IMPROVED ENERGETIC RECOVERY OF SKELETAL MUSCLE IN RESPONSE TO ISCHEMIA AND REPERFUSION INJURY FOLLOWED BY IN VIVO ^{31}P-MAGNETIC RESONANCE SPECTROSCOPY

JONAS LUNDBERG, M.D., ANN LINDGÅRD, B.Sc., ANNA ELANDER, M.D., Ph.D., and BASSAM SOUSSI, Ph.D.*

It is of great clinical interest to improve postischemic tissue recovery during microsurgical transfers. The effect of singlet oxygen energy (SOE) as photon illumination at 634 nm on rat skeletal muscle during ischemia and postischemic reperfusion was investigated noninvasively and continuously by in vivo ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS). A model of pedicled rat rectus femoris muscle was used, where phosphorous metabolites were followed before onset of ischemia (control), after 4 h of ischemia, and after 1 h of reperfusion. Two groups were studied: one control group (n = 10), and one SOE-treated group (n = 10). Blood perfusion was measured by laser Doppler flowmetry (LDF) during the study. After 4 h of ischemia, ATP levels were 72% and 51% of normal control values in the illuminated group and the control group, respectively ($P < 0.05$). After 1 h of postischemic reperfusion, phosphocreatine (PCr) recovered to 79% and adenosine triphosphate (ATP) to 71% in the illuminated group, whereas in the control group, the recovery was 57% and 51%, respectively ($P < 0.05$). It is concluded that singlet oxygen energy has a beneficial effect on the energy state of skeletal muscle during ischemia and postischemic reperfusion.

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Reconstructive centers have reported a success rate of microsurgical tissue transfers of 90–97%.[1–5] Furthermore, a reexploration rate of 6–26%, where 40–100% of these are saved, has been shown.[6] The high level of success does not show the rate of focal necrosis in the muscle. It only shows a tissue transfer that for most instances succeeded in reaching its goal, namely to cover a defect. But when using the free vascularized muscle for a functioning unit after the transfer, it is even more important to have viable muscle. Many attempts to limit ischemia/reperfusion injury by different substrates have been tried on skeletal muscle. Several authors demonstrated positive effects of muscle survival and function. None of these substrates are available for clinical use.[7–11]

The preserving method should be easy to handle, should not extend ischemia time, and should be effective in the clinical situation. One possible method might be hypothermia. In a previous in vivo rat rectus femoris study, hypothermia-treated animals showed a positive postischemic effect on the energy state in the skeletal muscle.[12]

A new noninvasive method might be singlet oxygen energy (SOE). Singlet oxygen, O_2 ($^1\Delta_g$), a highly reactive form of oxygen, can be produced photochemically by energy transfer from an excited photosensitizer.[13] The energy emitted from singlet oxygen upon relaxation to its triplet ground state oxygen, O_2 ($^3\Sigma_g^-$), is captured as photons at 634 nm and is here referred to as singlet oxygen energy (SOE). We previously showed up to a 60% decrease in secretion of reactive oxygen species (ROS) after 2 min illumination with SOE on human monocytes stimulated with phorbol myristate acetate (PMA).[14] We also showed a decreased degradation of phosphorus metabolites when illuminating preservation mediums with singlet oxygen energy (SOE), containing rat rectus femoris muscle, during 5 h of ischemia

Bioenergetics Group, Wallenberg Laboratory, Departments of Surgery and Plastic Surgery, Sahlgrenska University Hospital, Göteborg University, Göteborg, Sweden

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*Correspondence to: Bassam Soussi, Ph.D., Wallenberg Laboratory, Sahlgrenska University Hospital, 413 45 Göteborg, Sweden. E-mail: soussi@wlab.gu.se

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(Lindgard et al., unpublished findings). This spurred us to further investigate the effect of illumination in an in vivo model for ischemia and reperfusion. Thus, the aim of this study was to investigate the effect of SOE illumination at 634 nm in an in vivo model of rat rectus femoris muscle, using in vivo ^{31}P-magnetic resonance spectroscopy (^{31}P-MRS) continuously and noninvasively during the experiment period. For evaluating the degree of blood perfusion during ischemia and reperfusion, laser Doppler flowmetry (LDF) was used.

