Improved bioenergetic recovery during experimental ischemia and reperfusion by irradiation

Ann Lindgård

GÖTEBORG UNIVERSITY

Bioenergetics Group, Department of Surgery,
Wallenberg Laboratory & Lundberg Laboratory for Bioanalysis,
Sahlgrenska Academy, Göteborg University

ISBN 978-91-628-7294-6

Göteborg 2007

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These papers have already been published or are in manuscripts at various stages (in press, submitted, or in a manuscript).

Cover:
"Point of view IV"
(mixed technique on paper)

Printed by Intellecta Docusys AB
Göteborg, Sweden 2007
ISBN 978-91-628-7294-6

ABSTRACT

Prolonged ischemia and reperfusion frequently occur during clinical operations. The bioenergetic status decreases during ischemia and reactive oxygen species (ROS) are formed during reperfusion, which may lead to irreversible tissue injury. During prolonged ischemia, such as in complex microsurgical operations, tissue injury should be minimized to improve the chance of full recovery and function. Irradiation has previously been shown to improve functional recovery of cold-stored rat hearts via conservation of ATP. In this thesis, we used photons at 634 nm produced from a singlet oxygen system to investigate whether irradiation improves the bioenergetic status in skeletal muscle and graft hearts and decreases ROS in monocytes, and thus decreases tissue injury.

The effect of irradiation on bioenergetic status was examined in rat rectus femoris muscle *in vitro* following 5 h ischemia. Phosphocreatine (PCr), ATP and inorganic phosphate (Pi) levels were measured using high resolution 11.75T ^{31}P magnetic resonance spectroscopy (MRS). PCr and ATP were significantly higher in the irradiated groups than in the non-irradiated group, but no difference in Pi was observed. The effect of irradiation on bioenergetic status was examined in rat rectus femoris muscle *in vivo* following 4 h ischemia and 1 reperfusion. ATP, PCr and Pi levels were measured using 2.35T ^{31}P MRS. After 4 h ischemia, ATP levels in the irradiated group were significantly higher than in the non- irradiated group, but no difference in PCr/(PCr+Pi) levels were observed. After 1 h reperfusion, ATP and PCr/(PCr+Pi) levels in the irradiated groups were significantly higher than in the non-irradiated groups. Blood-perfusion was measured using laser Doppler flowmetry and did not differ between the groups. The effect of irradiation on xenografts was examined *in vivo* following heart xenotransplantation from hamster to rat. PCr and ATP levels were measured daily using 2.35T ^{31}P MRS over 4 days. Irradiation of xenografts before reperfusion preserved the energetic status of hamster grafts, as demonstrated by a significantly higher PCr/ATP ratio in the irradiated group than in the non-irradiated group on the first postoperative day. However, irradiation did not delay the rejection process in this experimental model. Finally, the effect of irradiation was examined in human monocytes. Intracellular ROS, nitric oxide (NO) release and ATP were measured using chemiluminescence assays. iNOS and eNOS mRNA levels were measured using reverse transciption PCR. NO levels were significantly higher in the irradiated group than in the non-irradiated group, but no differences in iNOS or eNOS mRNA were observed. Intracellular ROS release was significantly lower in the irradiated group than in the non-irradiated group, and there was no difference in ATP levels. The salvaging effect of irradiation may have a wide range of clinical applications, for example in complex microsurgery and transplantation surgery.

Key words: energy metabolism, skeletal muscle, monocyte, ischemia, reperfusion, NMR, singlet oxygen energy, light, LED

LIST OF PUBLICATIONS

This PhD thesis is based on the following publications, which are referred to in the text by their Roman numerals:

Paper I Lindgård A., Lundberg J., Rakotonirainy O., Elander A. and Soussi B. Preservation of rat skeletal muscle energy metabolism by illumination. *Life Sciences 72 (23), 2649-2658, 2003.*

Paper II Lundberg J., Lindgård A., Elander A. and Soussi B. Improved energetic recovery of skeletal muscle in response to ischemia and reperfusion injury followed by *in vivo* ^{31}P-Magnetic Resonance Spectroscopy. *Microsurgery 22 (4), 158-164, 2002.*

Paper III Lukes D., Lundgren A., Wilton J., Lindgård A., Omerovic E., Rakotonirainy O., Parra AK., Olausson M. and Soussi B. Singlet Oxygen Energy illumination during ischemia preserves high energy phosphates in a concordant heart xenotransplantation model**.** *Laser Physics 13 (1), 84-90, 2003.*

Paper IV Lindgård A., Hultén LM., Svensson L. and Soussi B. Irradiation at 634 nm releases nitric oxide from human monocytes. *Lasers in Medical Science* 22 (1), 30-36, 2007.

