$ via a mechanism, which involves the $RN^+=O$ intermediate [20]:

$$RNO^{\cdot} + HO_2^{\cdot} \rightleftharpoons RN^+=O + HO_2^{\cdot} \quad (1)$$

$$RN^+=O + O_2^{\cdot -} \rightarrow RNO^{\cdot} + O_2 \quad (2)$$

Because nitroxides readily enter cells and are neither immunogenic nor cytotoxic, they could substitute for and augment SOD protective activity both extra- and intracellularly. Subsequent studies showed indeed that they protect cells and laboratory animals from oxidative stress, induced by diverse insults such as: H_2O_2, hypoxanthine/xanthine oxidase, organic peroxides [25–27], cytotoxic xenobiotics [28,29], tumour necrosis factor [30] ionizing radiation [25,31–34], ulcerative reagents [35,36], sepsis [37], ultraviolet irradiation [38–40] and post-ischemic reperfusion injury [41]. Moreover, nitroxides were found to provide protection under anoxia, indicating the operation of alternative mechanisms independent of superoxide [28,42]. The present study focuses on the antioxidative activity of nitroxides in an experimental model of obligatory I/R in rat limb. It attempts only to characterize 'time window of injury', distinguish between ischemia and reperfusion components of damage and examine the potential of intervention by nitroxide antioxidants in regional I/R model of animal limb. A future extension of the research requires a comparison of efficacy by various nitroxide doses, times of administration (before and after ischemia) and modes of drug delivery (single bolus, repetitive or delayed release). Currently, we lack sufficient knowledge of the kinetics of damage development, the damage-inducing species or even the schedule of appearance of damage markers. Therefore, such a comprehensive project, which requires numerous laboratory animals, is beyond the scope of the present study.



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Materials and methods

4-OH-2,2,6,6-tetramethyl piperidine-1-oxyl (TPL), sodium dodecyl sulphate (SDS) and ferricyanide were obtained from Sigma Chemical Co. (St. Louis Mo) Ketamine was obtained from Fort dodge (Iowa, USA); Xylazine was obtained from Bio Labs (France).

Experimental design

All procedures were approved by the Hadassah-Hebrew University Animal Ethics Committee. The rat's hind limb was used as an in vivo model for ischemia-reperfusion injury. Under general anaesthesia, animals' hind limbs were subjected to ischemia using tourniquets. Immediately before reperfusion (tourniquet release) the animals received an intraperitoneal (i.p.) injection of saline without and with TPL at 80 mg/kg body weight (BW), which yields a nominal initial concentration of 450 μM nitroxide. I/R injury was evaluated physiologically, histomorphometrically and biochemically. Animals subjected to 1.5 and 4 h of ischemia underwent electromyography for evaluation of physiological damage. Additional groups of animals were subjected to 2 h of ischemia. Blood samples were collected at 0, 3, 8 and 24 h following the ischemia for haematological and biochemical analysis. Muscle samples were harvested for histomorphometric analysis 24 h following ischemia.

In brief, 90 male Sabra rats weighing 250–460 g were housed separately and allowed free access to food and water. The animals were randomly divided into experimental, control and sham groups numbering 6–10 animals. Animals were anaesthetized with a mixture of xylazine 2% (0.15 mL) and ketamine (0.85 mL) administered i.p. at 1 mL/kg BW. Under general anaesthesia the hind limbs of the animals were subjected to 1.5, 2 or 4 h of ischemia using a 1/8 inch rubber band tightened proximally on the thigh of the rat. Before removing the tourniquet saline, either without or with TPL (80 mg/kg BW), was administered i.p. Reperfusion was monitored by return of blood flow to the ischemic limb. Physiological injury was evaluated with EMG and blood analysis. Prior to collection of blood samples, animals were i.p. injected with 0.1 mL heparin (5000 u/mL). Blood was drawn directly from the heart and sent for complete differential cell count and for assay of lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) levels.

Electromyography (EMG) was monitored using a Mystro programmable electrophysiological system (Medelec), which stimulated the limb with a series of eight stimuli at 3, 5 and 10 Hz, while recording its response.

Light microscopy and histomorphometry analysis

The extent of tissue inflammation can be evaluated by changes in local immune cells numbers. Quantitation of cell nuclei provided a measure for infiltration of inflammatory cells. Tissue samples from muscle were harvested from sham, control (24 h following the end of 2 h ischemia) and experimental (24 h following the end of 2 h ischemia and administration of TPL) groups and put into 4% buffered phosphate formalin. Samples were embedded in paraffin, sectioned (5 μm thickness) and stained with haematoxyline and eosin. Acquisition of images was performed using a light microscope (Olympus BH2 with S Plan FL2 lens), Sony CCD videocamera (Microtechnica M852) with a resolution of 568×764 and Sony Trinitron monitor with image resolution 512×512 and colour resolution of 256° for the three basic colours of the screen (red, green, blue). Images were digitized and analysed for the number of cell nuclei using Image Pro plus (Media Cybernetics, MD). Image analysis was carried out in a pre-defined image window and by colour threshholding representing the 'actual objects' in a given window.

