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Preservation of rat skeletal muscle energy metabolism by illumination

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Abstract

Skeletal muscle viability is crucially dependent on the tissue levels of its high energy phosphates. In this study we investigated the effect of the preservation medium Perfadex and illumination with Singlet Oxygen Energy (SOE). Singlet oxygen can be produced photochemically by energy transfer from an excited photosensitizer. The energy emitted from singlet oxygen upon relaxation to its triplet state is captured as photons at 634 nm and is here referred to as SOE. Rat hind limb rectus femoris muscles were preserved for five hours at 22 °C in Perfadex, saline, SOE illuminated Perfadex or SOE illuminated saline. Extracts of the muscles were analysed by ³¹P NMR. Data were analysed using two-way analysis of variance and are given as mean values (μmol/g dry weight) ± SEM. The ATP concentration was higher ($p = 0.006$) in saline groups (4.52) compared with Perfadex groups (2.82). There was no statistically significant difference in PCr between the saline groups (1.25) and Perfadex groups (0.82). However, there were higher ($p = 0.003$) ATP in the SOE illuminated groups (4.61) compared with the non-illuminated groups (2.73). The PCr was also higher ($p < 0.0001$) in the SOE illuminated groups (1.89) compared with the non-illuminated groups (0.18). In conclusion, Perfadex in this experimental model was incapable of preserving the high energy phosphates in skeletal muscle during 5 hours of ischemia. Illumination with SOE at 634 nm improved the preservation potential, in terms of a positive effect on the energy status of the muscle cell.

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Introduction

Successful muscle preservation is important during time-consuming microsurgical operations. During surgery the muscle is subjected to ischemia and ATP synthesis via oxidative phosphorylation will not be possible due to reduced oxygen supply [20]. The high energy phosphates ATP and PCr will decrease and put the tissue in great risk. Difficulties during the transfer of the muscle transplant can lead to long periods of ischemia and are known to adversely affect the viability of the muscle. Therefore better methods for preservation of skeletal muscle are needed in order to increase the success of complicated muscle transplantations.

The energetic status of the muscle tissue is a good marker for the viability of the muscle where high concentrations of ATP and PCr indicate good viability [4]. As long as the energy is retained in the cell, ischemia followed by reperfusion may lead to restoration of the muscle.

Skeletal muscle can withstand 4 hours of ischemia with almost full recovery [20]. Longer ischemia needs to be accomplished with some muscle preservation method. The preservation should help to reduce cell injuries caused by lack of energy and reactive oxygen species (ROS) produced in the mitochondria.

A commonly used preservation method is flushing the tissue with a preservation medium during the ischemia. Perfadex has been used for preserving lung [22], pancreas [15], blood vessels [8] and in the investigation of the importance of calcium in long-time preservation of vessels [9]. Perfadex is a colloid containing, lightly buffered extracellular low K^+ electrolyte solution. No studies have earlier been done to explore the potential of Perfadex to preserve skeletal muscle. In this study we investigated the effect of Perfadex on skeletal muscle during five hours of ischemia.

An innovative preservation method is illumination with singlet oxygen energy (SOE) light. Singlet oxygen $O_2(^1\Delta_g)$ can be produced photochemically by energy transfer from an excited photosensitizer. The energy emitted from singlet oxygen upon relaxation to its triplet ground state $O_2(^3\Sigma_g^-)$ is captured as photons at 634 nm and is here referred to as SOE. The use of light in various forms is proving to be an interesting new tool in medicine. Light-emitting diodes (LED) have documented benefits in a study by Whelan where illumination with LED decreased wound healing time and produced improvement of 40% in musculoskeletal training injuries [23]. Morrone examined traumatised muscles treated with Ga-Al-As diode lasers (780 nm) and reported a better qualitative and quantitative healing process than in spontaneous healing [16]. Furthermore, it is documented that the mitochondrion is a receptor to monochromatic near infrared light and that laser light around 600 nm may increase the respiratory metabolism in some cells [10].