TABLE OF CONTENTS

ABBREVIATIONS

ADP	Adenosine diphosphate
AG	Aminoguanidine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine 3',5' –monophosphate
eNOS	Endothelial nitric oxide synthase
FID	Free induction decay
G-6-P	Glucose-6-phosphate
IMP	Inosine monophosphate
iNOS	inducible nitric oxide synthase
LDF	Laser doppler flowmetry
LED	Light emitting diode
MRS	Magnetic resonance spectroscopy
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
PCr	Phosphocreatine
PDE	Phosphodiester
PDT	Photodynamic therapy
Pi	Inorganic phosphate
PMA	Phorbol myristate acetate
PME	Phosphomonoester
ROS	Reactive oxygen species
SOE	Singlet oxygen energy
T	Tesla

INTRODUCTION

Ischemia and reperfusion injury

Ischemia (reduced blood flow) followed by reperfusion (restored blood flow) occurs in a wide range of organs, including heart, lung, kidney and gut, and in skeletal muscle during reconstructive plastic surgery, thrombolytic therapy, orthopedic surgery, transplantation and limb trauma such as crush injury and fractures. In microsurgery and reconstructive plastic surgery the duration of ischemia is now being pushed to the limits of tissue ischemic tolerance, particularly because of the development of improved microsurgical techniques for revascularization of severed limbs (1). Sensitivity to ischemia varies according to the type of musculature or organ. This variation is determined by factors such as energy demand. Of all tissues of the extremities, skeletal muscle is the least tolerant of ischemia (2): it tolerates up to 4 h ischemia while nerve (8 h), fat (13 h), skin (24 h), and bone (4 days) are less sensitive (2). Ischemic periods in skeletal muscle that exceed 6 h followed by reperfusion are also associated with high mortality due to release of toxic metabolites into the systemic circulation (2). Therefore, it is important to find preservation methods for skeletal muscle that decrease injuries caused by ischemia and reperfusion.

Energy status

The events during ischemia and reperfusion are basically similar in different organs and tissues, but the time scale of the event varies. However, the mechanisms for generation and consumption chemical energy are assumed to be identical at the molecular level irrespective of organ.

Energy within the cell is stored as adenosine triphosphate (ATP), and is required in energy demanding processes such as maintenance of membrane potential mainly via the sodium-potassium pump (Na^+/K^+ ATPase) (3). The pump keeps the intracellular Na^+ concentration low while maintaining high intracellular K^+ concentration (3, 4). ATP is synthesized from glucose in three steps: glycolysis, Krebs cycle and oxidative phosphorylation (Figure 1). The ATP molecule stores the energy in the form of a high-energy phosphate bond. When the ATP molecule is cleaved, the products are adenosine

diphosphate (ADP), inorganic phosphate (Pi), and energy. As the reaction is reversible, ADP can be recharged by oxidation back to ATP.