Tissue protein and dry weight measurements

Additional muscle samples were harvested, weighed, immediately frozen by liquid N_2 and kept at $-70^{\circ}C$. Later, part of the tissue was thawed and placed in an $80^{\circ}C$ oven. The samples were then weighed repeatedly every 6 h for several days until no change in their weight was observed. Thus their net dry weight was determined and water content was calculated. The other part of the tissue was homogenized in liquid N_2 with 250 μL of 0.1% SDS, centrifuged at 10 000 rpm for 10 min and the supernatant was collected and diluted. Protein was determined spectrophotometrically at 595 nm by Bradford assay using a Uvikon 860, Kontron.

EMG. Electromyography was used in an attempt to detect a measurable effect of I/R on the muscle response to repetitive nerve stimulation (RNS) [43]. The amplitude of the fourth or fifth response to a train of low frequency nerve stimuli of the compound muscle action in patients of Myasthenia gravis falls from the initial value. We stimulated the ischemic limb with a series of eight stimuli at 3, 5 and 10 Hz frequencies and measured the ratio between the amplitude of the fifth and first response waves, before and after 1.5 h and 4 h of ischemia.

Monitoring [nitroxide] by EPR

Electron paramagnetic resonance (EPR) spectra were recorded using an x band Varian E9 or JEOL JES-RE3X spectrometers operating at 9.5 GHz with

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centre field set at 3269 G, 100 kHz modulation frequency, 1 G field modulation amplitude and 20 mW incident microwave power. Blood samples taken from the rat at various points of time after i.p. injection at the end of the ischemic stage, were filled up into a gas-permeable teflon capillary, which was inserted into a quartz tube placed within the EPR spectrometer cavity. The EPR spectra were scanned at room temperature at various time points and the residual [TPL] was calculated from the EPR signal intensity using standard solutions of the nitroxide. To determine the concentration of the hydroxylamine, ferricyanide at 2 mM final concentration (higher concentrations significantly broaden the EPR spectral lines) was added to reoxidize TPLH and the samples were scanned again.

Statistics

Mean and SEM were used to describe the data. ANOVA test was used to compare between the values measured for the sham (no I/R) and the control (I/R saline alone) groups. For comparison of data over time, two-way factorial ANOVA test was used to compare results obtained for the control (saline) and experimental (TPL) groups at different points of time. The values of p obtained for analysis of each experiment are specified in the Figure legends of Figures 6 and 7 and in the Results section. It should be noted that the common statistical tools, including two-way factorial ANOVA test, are poorly suited for the characterization of the statistical significance of a transient change. For instance, where the values measured at initial and/or final points of time, for the control group equal the respective ones found for the experiment group, the analysis might overlook the transient change. Unless one knows in advance the exact schedule of the transient phenomenon, the narrower the time window of a transient change the poorer would be the statistical significance of the test.

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Results

The regional inflammatory response induced by I/R is reflected in the histological photographs of the muscle demonstrating infiltrating nuclei and necrotic markers, as shown in Figure 1. Muscle tissue from rat hind limb subjected to regional ischemia demonstrates a marked infiltration of cell nuclei (Figure 1B) as compared with untreated normal tissue (Figure 1A). The administration of TPL had no effect on the number of nuclei as demonstrated in Figure 1C.

Histomorphometry

Additional quantitative presentation of tissue damage is provided by the computerized histomorphometric analysis of the histological photographs of the muscle tissue, as displayed in Figure 2. The extent of I/R-induced oedema as reflected by the change in tissue water content did not differ between tissue of ipsilateral (TPL-treated) and contralateral (control) hind limbs, as seen in Figure 3.