We have previously shown that illumination with Singlet Oxygen Energy (SOE) at 634 nm reduced the production of reactive oxygen species with up to 60% in isolated human monocytes [6]. More recently, we showed that SOE improved the energetic recovery in vivo in skeletal muscle at postischemic reperfusion by in vivo ^{31}P -NMR spectroscopy [14].

We have also shown that SOE preserved high energy phosphates levels in an in vivo experimental transplantation model thus counteracting the oxidative damage effect of short-term ischemia [12]. The correlation between the ischemic reperfusion syndrome and ROS generation is today well known [11,17].

Until now, no studies have ever been done in vitro on skeletal muscle on the effect of illumination with SOE during ischemia and in combination with a preservation medium. We hypothesized that illumination with SOE may improve the energetic status of skeletal muscle during ischemia.

The aim of this study was to evaluate the effect of Singlet Oxygen Energy and Perfadex as a preservation method on skeletal muscle during five hours of ischemia. ^{31}P NMR spectroscopy was used in this study to detect the energy metabolites adenosine triphosphate (ATP), phosphocreatine (PCr), inorganic phosphate (Pi), glucose-6-phosphate (G-6-P) and inosine monophosphate (IMP).

Materials and methods

Animals

The Ethics Committee of Göteborg University, Gothenburg, Sweden, approved the experiment. Female Sprague–Dawley rats (B&K Universal AB, Sollentuna, Sweden) weighing approximately 300 g were used. The rats had free access to Purina chow and tap water before the experiment. The rats were anaesthetised with sodium pentobarbital (70 mg/kg body weight) intraperitoneally. We used a rat rectus femoris model [13], using standard microsurgical technique, developed to resemble the clinical situation when transferring free vascularised muscle flaps. The rectus femoris muscle was dissected free from surrounding tissue and the ischemic period started when the vessels were cut.

Preservation model

The preservation medium Perfadex (XVIVO Transplantation Systems AB) was stored at 8 °C. It was adjusted with Addex-THAM (trihydroxymethylaminomethane) (0.24 mol/L; Pharmacia AB, Sweden) to pH of 7.40. The composition of Perfadex is seen in Table 1. Physiologic saline solution, 0.9% NaCl, was used as a comparable preservation medium.

The six control muscles were non-ischemic and frozen in nitrogen immediately after the dissection. The muscles were divided into four groups, six rats in each group, all preserved in the dark for five hours in 22 °C:

1. Rectus femoris muscles preserved in saline.
2. Rectus femoris muscle preserved in Perfadex.

Table 1
Composition of Perfadex

Component	Concentration
Na ⁺	138 mmol/l
K ⁺	6 mmol/l
Mg ²⁺	0.8 mmol/l
Cl ⁻	142 mmol/l
Phosphate	0.8 mmol/l
Sulphate	0.8 mmol/l
THAM-buffer	0.24 ml/l
Dextran 40	50 g/l
Glucose	5 mmol/l
Osmolarity	325 mOsm/l
pH	7.40

3. Rectus femoris muscles illuminated with SOE and preserved in saline.
4. Rectus femoris muscles illuminated with SOE and preserved in Perfadex.

All muscles were then frozen in liquid nitrogen, freeze-dried (Lyovac GT2, Leybold–Heraeus) for eight hours and then stored in sealed plastic cryotubes at $-80\text{ }^{\circ}\text{C}$ until extraction.

Illumination with SOE

The SOE was produced by Valkion equipment (Polyvalk AB, Sweden) as photons via a fibre optic cable (cable length 142 cm, diameter of the end of the 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 a good heat and a light resistance and can be applied on metal surface. As light source, 6 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 less compared to the use of a halogen lamp and consequently the requirements for the coating are also 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 around 90%. Circulating air through a water flask generated the humidity. 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 to escape. The Singlet Oxygen Energy corresponds to light energy with a wavelength of 634 nm. The illuminations with SOE of muscles preserved in the solutions were done three times (ten minutes each) during ischemia, initially, at half time and during the last ten minutes of ischemia. During the illuminated periods the fibre optic cable was moved over the surface of the preservation media, which contained the rectus femoris muscle (Fig. 1).