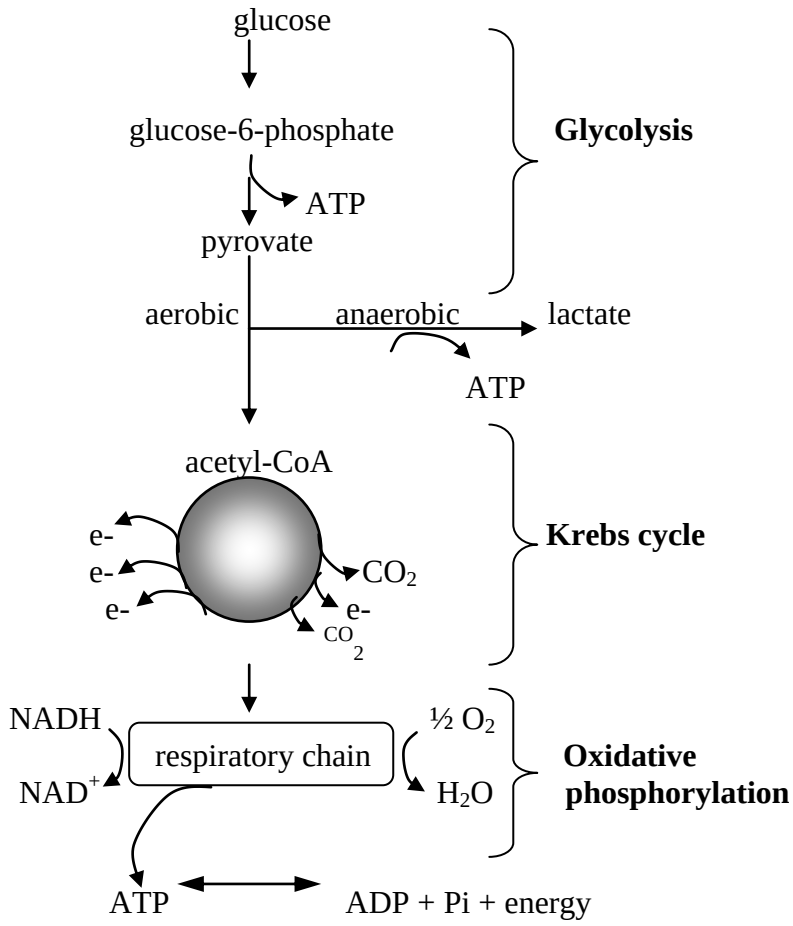

Figure 1. Glycolysis, Krebs cycle and the electron transport chain (3). Glycolysis occurs in the cytosol of the cell. It is an anaerobic process that yields ATP and pyrovate. In the presence of oxygen, pyrovate enters into the Krebs cycle. The Krebs cycle occurs inside the mitochondria matrix. It is an anaerobic process that yields carbon dioxide, reduced coenzymes, and a large flow of electrons used in the respiratory chain. Oxidative phosphorylation occurs in the membrane of the cristae inside mitochondria. It is an aerobic process that yields water and ATP.

Skeletal muscle cells also stores energy in creatine phosphate (PCr) by the creatine kinase reaction when ATP levels are high (5). As this reaction is reversible, ATP is replenished when its level is depleted, such as during ischemia. Thus, the creatine kinase reaction

controls cellular energy homeostasis and plays a particularly important role in tissues such as skeletal muscle with large and fluctuating energy demands.

$$ATP + Cr \xleftrightarrow[\text{creatine kinase}]{} PCr + ADP + H$$

During ischemia, the lack of oxygen will halt the production of ATP through oxidative phosphorylation, and ATP levels are maintained by the creatine kinase reaction. However after approximately 1 h of ischemia, PCr levels are depleted and ATP is produced by anaerobic glycolysis with the formation of lactic acid. After longer ischemia (>2 h), the energy demand exceeds the energy supply (6) and ATP is reversibly catabolized in the purine nucleotide pathway (Figure 2). After prolonged ischemia (>4 h), hypoxanthine is irreversible catabolized into xanthine, making a complete energetic recovery impossible upon reperfusion (Figure 2) (2, 6). After severe (6 h) of ischemia, ATP is almost completely depleted (7) and as a result the energy-dependent sodium-potassium pump will stop. Potassium leaks out of the cell while sodium, chloride and water enter, causing cell swelling (8).