MATERIALS AND METHODS

Animals

Twenty male Sprague-Dawley rats (B&K Universal, Sollentuna, Sweden) weighing 510–690 g were randomly assigned to the study groups. The rats were fed Purina Rat Chow and tap water ad libitum, and remained in normal cages until the experiments were performed. The experimental protocol was reviewed and approved by the Committee for Research Ethics at Göteborg University.

Rectus Femoris Model

The rat rectus femoris model was previously described,[15–17] and was detailed in a modified model developed in our laboratory.[12] The rats were anesthetized with mebumal (36 mg/kg body weight) intraperitoneally, and anesthesia supplementation (mebumal, 2 mg/kg body weight) was administered intraperitoneally as required. The left rectus femoris muscle was used in each rat, leaving the right side unexposed.

Dissection was performed using the standard microsurgical technique. The only vessels remaining after dissection were the pedicle to the rectus femoris muscle originating from the femoral vessel. Saline-soaked dressings were used to enwrap the rectus femoris muscle and to cover the open wound.

Experimental Setup

Ischemia was induced by a plastic microvascular clamp (Vascu Statt® original, 1001-503, angled 45° mini, clamping pressure 10–15 g, Vingmed, Svenska AB, Järfälla, Sweden) on the femoral vessels proximal and distal to the pedicle to the rectus femoris in both groups. The clamp was released after 4 h of ischemia. Lidocaine hydrochloride (Xylocain, 20 mg/ml, AB Astra, Södertälje, Sweden) was used locally to prevent postischemic spasm.

Treatment With SOE

SOE was produced by Valkion equipment (Polywalk AB, Sweden) as photons via a fiberoptic cable (cable length, 142 cm; diameter of end of fiberoptic cable, 3 mm). In the Valkion equipment, singlet oxygen was generated through a photosensitization process. The photosensitizer used was phthalocyanine, zinc (II), a blue-reddish dye, being one of the few sensitizers able to perform in a gaseous atmosphere. It also has good heat and light resistance and can be applied on a metal surface. As light source, six light-emitting diodes (LED) were used. There are different techniques to make a coating of the sensitizer on a metal surface. When using diodes as a light source, the heat development during the process is a lot less compared to the use of a halogen lamp, and consequently the requirements for the coating are less severe. The activation chamber developed to produce the singlet oxygen consisted of an aluminium plate, coated with the sensitizer. The medium where the singlet oxygen was generated was air with a relative humidity of around 90%. Humidity was generated by circulating air through a water flask. The lifetime of singlet oxygen in this medium is about 2 μs. Between the aluminium plate and the light source, a seal prevented the activated light from escaping. The SOE corresponds to light energy with a wavelength of 634 nm.

The rectus femoris muscle was located in its original in vivo position. SOE illumination was made directly on the muscle via the fiberoptic cable for 5 min each time: before the start of ischemia, at the end of ischemia, and three times during reperfusion. The end of the fiberoptic cables were positioned 5 mm from the rat rectus femoris muscle. During illuminated periods, the fiberoptic cables were moved over the entire surface of the rectus femoris muscle (Fig. 1A,B).

Study Groups

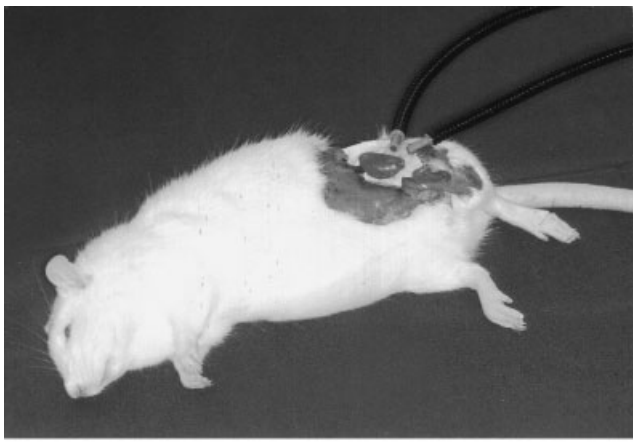
Twenty rats were randomly divided into two groups, with 10 in each.