Extraction

The muscles were dissected free from blood and connective tissue and minced to powder that was weighed (Mettler AC 88, Zurich, Switzerland). The powder was placed on ice in plastic-cryotubes and the metabolites were extracted with 1.5 M perchloric acid containing 1 mM EDTA, prepared in Deuterium oxide (isotopic purity, Dr. Glaser AB Basel). The extraction continued for 20 minutes in an ice bath. After 15 minutes the tubes were mixed on a Vortex mixer and placed in the ice bath again for five more minutes. The precipitate was separated by centrifugation, 2000 g at $+4\text{ }^{\circ}\text{C}$ for 10 minutes. The supernatant was neutralised by addition of a 4 M potassium hydroxide solution. A mixture of Bromthymol Blue and Phenol Red was used to titrate to pH of 7, which was controlled with a pH meter (Orion, model 410A, Labassco). The samples were placed in an ice bath for 30 minutes and centrifuged, 2000 g at $+4\text{ }^{\circ}\text{C}$ for 10 minutes, to eliminate the potassium chloride. The samples were frozen in plastic-cryotubes in liquid nitrogen and were stored at this temperature until analysis.

NMR analysis

The sample volume was 3 ml, corresponding to approximately 190 mg freeze-dried muscle. The ^{31}P NMR measurements of α -, β - and γ -ATP, PCr, Pi, G-6-P and IMP were identified from their

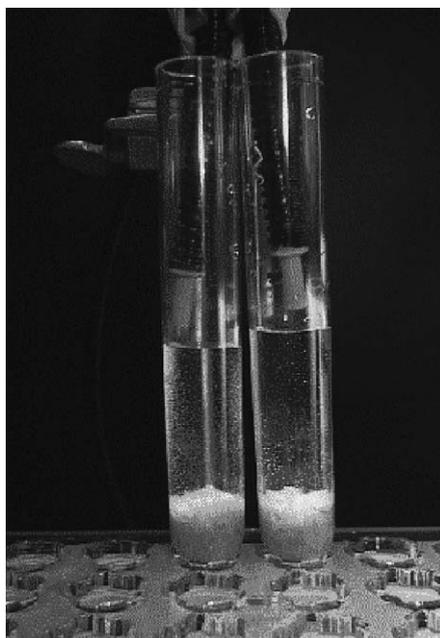


Fig. 1. Illumination with SOE via fiber optic cables.

chemical shift on a Bruker DMX 500 giving an operating frequency of 500.13 MHz for the ^1H nucleus. The samples were placed in 10 mm standard NMR sample tubes and the analyses were performed at + 4 °C. For the field frequency stabilisation a deuterium lock was used and the homogeneity of the magnetic field was optimised with the lock signal. Auto adjust shim was used to receive the optimum values during the data acquisition. The spectra were obtained by accumulation of 512 free induction decays (FID) with a repetition time of 4.5 seconds. A line broadening of 4 Hz was applied to the free induction decay before Fourier transformation. Total run time was 52 minutes and chemical shifts were referenced to PCr at 0 ppm. The β -ATP peak was chosen for ATP content calculation. The peak areas from respective metabolites were determined by comparing the areas of the individual peaks with the area of an internal standard, phenylphosphonic acid.

Statistical analysis

The experiment was regarded as a 2×2 factorial experiment with saline/Perfadex and SOE illuminated/non-illuminated as factors. Data were analysed using two-way analysis of variance with F-tests of as well main effects as of interactions [5]. Data are expressed as mean \pm SEM (standard error of the mean). Differences were considered statistically significant when $p < 0.05$. The SEM of the F-tests for PCr was based on three of the groups, deleting data for non-illuminated muscles preserved in Perfadex since all values in that group were zero. In the F-tests of PCr, only 15 degrees of freedom were used in the denominator.