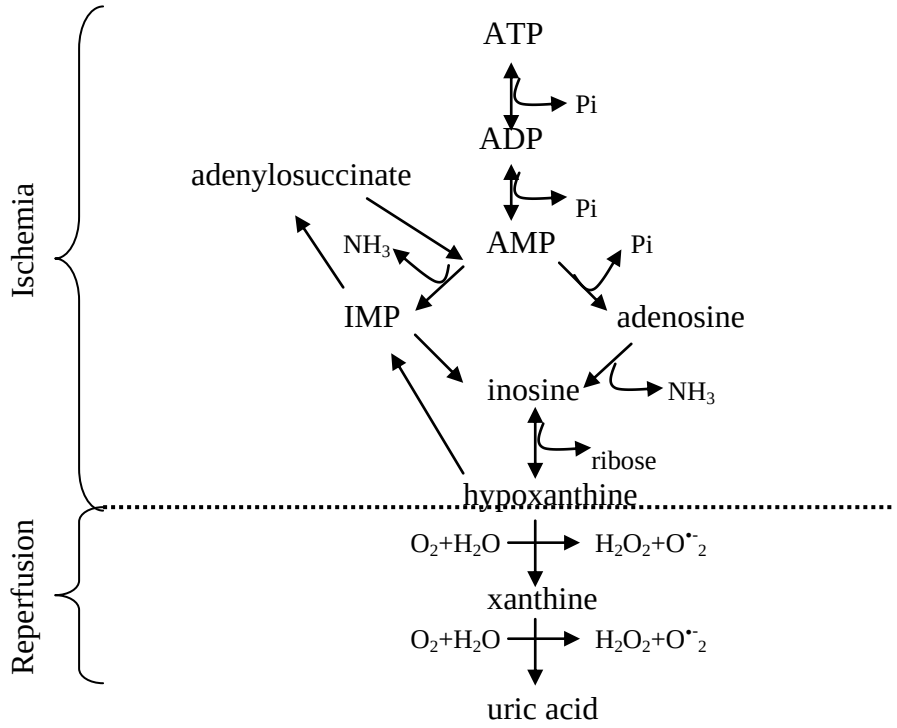

Figure 2. Purine nucleotide pathway during ischemia (>4 h) and reperfusion (9). H_2O_2 hydrogen peroxide, $O^{\bullet-}_2$ superoxide radical.

Reperfusion after short periods of ischemia (minutes) is a benign process. However, after prolonged ischemia (4-6 h), reperfusion results in the release of toxic products into the systemic circulation (2). Reperfusion after 6 h of ischemia is also associated with high mortality due to multiple organ failure (2). The effect of ischemia and reperfusion on skeletal muscle has been well described in several studies (Table 1). By comparing ATP levels and histology after different periods of ischemia, it is clear that the energetic status as a measure of cell viability correlates with the histology.

Table 1. The effects of ischemia and reperfusion on ATP level and histology in different experimental models of skeletal muscle.

Ischemia	**Reperfusion**	**ATP (% of control) / Histology**	**Model**
2 h	1 h	81	rat (7)
2 h	1 day	5% necrosis, few inflammatory cells	rabbit (10)
3 h	5 h	41	rat (1)
3 h	2 days	fiber fragmentation, inflammation	rat (11)
4 h	1 h	32	rat (12)
4 h	3 days	46% necrosis	rat (13)
5 h	45 min	31	dog (14)
5 h	2 days	90% necrosis	dog (15)
6 h	1 h	0	rat (7)
6 h	1 day	all muscle fibers are injuried	rabbit (10)

Reactive oxygen species

Reactive oxygen species (ROS) are an important component in host defense (16) but can also inflammation, lipid peroxidation and apoptosis (16, 17). ROS is a collective term that includes oxygen radicals such as superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$), and some non-

radical derivatives of oxygen (that can lead to formation of free radicals) such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1\Delta_g$) (18, 19).

A free radical contains one or more unpaired electrons and is highly reactive. When two radicals meet the product is a nonradical. However, when a radical reacts with a nonradical, a new radical results and initiates chain reactions (20). Intracellular ROS release is an important component in host defense against infectious agents. The extracellular ROS release may damage surrounding tissues and therefore promoting inflammatory processes and DNA damage (16, 19). Under normal conditions, ROS are formed in the electron transport chains within mitochondria (18) but are cleared from the cell by the scavenger system superoxide dismutase (SOD), catalase and glutathione peroxidase (21).

The formation of ROS in skeletal muscle have been shown in an *in vivo* study (22). ROS are generated during early ischemia but neutralized by the endogenous scavenger system. However, the main formation of ROS occurs during the initial reperfusion phase (23), when blood containing oxygen and water re-enters the tissue, forming mainly the highly reactive superoxide radical (24, 25) (Figure 2).

Nitric oxide

Nitric oxide ($NO^{\cdot}$) has an unpaired electron and is a free radical, and belongs to the collective term reactive nitrogen species (18). NO is a key biological messenger, playing a role in a variety of biological processes such as vasodilation and the modulation of the immune system (26-28). NO is formed during the conversion of L-arginine to citrulline and the reaction is catalyzed by NO synthase (NOS) (28). At least three isoforms of NOS have been identified: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) (28, 29).

NO is also a major mediator of tissue damage during ischemia and reperfusion (28). NO react with superoxide (formed in abundance during reperfusion) to form peroxynitrite ($ONOO^-$), which is protonated to form peroxynitrous acid (HOONO) (28). Dissociation of peroxynitrous acid yields the highly cytotoxic species nitrogen dioxide (NO_2) and

hydroxyl (OH•) (28). There is little and contradictory information about NO in skeletal muscle, with some studies suggesting that NO is cytotoxic while other show NO is beneficial (28, 30-32). In myocardial ischemia- and reperfusion studies, it seems that low doses of NO are protective and high doses are harmful (33).