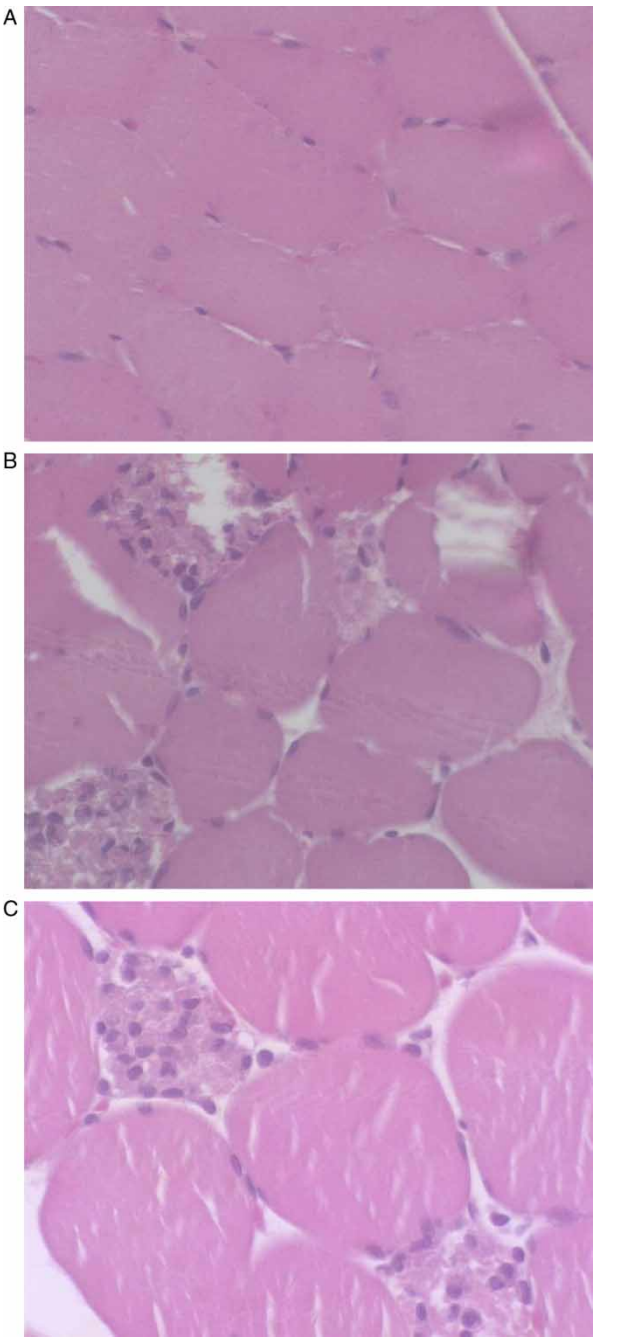

Figure 1. Histological pictures of muscle tissue demonstrating the effect of I/R-induced infiltration of cells. (A) Sham, no I/R; (B) I/R, saline alone; (C) I/R, saline +TPL. The samples from the rat hind limb were kept in 4% buffered phosphate formalin and stained with haematoxylin and eosin.

Electromyography (EMG)

Reportedly, ischemic muscles exhaust more easily than control muscle. Stimulation of ischemic limb

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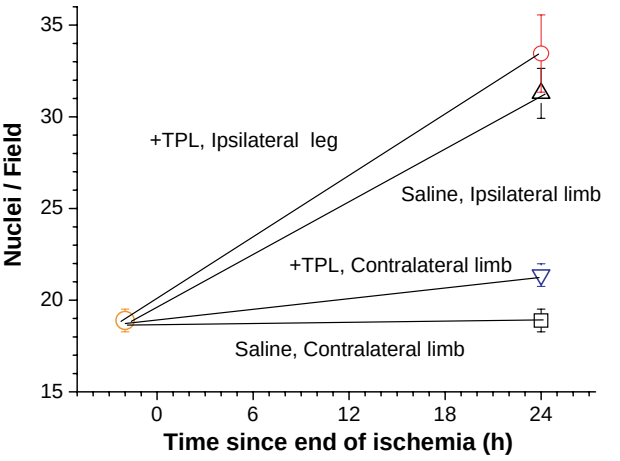

Figure 2. Regional inflammatory response following I/R. The post-ischemic increase of nuclei number in muscle tissue. Histomorphometric analysis of muscle tissue from ipsilateral and contralateral hind limbs of rats following 2 h of regional ischemia; 24 h later, samples were harvested, processed and their nuclei content was quantitated. Error bars represent SEM, $n=8$.

with a series of eight stimuli at frequency of 3, 5 and 10 Hz, resulted in appropriate response waves that were recorded. The ratio of the 5th to 1st response waves usually reflects changes in muscle response. The ratio of response waves of normal and ischemic (1.5 or 4 h) muscles is presented in Figure 4. No significant difference in response waves was noted between normal and 1.5 or 4 h ischemic muscles. Increases in stimuli frequency from 3–10 Hz did not change the ratio of the 5th to the 1st wave, as demonstrated in Table I.

We examined if the ischemic muscle could be exhausted more easily than the normal muscle, but did not detect a difference between these groups in these frequencies (Table I). Hence we did not study the TPL effect on the EMG test.