In group 1 (control group), the rectus femoris model was subjected to room temperature (24 ± 0.5°C), 4 h of ischemia, and 1 h of reperfusion.

In group 2, the rectus femoris model was subjected to SOE at room temperature (24 ± 0.5°C), 4 h of ischemia, and 1 h of reperfusion.

Experimental Protocol

Two experimental protocols were used: one for in vivo ^{31}P-MRS measurements, and one for laser Doppler measurements. For both the ^{31}P-MRS series and the



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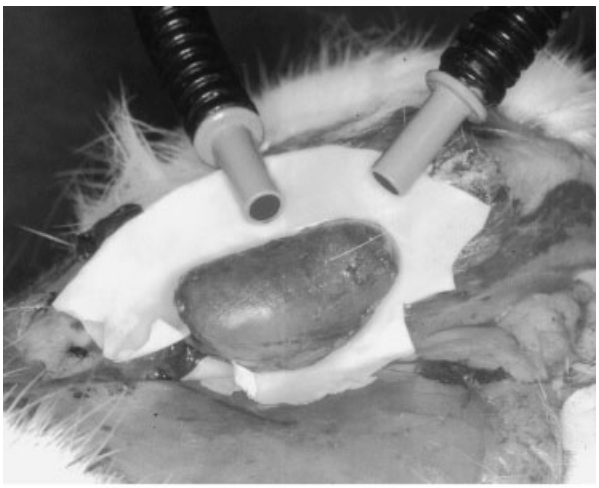

B

Figure 1. ***A****: Rat rectus femoris model in original in vivo position, exposed to SOE illumination via two fiberoptic cables positioned 5 mm from muscle flap. During illuminated periods, fiberoptic cables were moved over entire surface of rectus femoris muscle.* ***B****: Close-up of rectus femoris muscle exposed to SOE illumination.*

laser Doppler series, the rats were analyzed three times: before ischemia (control), after 4 h of ischemia, and after 1 h of reperfusion.

Temperature

The surface temperature of the muscle was measured at the same points as the in vivo ^{31}P-MRS and laser Doppler series: before onset of ischemia (control), after 4 h of ischemia, and after 1 h of reperfusion. The measurements were made on both bottom and top surfaces of the rectus femoris muscle. The final value was the mean of the two measurements. A single temperature probe with microtip (sensor 442, Perimed, Järfälla, Sweden) was used on each surface of the muscle at a time (upper and down surfaces, respectively), connected to a temperature unit (PF 5020, Perimed). The body temperature of the rat was maintained with a heating blanket and monitored with a rectal probe. Room temperature was 24°C (±0.5°) throughout the experiment.

In Vivo ^{31}P-Magnetic Resonance Spectroscopy

All ^{31}P-MRS experiments were performed on a Bruker Biospec BMT 24/30 horizontal magnet with an X-32 acquisition system at 2.35 Tesla (T). Rat rectus femoris muscle was brought "outside" the rat with the pedicle vessels still intact and placed on a 1-cm-diameter surface coil dual-tuned to ^{1}H and ^{31}P (100 MHz and 40 MHz) frequencies. The homogeneity of the magnetic field was optimized by adjustment of the current of the shim coils while the free induction decay (FID) of the water signal was observed. Acquisition parameters for ^{31}P-MRS include a 2,500-Hz sweep width, 4K data points, and 1,024 averages with a repetition time of 2 s, resulting in 34 min, 8 s of total acquisition time. Observed FIDs were Fourier-transformed, and first-order phase correction was done. Phosphocreatine (PCr), adenosine triphosphate (ATP), and inorganic phosphate (Pi) peaks were identified in the spectra and integrated accordingly. Saturation correction was done with a spectrum observed with a 16-s repetition time.