Prevention of ischemia and reperfusion injury

Preservation solutions

Preservation solutions are used to counteract the unwanted effects of ischemia such as cell swelling, impaired energy metabolism, and ROS formation. Cell swelling is suppressed by the addition of osmotically active substances whose molecular mass is sufficiently large to prevent entry into cells, and include saccarides (glucose, mannitol) and impermeant anions (phosphate, sulfate, lactobionate) (34). The composition (e.g. the ratio of sodium/potassium, and the concentration of magnesium and calcium), and can be either intra- or extracellular like, depending on what the solution is mainly designed to be used for (34, 35).

The most commonly used preservation method today is flush perfusion of the organ with a cold preservation solution to remove blood and obtain a rapid core cooling (hypothermia) of the organ (36) for decreased cell metabolism (34, 37, 38). However, flush perfusion of small skeletal muscle is generally not performed in clinical plastic microsurgery as the perfusion-pressure during flushing can damage the microvasculature (39). A preservation method is thus required that does not injure the microvasculature, is easy to perform, maintains the energy levels, decreases the production of ROS and ultimately increases the chances of recovery after surgery.

In the 1970s, Collins developed a preservation solution that was later modified in Europe, and is now known as the EuroCollins (EC) solution (35, 40). This solution was used for liver, kidney and pancreas in the US until 2002 and is still used in Europe (40). Belzer and Southard developed the University of Wisconsin (UW) solution in the late 1980s for liver, kidney, and pancreas, which later became the golden standard (40, 41). Today, three other solutions are also commonly used: Celsior and IGL-1 (which are modified UW

solutions), and histidine-trytophane-ketoglutarate (HTK) which was originally developed as a cardioplegia solution (35, 40, 42).

In addition, the preservation solution Perfadex is designed mainly for the storage of lung transplants (43-45), but is also preserves contractility of rat vascular smooth muscle (36) and porcine pancreatic function (46) after cold ischemia. No previous studies have explored the potential of Perfadex to preserve skeletal muscle.

Antioxidants

An antioxidant is defined as a substance that significantly delays or prevents oxidation of a molecule found *in vivo* (18). Treatment of tissue with different antioxidants and scavengers is an obvious therapeutic approach during ischemia and reperfusion due to the formation of ROS (12, 47). Antioxidants synthesized in the human body includes superoxide dismutase (SOD), catalase and glutathione peroxidase (20). SOD dismutates superoxide radicals ($O_2^{\bullet-}$) to form hydrogen peroxide (H_2O_2) and oxygen (O_2). Catalase catalyses the breakdown of H_2O_2 to form water and O_2 (48). Glutathione peroxidase also breakdown H_20_2 (20, 21). Dietary antioxidants includes for instance the fat-soluble α-tocopherol (vitamin E) and ascorbate (vitamin C) (12, 20, 23). There are also synthetic antioxidants, such as indeno-indol derivate, which is very potent and can limit lipid peroxidation 100 times more efficiently than α-tocopherol (47, 49). In addition, several molecules can function as antioxidants such as uric acid (18). Results also show a strong antioxidant activity using irradiation (48).

Irradiation

The beneficial effects of light to treat various diseases have been known since ancient times (50). Light is used today in dermatology, in photodynamic therapy for cancer, to accelerate wound healing, and to improve musculoskeletal training injuries (51-53).