Results

The peaks of ATP, PCr, Pi, G-6-P and IMP have been identified with a good signal to noise ratio and are given as mean values ($\mu\text{mol/g}$ dry weight) \pm SEM. The high energy phosphates ATP and PCr were determined in the control group (not subjected to ischemia, Fig. 2) to be 18.0 ± 1.3 and 82.5 ± 3.8 respectively. The concentrations of the energy metabolites Pi, G-6-P and IMP were 37.5 ± 1.9 , 0.3 ± 0.1 and 0.4 ± 0.3 , respectively.

After five hours of ischemia all energy metabolites analysed changed independently on group affiliation and are given as mean values ($\mu\text{mol/g}$ dry weight) \pm SEM in Table 2.

Using two-way analysis of variance there was no statistically significant interaction between muscles preserved in saline/Perfadex and SOE illuminated/non-illuminated muscles for the energy metabolites studied, except for Pi. This interaction is yet to be explained. The analysed groups expressed as mean values are shown in Table 2.

After five hours of ischemia the mean ATP value in saline groups decreased to 4.52 and to 2.82 in Perfadex groups, with a statistically significant difference of 1.7 ± 0.6 , $p = 0.006$. The mean ATP value of groups illuminated with SOE decreased to 4.61 and to 2.73 in non-illuminated groups, with a statistically significant difference of 1.9 ± 0.6 , $p = 0.003$. Illustrative spectra are shown in Fig. 3.

The mean PCr value in saline groups decreased to 1.25 and to 0.82 in Perfadex groups, with a non-significant difference of 0.4 ± 0.2 , $p = 0.052$. The mean PCr value in groups illuminated with SOE decreased to 1.89 and to 0.18 in non-illuminated groups, with a statistically significant difference of 1.7 ± 0.2 , $p < 0.0001$.

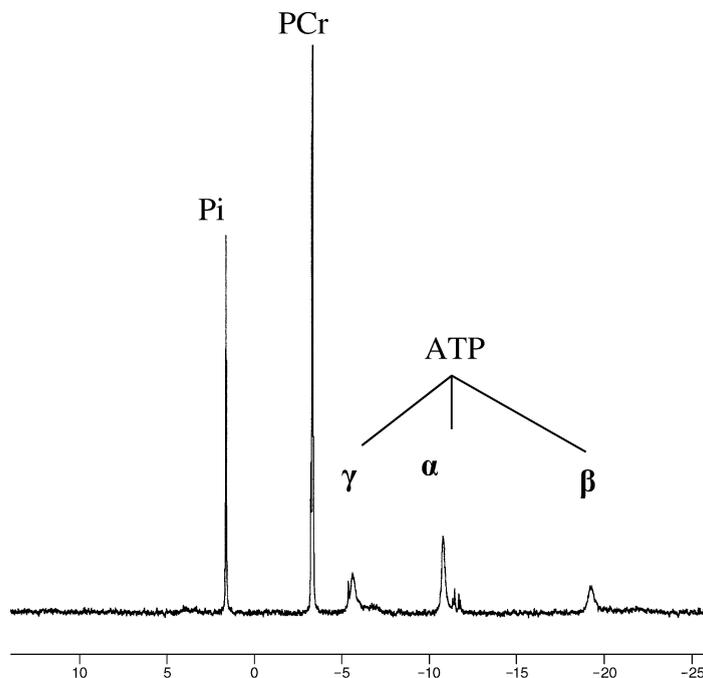


Fig. 2. In vitro ^{31}P NMR spectra from a control rat rectus femoris muscle showing the peaks of adenosine triphosphate (ATP), phosphocreatine (PCr) and inorganic phosphate (Pi).

Table 2

Energy metabolites in rat rectus femoris muscles after five hours ischemia in saline and Perfadex preserved groups (SOE illuminated and non illuminated)

	ATP	PCr	Pi	G-6P	IMP
Saline + SOE	5.0 ± 0.6	2.1 ± 0.2	118 ± 3.3	4.7 ± 0.3	13.9 ± 0.5
Perfadex + SOE	4.2 ± 0.6	1.6 ± 0.2	112 ± 3.3	4.1 ± 0.3	13.9 ± 0.5
Saline	4.0 ± 0.6	0.4 ± 0.2	114 ± 3.3	4.4 ± 0.3	14.1 ± 0.5
Perfadex	1.5 ± 0.6	0 ± 0.2	124 ± 3.3	3.6 ± 0.3	14.6 ± 0.5
P value A	0.006	0.052	0.533	0.037	0.632
P value B	0.003	< 0.0001	0.259	0.192	0.382