Laser Doppler Flowmetry

Microcirculation was measured with laser Doppler flowmetry (LDF; Periflux System 5001, Perimed, Sweden), using a probe with a microtip 0.5 mm in diameter (Periflux 418-1). Measurements are expressed in arbitrary perfusion units (PU). The probe was calibrated with a motility standard, from Perimed, of 250 PU. Measurements were taken from the distal 2/3 of the rectus femoris muscle, i.e., the same area where the ^{31}P-MRS measurements took place. Each measurement was repeated 5 times at different areas of the distal 2/3 of the muscle, because of variations from these areas. The final value was the mean of the five measurements.

Statistics

Results are presented as mean ± SEM. Student's *t*-test was used to calculate statistical significance. Probabilities of less than 0.05 were regarded as significant.

RESULTS

Analysis of phosphorus metabolites, PCr, Pi, α- β-, and γ-ATP and intracellular pH was performed at three

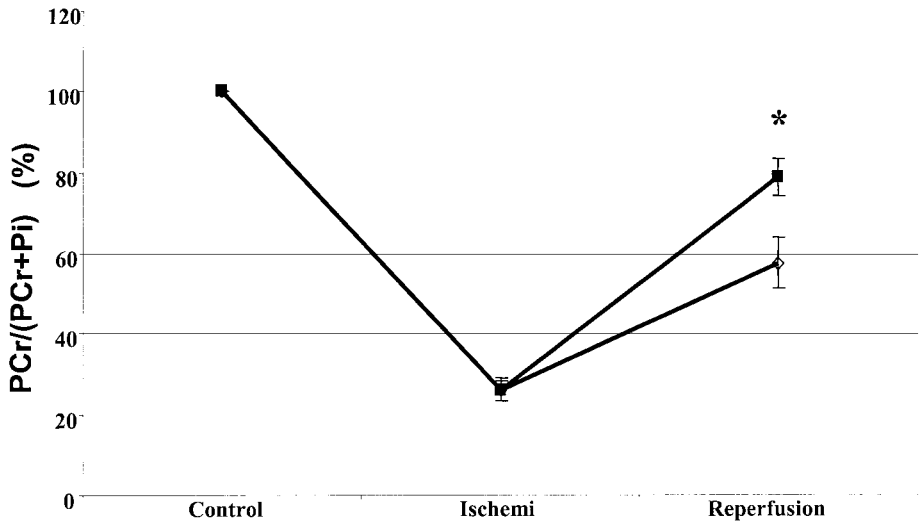

*Figure 2. Percentage of PCr/(PCr + pi) calculation from in vivo ^{31}P-MRS. Comparison between control (open diamonds) and SOE (solid squares) groups. Bars are presented as percent of control value. Level of significance is given between groups in the figure; *P <0.05.*

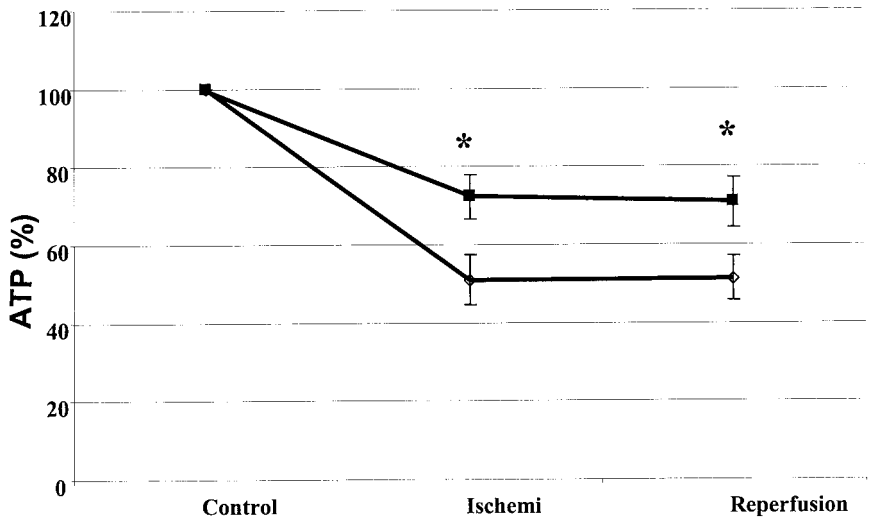

*Figure 3. Percentage of ATP calculation from in vivo ^{31}P-MRS. Comparison between control (open diamonds) and SOE (solid squares) groups. Bars are presented as percent of control value. Level of significance is given between groups. *P < 0.05.*

time points: before onset of ischemia (control), after 4 h of ischemia, and after 1 h of reperfusion by ^{31}P-MRS. The changes during ischemia and reperfusion of PCr/(PCr + Pi) and β-ATP were calculated as percent of normal control values and are presented in Figures 2 and 3. Changes in pH are presented in Figure 4.