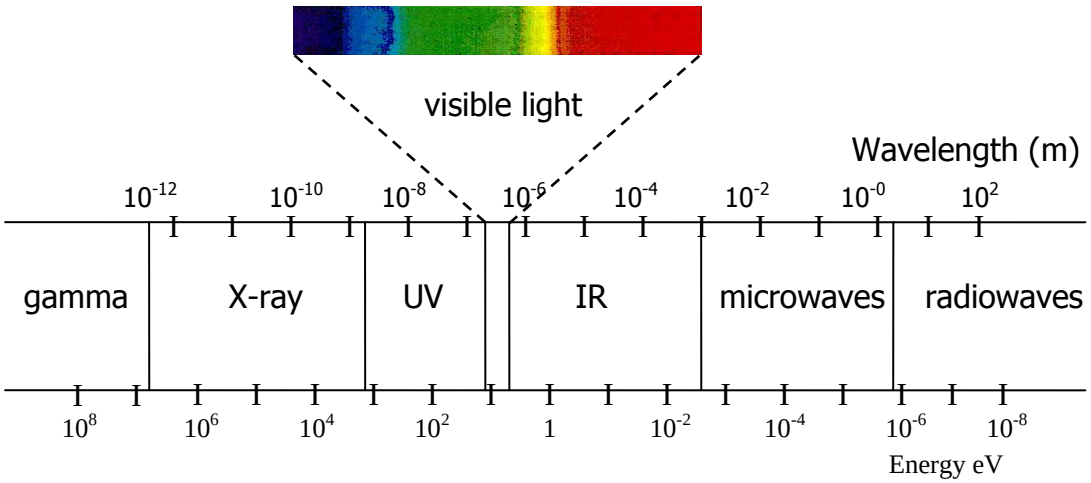

Figure 3. Visible light is electromagnetic radiation (photons) of wavelengths approximately 400 nm to 700 nm. The wavelength is inversely correlated to the energy.

The nomenclature used to describe light treatment varies, and the most common terms are listed in Table 2. The papers included in this thesis have used the terms SOE light and irradiation, and here I have chosen to use irradiation.

Table 2. Nomenclature used in the literature to describe irradiation treatment.

Low level laser	Light emitting diode	Laser pulse	Low energy laser
Red laser	Laser therapy	Low power laser	Red light therapy
SOE light	IR diode	Low intensity laser	Pulsed radiation
Infrared	Biostimulating laser	Bioregulating laser	Soft laser
Irradiation	Photobiostimulation laser	Near infrared	Low power laser light
Low reactive level	Photobiomodulation	Medical laser	Therapeutic laser

Various forms of irradiation have been studied experimentally (Table 3). For example, irradiation at 634 nm reduced the production of extracellular ROS in human monocytes mainly via the inhibition of NADPH oxidase (48). Irradiation has also previously been shown to improve functional recovery of cold stored rat hearts via conservation of ATP (54).

Red light is considered to reach lower depths in skin tissue than shorter wavelengths (55) and a study on human skin has shown a depth of penetration of almost 15 mm when irradiating with 694 nm (56). In experimental studies on cell cultures, wavelengths in the visible and the infrared region can pass through a monolayer cell culture (57).

There is also evidence that irradiation can release NO from cells by release of a irradiation-sensitive store of NO (54, 58-60).

Table 3. Studies of irradiation performed in the wavelength 340-1064 nm in different experimental models.

Nomenclature	**nm**	**Effect**	**Model**
UVA	340	increased membrane conductance	mammalian cells (61)
Laser pulse	355	reduction of cytochrome c	horse (62)
He-Cd laser	441	NO release	hemoglobin (60)
Argon	514.5	promted ATP synthesis	guinepig mitochondria (63)
Laser pulse	532	monitoring respiratory chain	hepatocytes (64)
Red laser	630	induced iNOS and increase cGMP	rabbit (65)
He-Ne	632	prevented restenosis	rabbit (66)
He-Ne	632.8	no induced micronucleus formation	lymphocytes (67)
He-Ne	632.8	decreased number of mitochondria	human lymphocytes (68)
He-Ne	632.8	enhanced de novo protein synthesis	muscle myoblast (69)
He-Ne	632.8	increased ADP/ATP exchange	rat mitochondria (70)
He-Ne	632.8	increased ATP levels	HeLa cells (71)
He-Ne	632.8	increased ATP synthesis	rat mitochondria (72)
He-Ne	632.8	increased membrane potential	rat hepatocytes (73)
He-Ne	632.8	promoted survival	mice muscle (74)
He-Ne	632.8	increased protein synthesis	rat hepatocytes (75)
He-Ne	633	accelerated Ca^{2+} uptake	bull mitochondria (76)
SOE	634	decreased ROS	monocytes (48)
Argon	660	increased ATP at preservation	heart (54)
Argon	660	increased oxygen consumption	rat mitochondria (77)
LED	670	improved wound healing	human (52)
LED	670	increased cytochrome oxidase activity	neurons (78)
Ga-Al-As	780	improved recovery	rat muscle (79)
IR	803	reduced infarct size	dog, rat (80)
Ga-As	810	reduced infarct size	dog, rat (81)
IR diode	820	increased cell attachment to glass	HeLa cells (82)
IR	1064	inhibited neointimal hyperplasia	rabbit (83)

AIMS

The overall aim of this thesis was to investigate whether irradiation can reduce damage caused by ischemia and reperfusion.