Data analysed using two-way analysis of variance expressed as mean ($\mu\text{mol/g}$ dry weight) \pm SEM. Six muscles in each group. SEM is calculated from the analysis of variance, assumed that there is equal variance in all groups. P value A refers to differences between saline and Perfadex preserved groups. P value B refers to differences between SOE illuminated and non-illuminated groups.

When ATP and PCr are degraded in the muscles, Pi is formed. The mean Pi value in saline groups increased to 116.2 and to 118.3 in Perfadex groups, with a non-significant difference of 2.1 ± 3.3 , $p = 0.533$. The mean Pi value in groups illuminated with SOE increased to 115.4 and to 119.2 in non-illuminated groups, with a non-significant difference of 3.8 ± 3.3 , $p = 0.259$. The interaction effect estimated as half of the difference between these differences was 8.2 ± 3.3 , $p = 0.020$.

The mean IMP value in saline groups increased to 13.99 and to 14.23 in Perfadex groups, with a non-significant difference of 0.2 ± 0.5 , $p = 0.632$. The mean IMP value of groups illuminated with SOE increased to 13.89 and to 14.33 in non-illuminated groups, with a non-significant difference of 0.4 ± 0.5 , $p = 0.383$.

The mean G-6-P value in saline groups increased to 4.55 and to 3.86 in Perfadex groups, with a statistically significant difference of 0.7 ± 0.3 , $p = 0.037$. The mean G-6-P value in the groups

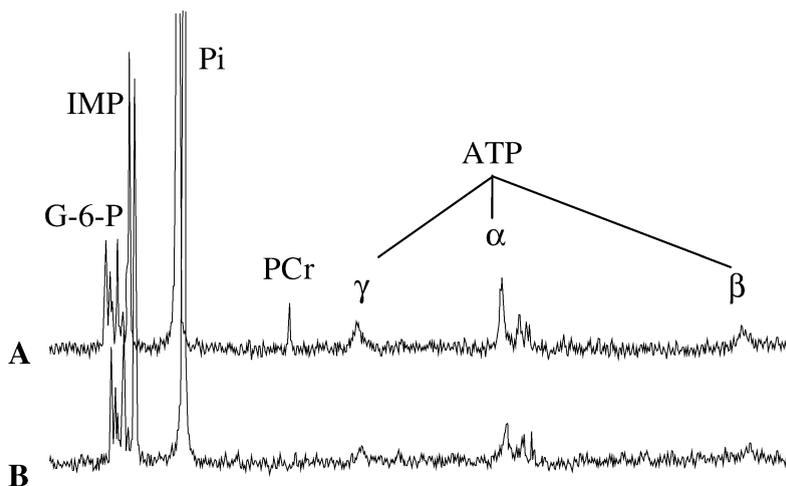


Fig. 3. In vitro ^{31}P NMR spectra from rat rectus femoris muscle after five hours of ischemia, in the SOE illuminated (A) and in the non-illuminated (B) groups. Differences are seen in the peaks of ATP and PCr where the peaks are higher in the spectra of the SOE illuminated group.

illuminated with SOE increased to 4.41 and to 4.0 in non-illuminated groups, with a non-significant difference of 0.4 ± 0.3 , $p = 0.192$.

Discussion

The first objective of this study was to investigate if Perfadex is a good preservation medium for skeletal muscle, as it is for lung, pancreas and blood vessels [8,15,22]. The second objective was to evaluate the effect of illumination with SOE on skeletal muscle in preservation medium. The muscle energy metabolites were analysed by high resolution ^{31}P NMR spectroscopy. Using this method we were able to detect ATP, PCr, Pi, IMP and G-6-P.