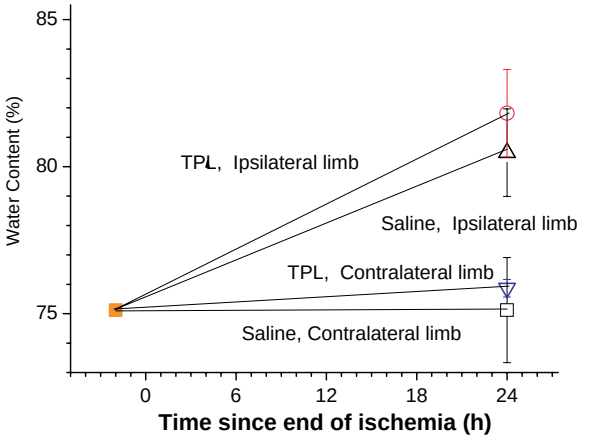

Figure 3. Post-ischemic change in the water content of rat limb muscle. To monitor muscle oedema, samples excised from muscle tissue of ipsilateral and contralateral hind limbs of rats following 2 h of regional ischemia. The samples were dried at 80°C and the water content was calculated from their respective weights before and after drying. Error bars represent SEM, $n=8$.

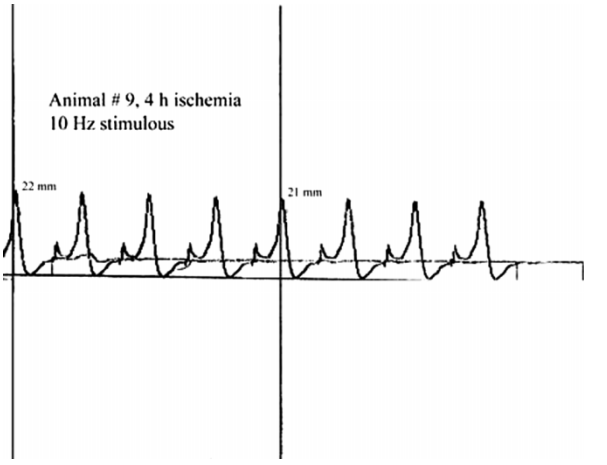

Figure 4. A typical record of the amplitudes measured for the first and the fifth waves.

Systemic response

Unlike the I/R-induced regional response, which persisted for days, the systemic response demonstrated a transient change over a few hours. The change in markers of the physiological response transiently peaked following I/R and disappeared later as the pre-ischemic values were recovered. The physiological response to various insults including I/R can be reflected in blood counts and serum enzyme levels. The differential blood counts of sham, saline (control) and TPL-treated animals are presented in Figure 5 and 6. While neither the total WBC counts nor the platelet levels changed following ischemia (results not shown) the respective fractions of granulocytes and lymphocytes were transiently modified (Figure 5). Following 2 h of regional ischemia the lymphocyte fraction progressively decreased while that of the granulocytes increased. The results displayed in Figure 5 show that I/R-induced effect on the differential counts of granulocytes and lymphocytes was manifested only following the start of reperfusion but not during the ischemic phase. About 8 h following the start of reperfusion the fraction of lymphocytes was reduced almost 2-fold whereas that of the granulocytes demonstrated almost 6-fold increase. Both fractions of WBC relaxed to their normal preischemic values after 24 h (Figure 5). The adminstration of the nitroxide had no effect on the I/R-induced changes in granulocytes and lymphocytes levels or the rate of recovery (Figure 5).

The effect of I/R on RBC, hematocrit and haemoglobin in the absence and the presence of nitroxide is presented in Figure 6. An increase in their levels started during the ischemic period and continued upon reperfusion achieving maximal values at 8 h following the start of reperfusion. As for WBC, after 24 h full recovery was observed (Figure 6), however, unlike the case of WBC, the ischemia-induced effect

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Table I. Ratio of the amplitudes of the 5th to 1st waves elicited by repetitive nerve stimulation measured prior to, 1.5 and 4 h following ischemia.

	Baseline			1.5 hours ischemia			4 hours Ischemia		
Animal No.	3Hz	5Hz	10Hz	3Hz	5Hz	10Hz	3Hz	5Hz	10Hz
1	1.2	1	1.04	1.12	1.05	1.05	1	1.04	1.04
2	0.95	1	1.04	1	1	1	1	1.05	0.95
3	1	1	1	1	1	1	1	1	1
4	1	0.95	1	1.11	1	1.2	1	1	1
5	1	1.18	1.16	0.91	0.9	0.94	1	0.94	0.9
6	1	0.95	1	1	1	0.95	1	1	1
7	1	1	1	1.12	1.06	1	1	1.05	1.05
8	1	1	1	1	1	0.86	1.04	1	1.05
9	1	1	1	0.84	0.9	0.83	0.95	0.95	0.95
10	1	1.05	0.95	1.1	1	1	0.96	0.93	0.9
Averege	1.015	1.013	1.019	1.02	0.991	0.983	0.995	0.996	0.984