The specific aims of this thesis were:

- To evaluate the effect *in vitro* of irradiation at 634 nm and Perfadex as a preservation method during five hours of ischemia on rat rectus femoris muscle, using *in vitro* high resolution ^{31}P Magnetic Resonance Spectroscopy (Paper I).

- To investigate the effect *in vivo* of irradiation at 634 nm on rat rectus femoris muscle, using *in vivo* ^{31}P MR Spectroscopy. To investigate the effect of irradiation on the blood perfusion during ischemia and reperfusion, using Laser Doppler flowmetry (Paper II).

- To investigate whether exposure of hamster hearts to irradiation at 634 nm during and after the transplantation procedure could improve the energetic status, using *in vivo* ^{31}P MR Spectroscopy (Paper III).

- To investigate the effect of irradiation at 634 nm on human monocytes on the release of NO, release of intracellular ROS and ATP, using chemiluminescence. To investigate the effect of irradiation on iNOS and eNOS, using RT PCR (Paper IV).

METHODOLOGICAL CONSIDERATIONS

The Ethics Committee of Göteborg University, Göteborg, Sweden, approved the experiments. The animals were allowed to settle in the animal quarters for several days before the experiments. The animals were fed Purina chow and tap water ad libitum before the experiments. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US NIH publication 85-23, revised 1996.

Experimental models

Rat ischemia model – Paper I

Male Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden) weighing 300 g were used. The rats were anaesthetized with sodium phentobarbital 70 mg/kg body weight intraperitoneally and supplementary anesthesia 2 mg/kg body weight was administered intraperitoneally as required. The body temperature of each rat was maintained with a heating blanket and monitored with a rectal probe. Room temperature was 24°C (±0.5) throughout the experiments. At the end of the experiments, the animals received intracardial injection of sodium phentobarbital, followed by section of the heart.

The experimental model used in this study was designed to simulate clinical plastic microsurgery, where flush perfusion of organs is not generally performed to spare the microvasculature. The rat rectus femoris muscle model has been used in several studies (22, 32, 84). The model was modified and published by Lundberg (38). The rat rectus femoris muscle contains approximately 3% type I (red muscle, oxidative metabolism), 22% type IIA (intermediate muscle, oxidative and glycolytic metabolism) and 75% type IIB (white muscle, glycolytic metabolism) (85).

The left rectus femoris muscle was used, leaving the right side unexposed. The dissection started with an incision above and parallel to the inguinal ligament. The incision continued down the ventral side of the thigh to the patella. The femoral nerve was identified and cut proximal to the diversion to the rectus femoris muscle. The dissection

of the rectus femoris muscle was started from a point distal to the insertion to the patella in the medial side. The gracilis muscle and vastus medialis muscle were bluntly dissected free from the rectus femoris muscle, and at the lateral side of the patella the vastus lateralis muscle and gluteus superficialis muscle were relocated laterally by dissection. At this point, the rectus femoris muscle was free from the surrounding muscles. The insertion to the patella was then cut and the insertion to the pelvic girdle severed. The remaining blood supply to the rectus femoris muscle comprised the vessels originating from the femoral vessel. The ischemic period began when the vessels were cut. The muscles were immediately put in Perfadex or saline (0.9% NaCl).

Rectus femoris muscles were irradiated by slightly moving fibre optic cable over the surface of the preservation solutions, which contained the rectus femoris muscle (Figure 7). The irradiations of muscles preserved in the solutions were done (10 min each) initially, at half time and during the last ten minutes of ischemia. All muscles were then frozen in liquid nitrogen, freeze-dried (Lyovac GT2, Leybold-Heraeus) for 8 h and then stored in sealed plastic cryotubes at –80°C until extraction.

Rat ischemia and reperfusion model – Paper II

Male Sprague-Dawley rats were initially treated the same as in paper I. The experimental model used in this study was designed to resemble the clinical situation when transferring free vascularised muscle flaps. In surgery transferring of muscles is a well established method to reconstruct body parts and reconstruct muscle-function. During the transferring the muscle is exposed to ischemia and reperfusion.