PCr/(PCr + Pi) decreased to 26% of control value, in both groups after 4 h of ischemia. Apart from the ischemia values of PCr/(PCr + Pi), there was a significant difference of ATP between the two groups at ischemia. The control group (group 1) decreased to 51% of control value, and the SOE illuminated group (group 2) decreased to 72% of control value ($P < 0.05$). The control values of pH did not differ significantly between the two groups, where group 1 had a control value of 7.22 and group 2 had a value of 7.29.

The pH ischemia values were also similar. Group 1 decreased to 6.26, and group 2 to 6.35.

There was a significant difference between the two groups at 1 h of reperfusion regarding PCr/(PCr + Pi) and ATP, as demonstrated in Figures 2 and 3. PCr/(PCr + Pi) recovered to 57% of control value in group 1, and recovered to 79% of control value in group 2 ($P < 0.05$).

For ATP, the tendency was the same: 51% and 71% recovery of the control value in groups 1 and 2, respectively ($P < 0.05$). pH behaved similarly in both

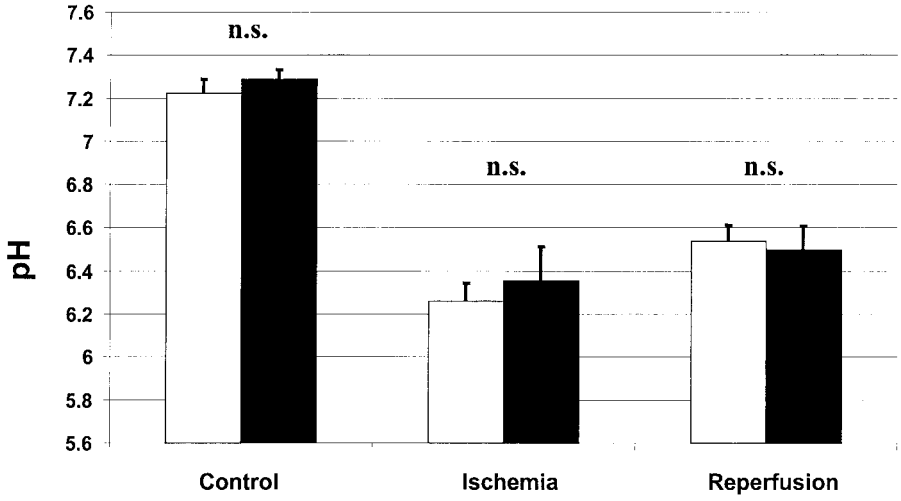

Figure 4. Intracellular pH calculation from in vivo ^{31}P-MRS. Comparison between control and SOE groups. Bars are presented in absolute values. Level of significance is given between groups. n.s., nonsignificant.