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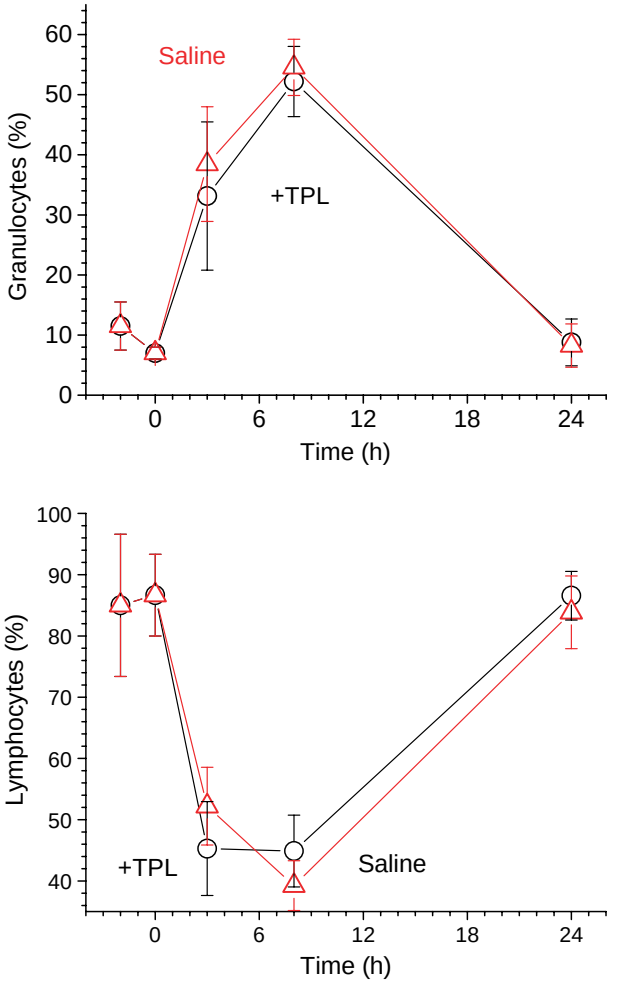

Figure 5. Effect of TPL on post-ischemic change of WBC differential count. Bottom: Lymphocytes; Top: Granulocytes. Rats' hind limbs were subjected to 2 h ischemia using tourniquets. Immediately before reperfusion (tourniquet release) the animals received an i.p. injection of saline either without (triangles) or with (circles) TPL 80 mg/kg BW. Blood was withdrawn at different times following the end of ischemia. Error bars represent SEM, n = 6.

constitutes a major part of the total I/R-Induced damage. For instance, most of the change in hematocrit content (Figure 6B) took place even before the administration of the nitroxide. The protective effect of TPL was small but demonstrated similar patterns for RBC, hematocrit and Hb. It was not surprising, therefore, that TPL mainly facilitated the recovery to pre-ischemic values, rather than preventing the transient increase. It should be noted that the commonly used statistical tools are less effective for the characterization of the statistical significance of a transient change. If the values at initial and final points of time measured for the control group do not differ from the respective ones found for the experiment group the analysis might provide too high *p*-values and overlook a genuine transient change.

The results displayed in Figure 7 demonstrate a transient increase in serum levels of CPK and LDH predominantly upon reperfusion. CPK and LDH levels, which hardly changed during ischemia, rapidly increased upon reperfusion, peaking at 3 h post-ischemia by 7-fold and 3.8-fold, respectively ($p < 0.001$, ANOVA). The transiently elevated levels rapidly relaxed to normal values within 24 h. The results demonstrate that TPL inhibited the post-ischemic release of cellular enzyme into the serum. Figure 7 demonstrates that in nitroxide-treated animals the levels of CPK increased only 3.3-fold ($p = 0.002$, 2-way factorial ANOVA) whereas LDH level increased 2.5-fold ($p = 0.057$, 2-way factorial ANOVA).

The fate of the nitroxide antioxidant

Generally, the nitroxide concentration in the tissue rapidly decreased both through clearance and via reduction to the respective hydroxylamine [21–24,44]. EPR spectroscopy, which is 'blind' to diamagnetic species can directly report on the spin loss. Blood samples were taken at various points of time and scanned for the EPR signal of TPL before and