The ischemic time was chosen with respect to previous results showing that the muscle can tolerate four hours of ischemia with partial recovery after reperfusion [20,21]. After six hours of ischemia there is irreversible damage in the muscle and no energetic recovery after reperfusion [7]. In the current study the ischemic time was set to five hours to make certain that it would still be detectable concentrations of ATP and PCr in the muscle. The temperature was $22\text{ }^{\circ}\text{C}$, which is based on clinical practise in plastic surgery, where the operating room temperature is $22\text{--}26\text{ }^{\circ}\text{C}$. In agreement with the nucleotide pathways we found that PCr consumption is followed by low concentration of ATP with a concomitant increase in degradation products such as Pi.

An optimal preservation medium for skeletal muscle should help to maintain high energy phosphates and consequently prolong the cell viability during ischemia. When preserving muscles in Perfadex, we found low concentration of ATP and no PCr. The results of Perfadex were unexpected and may be due to the experimental model used in this study. The muscles were stored in plastic tubes with the solution and not flushed. A better preservation effect with Perfadex likely occurs by flushing the tissue in combination with hypothermia. However, this experimental model was designed to simulate clinical plastic microsurgery, where flushing of organs are not generally done in order not to damage the small vessels.

The second objective of this study was to evaluate the effect of illumination with SOE to preserve skeletal muscle during five hours of ischemia. SOE should not be confused with singlet oxygen, which is a reactive form of oxygen before relaxation to its ground state triplet oxygen. SOE is the energy emitted by singlet oxygen during its relaxation to ground state oxygen, captured as photons at $\lambda\ 634\ \text{nm}$. The light source for SOE is light emitting diodes (LED), which has characteristics that make the suitable for clinical practise. LED can produce multiple wavelengths and produce no heat. We found higher concentrations of ATP and PCr in the SOE illuminated than in the non-illuminated muscles indicating that muscles illuminated with SOE have a higher viability during ischemia.

The main damage on muscle cells occurs on the onset of reperfusion [19] and studies indicate that generation of reactive oxygen species (ROS) contribute to reperfusion damage after ischemia [11,17]. However, also during ischemia, cellular damage occurs since the mitochondria may become a source as well as a target of free radicals [2,21]. In a parallel study we have shown a reduction in ROS generation in PMA activated monocytes exposed to SOE [6]. The antioxidant effect of SOE reported was partly attributed to inactivation of NADPH oxidase. In the present study, SOE might have reduced the production of ROS during ischemia, which might explain the higher concentrations ATP and PCr in the SOE illuminated muscles compared with non-illuminated muscles.

The results of the present study agree with those obtained in a previous study where illumination with SOE increased ATP and PCr during ischemia and reperfusion *in vivo* in skeletal muscle [14]. The results of the current study also agree with a study that reported that photoirradiation improved functional recovery of a cold stored rat heart via conservation of ATP and antioxidant enzyme activity [25].

It is well known that components of the mitochondria respiratory-chain such as cytochrome oxidase absorb in the red to IR range [1,3,10]. Agreement is found in a study where LED was applied at near IR (670 nm) on primary neurons [24]. Illumination with LED was found to stimulate cytochrome oxidase activity and reverse the down regulated enzymes to control levels. 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 [10].

Ischemia is known to affect the mitochondrial membrane-bound cytochrome c oxidase [18]. The function of cytochrome c oxidase is connected to cardioplin content in the mitochondrial membrane. After four hours of ischemia the function of cytochrome c oxidase is depressed, while cardioplin content decreases. This suggests a peroxidative attack of the membrane. A reduced cytochrome c oxidase activity would increase the risk for an incomplete reduction of oxygen and thus further formation of ROS. A stimulated respiratory chain could explain the protective effect of SOE. Furthermore, a protection of the muscle cell could also be explained by reduced production of ROS by NADPH oxidase as previously shown [6].

The exact mechanism of illumination with SOE is unknown and warrants further studies.

Conclusion

The results of the current study showed that Perfadex in this experimental model was unable of preserving high energy phosphates in skeletal muscle during five hours of ischemia. However, illumination with SOE of muscles preserved in Perfadex as well as saline, improved the energy status during ischemia.

Acknowledgements

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