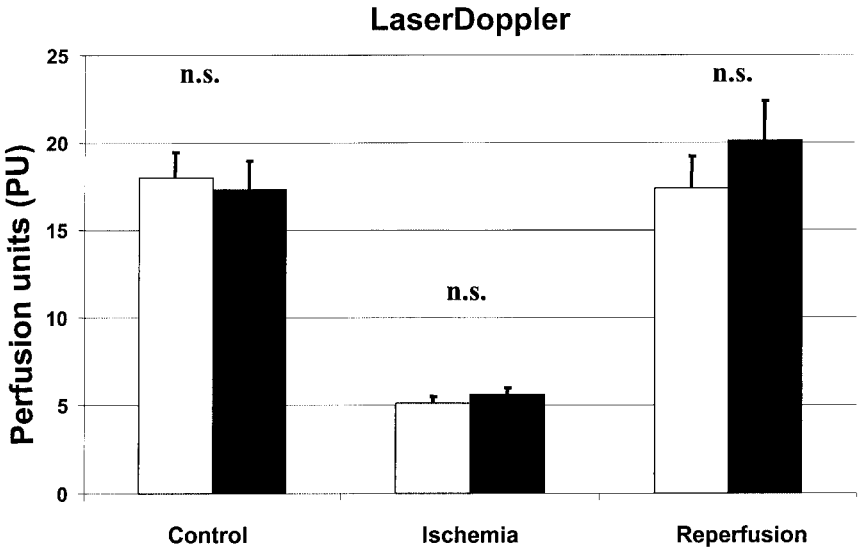

Figure 5. Laser Doppler. Comparison between control and SOE groups. Bars are presented as perfusion units (PU). Level of significance is given between groups. n.s., nonsignificant.

groups, where group 1 reached 6.54 and group 2 increased to 6.50 (no significance).

The blood perfusion in the muscle flap, measured by laser Doppler in perfusion units (PU), did not differ significantly between the two groups at any measuring point. The control values were 18 ± 1.4 and 17.3 ± 1.7 in groups 1 and 2, respectively (Fig. 5).

When applying the microvascular clamp, the perfusion value decreased to between 5–10 PU in the two groups. After 4 h of ischemia, the values were 5.1 ± 0.4 and 5.6 ± 0.4 in groups 1 and 2, respectively. Reperfusion for 1 h restored respective values to 17.4 ± 1.8 and 20.1 ± 2.2.

The mean temperature of the muscle flaps did not differ between groups during the experiment. The control values were 26.1 ± 0.3°C (group 1) and 26.3 ± 0.5°C (group 2), respectively. No difference was recorded between groups concerning body temperature during the experiment. Body temperatures were at control 31.4 ± 0.5°C and 31.7 ± 0.2°C for groups 1 and 2, respectively.

The temperature was measured for 5 min in front of the SOE light cable at the same distance as between the SOE light cable and the illuminated muscle in the experiment. No difference in temperature was recorded during the measurements, which is important, as an

increase in temperature could negatively influence the results.

DISCUSSION

We previously showed that illumination with SOE at 634 nm decreased the generation of reactive oxygen species (ROS), using activated human monocytes.[14] Furthermore, beneficial effects on the energetic status of the ischemic skeletal muscle in vitro (Lindgard et al., unpublished findings), and on the heart in vivo, were shown (Lukes et al., unpublished findings). This spurred us to investigate the effect of SOE on skeletal muscle subjected to ischemia and reperfusion, followed continuously and noninvasively by ^{31}P-MRS.

A pedicled rat rectus femoris muscle model, developed in an earlier study, was used.[12] In this model, ischemia time was set to 4 h and reperfusion to 1 h, since a moderate deep ischemia, and not a complete recovery, was shown after these time intervals. The muscle was analyzed for phosphorus metabolites by in vivo ^{31}P-MRS, by placing the rectus femoris muscle on the coil outside the rat body. The vessels were still intact and unstretched, which was important in order to achieve stable results.

In the group where rectus femoris muscles were illuminated with SOE, the energetic level demonstrated by ATP was higher than in the muscles in the control group during ischemia. Similar results showing higher concentrations of ATP in the SOE-illuminated groups were shown in a previous study, where SOE illuminated preservation medium or NaCl containing rat rectus femoris muscle subjected to 5 h of ischemia (Lindgard et al., unpublished findings). It has been suggested that the cellular damage that occurs during ischemia is generated by ROS.[18,19] The etiology of the positive effect of SOE could therefore be a higher capacity of the muscle to preserve energy or less production of ROS. In a study by Hulten et al.,[14] a reduction of generation of ROS by phorbol myristate acetate (PMA)-activated monocytes exposed to SOE was shown. This antioxidative effect was partly explained by inactivation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[14]

However, the greatest damage of ROS on muscle cells occurs at reperfusion.[6,20] We demonstrated a beneficial effect of SOE illumination at reperfusion in the treated group, where both ATP and PCr showed higher concentrations than control muscles.