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after addition of 2 mM $K_3Fe(CN)_6$, which oxidizes the hydroxylamine back to nitroxide. The time-dependence of [TPL] and of the total {[TPL]+[TPLH]} is displayed in Figure 8. The results indicate that TPL concentration in the blood transiently peaked about 1 h after injection and progressively depleted and that the kinetics of TPL depletion did not differ between control rats and those subjected to I/R. Figure 8 demonstrates also that out of the nominal concentration of 460 μM TPL initially administered only 1–2 μM persisted for a relatively short period during the 'time window' of the damage, whereas the rest, over 99–99.9%, was rapidly reduced to TPLH. Protective activity has been ascribed also to the reduced form of the nitroxide [45–47]. Yet, in most studies the hydroxylamine provided little to no protection, particularly against radiation injury [48,49], suggesting that the nitroxide radical rather than its reduced form provides protection [25,49]. It was proposed that TPLH may show some radioprotection as the radical form due to efficient reoxidation to the radical form *in vivo* [50]. Obviously, since both oxidation states always coexist in the tissue [22–24,51] it is not simple to distinguish between their contributions. Previous studies [52] as well as recent unpublished results showed that TPL at 1 μM and even sub-μM range protected DNA from peroxyl radicals-induced damage [52]. The present results might demonstrate a similar effect suggesting a catalytic, rather than stoichiometric reaction.

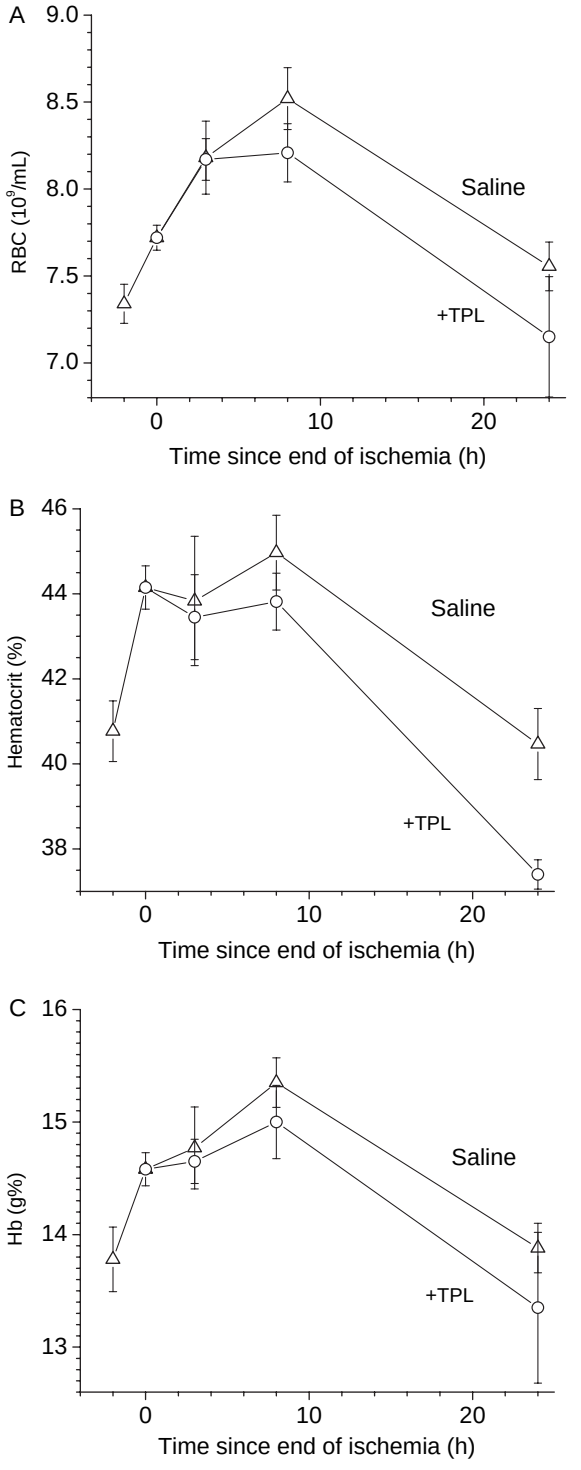

Figure 6. (*Continued*)

Discussion

Nitroxides have been previously shown to inhibit I/R damage in other experimental models such as isolated beating rat heart [53] and their protective effect supported the implication of ROS in the injurious processes while providing a valuable research tool for further studies. Similarly, nitroxides were anticipated to protect against regional I/R-induced in the rat limb. This research direction is important because I/R-induced injury still poses an unanswered clinical challenge in procedures involving replantation and free tissue transfer. The inevitable reperfusion following ischemia adversely generates ROS, which can exert deleterious effects. While interventions directed at modulating the production of ROS or their effects is promising, only limited progress has been made.

Figure 6. Effect of TPL on post-ischemic change of (A) RBC count, (B) hematocrit and (C) haemoglobin content. Rats' hind limbs were subjected to 2 h ischemia using tourniquets. Immediately before reperfusion (tourniquet release) the animals were i.p. injected with saline either without (triangles) or with (circles) TPL 80 mg/kg BW. Blood was withdrawn at various points of time following the end of ischemia. Error bars represent SEM, $n=6$. (A) The RBC count increased and peaked at 8 h following ischemia and recovered to pre-ischemic level during 24 h. TPL slightly inhibited the increase and facilitated the recovery ($p=0.124$, two-ways factorial ANOVA); (B) The hematocrit content increased and peaked at 8 h following ischemia and recovered to pre-ischemic level during 24 h. TPL slightly inhibited the increase and facilitated the recovery ($p=0.079$, two-ways factorial ANOVA). (C) The haemoglobin level increased and peaked at 8 h following ischemia and recovered to pre-ischemic level during 24 h. TPL slightly inhibited the increase and facilitated the recovery ($p=0.297$, two-way factorial ANOVA. See note regarding the statistical test in Methods).

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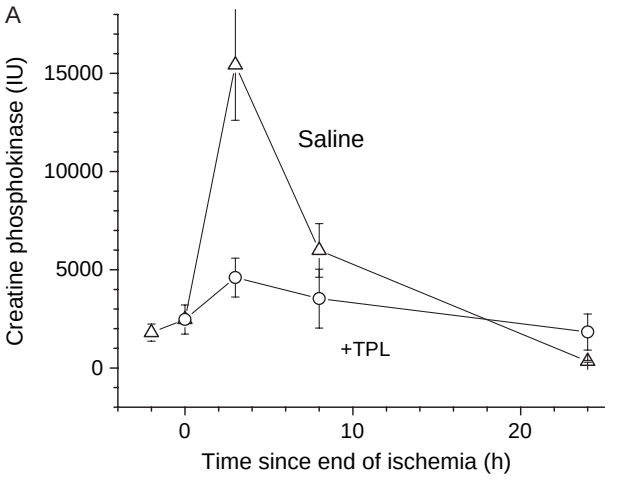


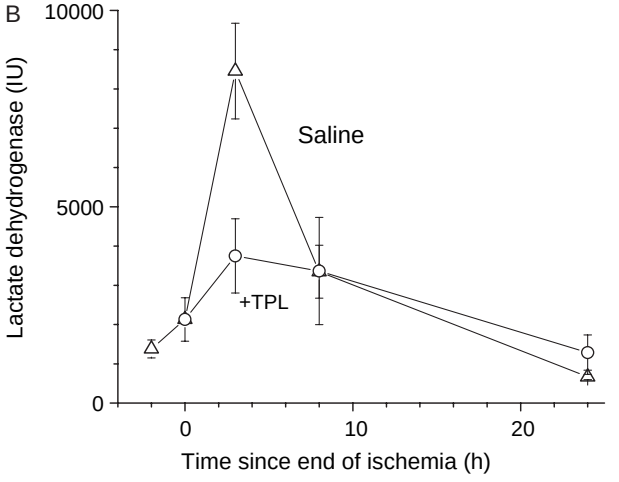
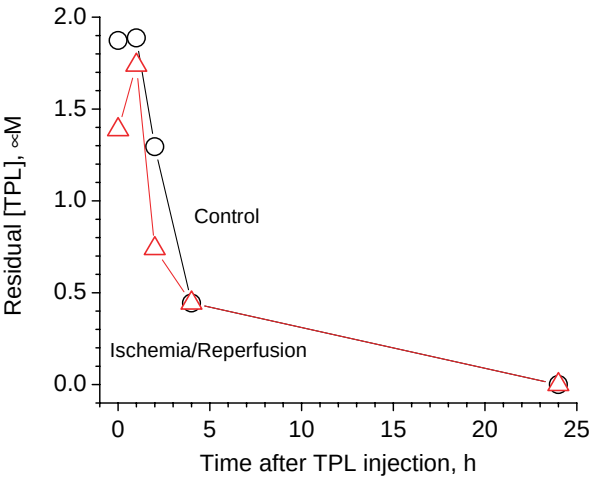


Figure 7. Effect of TPL on post-ischemic change in the blood levels of (A) CPK and (B) LDH. Rats' hind limbs were subjected to 2 h ischemia using tourniquets. Immediately before reperfusion (trouniquet release) the animals received an intraperitoneal injection of saline either without (triangles) or with (circles) TPL 80 mg/kg BW. Blood was withdrawn at various points of time following the end of ischemia. Error bars represent SEM, $n=6$. The sham ($t=-2$ h) and control groups ($t=0$ h) included 14 and 15 animals, respectively. The levels of CPK and LDH increased and peaked 7-fold and 3.8-fold, respectively, at 3 h following ischemia ($p<0.001$, ANOVA) and recovered to pre-ischemic level during 24 h. TPL inhibited the transient elevation (3.3-fold increase of CPK level, $p=0.002$, two-way factorial ANOVA and 2.5-fold increase of LDH level, $p=0.057$, two-ways factorial ANOVA).