Singlet oxygen is known to be a harmful, highly reactive oxygen species which is partly involved in the inflammatory process in biological systems.[21] We suggest that energy emitted from singlet oxygen, in its relaxation to its ground state triplet oxygen, is transferred as photons to another medium, in this study rat rectus femoris muscle. The photons of SOE have a wavelength of 634 nm.[14]

Biostimulation with a laser can be achieved by many different wavelengths. Laser light of 830 nm was shown to increase blood flow and dilatate arterioles in rat mesenteric microcirculation.[22] In this study, SOE at λ 634 nm did not affect the blood perfusion measured by LDF.

A method frequently used is photoirradiation by He-Ne laser with a wavelength of 633 nm, which has been shown to increase collagen production by human fibroblasts. It was also found to increase the rate of wound closure in rats and mice. An argon dye laser of 630 nm improved wound epithelialization, cellular content, granulation tissue formation, and collagen deposition in diabetic mice.[23] In pigs, He-Ne laser had no effect on wound healing.[24] Low-power laser irradiation of 632 nm enhanced the functional and morphological recovery of injured nerves.[25] The same authors found an accelerated mitosis in fibroblasts irradiated with a low-power-dose laser. At higher doses, cells were destroyed.

Rat heart irradiated by an argon-dye laser with a wavelength of 660 nm showed a postischemic recovery improvement of functional parameters.[26] Karu showed an increase of ATP synthesis in cultured cells after irradiation with a He-Ne laser of 633 nm.[27] Rat mitochondria stimulated with a He-Ne laser of 633 nm showed an increase of ATP synthesis of 70%.[24] As demonstrated in this study, SOE-illuminated rat skeletal muscle showed a higher level of end-ischemic ATP, which also was demonstrated in a study of isolated cardiomyocytes irradiated with a low-power laser.[26] Furthermore, a higher level of postischemic ATP concentration was shown in this study.

Rochkind and Ouaknine[25] found that free radicals, possibly singlet oxygen, was generated in cells during He-Ne irradiation. The assumed mechanism of enhanced cell respiration was proposed through light stimulation of porphyrins or cytochromes. Porphyrins are known to be excellent photosensitizers, and have absorption bands, e.g., at 630 nm. Cytochromes of the respiratory chain have absorption spectra similar to porphyrins.[25] Thus, singlet oxygen was proposed to be produced by a photosensitization process which, at small amounts, may be a biochemical intermediate in biological processes. At higher concentrations, singlet oxygen damages the cell.[21,25]

A stimulatory effect of the respiratory chain was also described in a review by Karu.[28] Photoacceptors absorb light, is changed to an electronically excited state from which molecular processes can lead to a measurable biological effect. These photoacceptors are proposed to be located in the terminal respiratory chain (cytochrome c oxidase). The enhanced cell respiration is explained by

an acceleration of electron transfer in the respiratory chain, due to a change in the redox properties of cytochrome c oxidase following photoexcitation of its electronic states.[28]

Mitochondria can be a source and a target of ROS during ischemia and reperfusion.[19,29] Previous studies showed that ischemia and reperfusion affect the mitochondrial membrane-bound cytochrome c oxidase.[30,31] The function of the enzyme is tightly connected to cardiolipin content, a polyunsaturated phospolipid of the mitochondrial membrane. After 4 h of ischemia, and after 4 h of ischemia and 1 h of reperfusion, the function of the enzyme is depressed, while cardiolipin content decreases, suggesting peroxidative attack of the membrane.

A reduced capacity of cytochrome c oxidase would increase the risk for an incomplete reduction of oxygen and thus further formation of ROS.[18]

Consequently, a stimulated respiratory chain could explain a protective effect of SOE. Furthermore, a protection of the muscle cell could also be explained by reduced production of ROS by NADPH oxidase after SOE illumination, as previously shown.[14]

In conclusion, singlet oxygen energy treatment of ischemic and reperfused rat skeletal muscle shows higher end-ischemic ATP levels. Overall energetic recovery is improved, as demonstrated by higher levels of ATP and PCr. This in vivo, noninvasive MRS evidence will encourage further research on the effects of SOE in situations of ischemia and reperfusion injury.

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