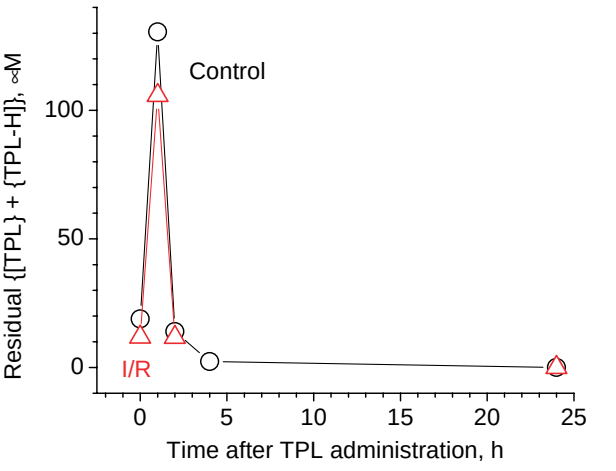


Figure 8. The time-dependence of [TPL] and total [TPL]+[TPLH] in the rat blood. Blood samples taken from the rat at various time points after i.p. administration of TPL at 80 mg/kg BW. The residual [TPL] was calculated from the EPR signal intensity using standard solutions of the nitroxide. To re-oxidize the hydroxylamine (TPLH) formed by reduction of TPL in the animal, $K_3Fe(CN)_6$ at 2 mM was added and the solution was re-scanned to determine the total {[TPL]+[TPLH]}. The broadening of the EPR line by $K_3Fe(CN)_6$ was taken into consideration for the calculation of the nitroxide concentration.

Hence, it was and is our opinion that using nitroxide antioxidant could prove beneficial.

Systemic damage

Beside injury induced during ischemia, reactive oxygen-derived species that are formed during the post-ischemic reperfusion phase, when oxygen arrives at hypoxic/anoxic tissue, can cause additional damage [54]. This component of damage was particularly expected to be affected by nitroxides, although they demonstrate protective activity also under anoxia. In the present study regional I/R stress led to transient systemic changes in the differential blood counts and the release of cellular enzymes into the serum, reflecting the time-dependent physiological response (Figures 5–7). Despite the relatively long (2 h) period of ischemia the systemic inflammatory/damage markers, which include haematological parameters and enzymes level, relaxed back to their respective pre-ischemic values within 24 h. The transient changes in the haematological parameters occurred predominantly during ischemia before the administration of TPL, which could affect the reperfusion-induced damage alone. This explains the limited protective effect demonstrated in Figure 6. Conversely, the increase in CPK and LDH levels, which was induced upon reperfusion, has been greatly inhibited by TPL. Since nitroxide antioxidants do not require pre-incubation or early administration but distribute instantaneously over the body, their effect allows the distinction of ischemia-induced from reperfusion-induced injury. The protective effect of nitroxides, which

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react neither with O_2, H_2O_2 or NO, nor with most diamagnetic species, indicates the involvement of paramagnetic deleterious species operative during the 'time window' of post-ischemic reperfusion injurious processes.

Regional damage

The regional damage induced by I/R in the ipsilateral hind limb of the animal is manifested by physiological changes including EMG changes seen as muscle fatigue, as changes in tissue water content as marker for tissue oedema [55] and as infiltration of cell nuclei, did not recover during 24 h reperfusion (Figures 1–3). Similarly, the regional histological changes demonstrating an extensive damage (Figure 1) were not reversed even after 24 h. The nitroxide did not provide any protection against this injury either. Nitroxide antioxidants neither restitute nor repair impaired tissue sites but rather protect from injury inflicted by deleterious species *in-situ.* In the present case, TPL was administered upon the start of reperfusion rather than before or during ischemia alone. Hence, the nitroxide was anticipated to affect post-ischemic reperfusion injury alone, rather than ischemia-induced injury. The failure of TPL to affect the regional damage might be due to a too-low residual concentration (at μM to sub-μM range) resulting from rapid loss through clearance and reduction. This suggests a need for a continuous or repetitive administration of the nitroxide over a longer time-window. Alternatively, the lack of protection might suggest that the persistent damage is primarily ischemia-borne injury, rather than I/R.

Hence, a comprehensive study optimizing doses, schedule and modes of delivery (including pre-ischemic delivery as well as continuous and delayed administration) is needed. Alternatively, selection of a more effective nitroxide might be needed. Nitroxide constitute a large family of stable radical derivatives differing by their charge, lipophilicity, size, redox potential and ring substituents. This offers a wide and promising selection but requires far more experimental work. Moreover, the fact that nitroxides have several mechanisms underlying their antioxidant activity including catalytic, pseudo-catalatic and stoichiometric enables a further research of their potential use for controlling the damage in the ischemic hind limb model [20,26,46,56–59].

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