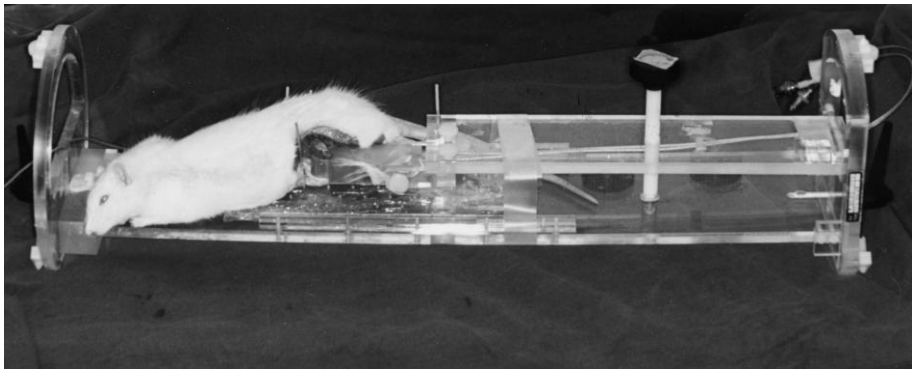

Figure 4. Anaestheised rat with the rectus femoris muscle lying on the surface coil, ready for MRS.

The reason using rectus femoris muscle was that the muscle has a long pedicle so that it could be measured isolated without interference from the body. This is a condition for continuous and non invasive measurements to be made by *in vivo* MRS (Figure 4).

The model was same as in paper I except that the femoral vessel was intact. Some of the tissue surrounding the rectus femoris muscle pedicle was left in order to prevent the pedicle from drying. During ischemia the femoral vessel was clamped proximal and distal of the pedicle in order not to injury the thin vessels. The MRS measurements were done on the distal twp thirds of the rectus femoris muscle. This area was chosen because it was the only part of the muscle which could be evaluated in this experimental set-up by *in vivo* MRS. The MRS measurements were done according to the chart flow in Figure 5.

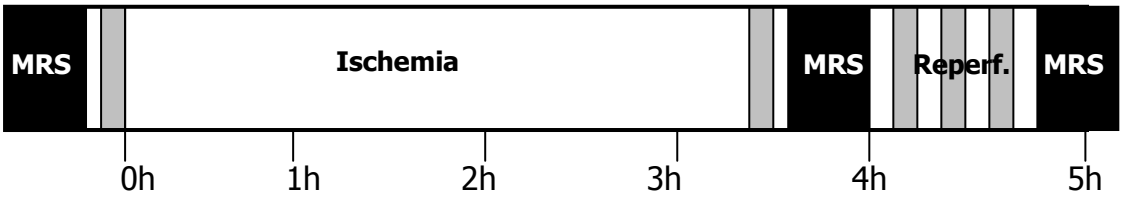

Figure 5. Chart flow of MRS measurements and irradiation in paper II. The MRS measurements (black boxes) were done before the onset of ischemia, during the end of ischemia and after reperfusion. The received MRS value is a medium value for the 34 minutes of measurements. The irradiations (grey boxes) were 5 min each, and were done before the onset of ischemia, during the end of ischemia and 3 times during reperfusion.

Rectus femoris muscles were irradiated by slightly moving the fibre optic cable close over the surface of muscle (Figure 7).

Transplantation of rat and hamster heart – Paper III

Male inbred Lewis rats (Mollegaard, Skensved, Denmark) weighing 200-220 g were used as recipients, and male Golden Syrian Hamsters (B&K Universal AB, Sollentuna, Sweden) weighing 70-80 g were used as donors. The animals were anesthetized with a combination of Hypnorm (Fentanyl; 0.6 ml/kg) and Stesolid (Diazepam; 2.5 mg/kg) given i.p. Thereafter, Temgesic (Buprenorhine) was administered in a dose corresponding

to 0.01–0.05 mg/kg. During the operative procedure, a constant body temperature (37 ±0.5°C) was maintained by a homoeothermic blanket system (Harvard Apparatus) consisting of a warming pad and a rectal probe for the regulation of the heat output. After the initialization of the cooling of the hearts in the donor animals, the system was switched off. The same system was used for the recipient operation.

The *in vivo* xenotransplantation heart model was used to investigate the effect of irradiation when transplanting across species barriers. The reason using the heart transplantation model was that there is a synergy between the initial injuries of ischemia/reperfusion and acute rejection. Minimizing the injury is thus of importance, and targeting ROS generation can be one key. The immunological mechanisms at xenotransplantation have a serious impact on the survival. However, the immunological-, pathogenic-, and ethical barriers are further discussed by Lukes (86). The significance of the ischemia/reperfusion injury in xenotransplantation is largely unknown. However, reports support that at least a short ischemia/reperfusion injury in xenotransplants does activate the endothelium, making it more immunogenic in the immediate postoperative period (87).

A non-suture cuff technique was used (88). The ischemia time outside the donor, i.e. the time between excision of the heart and the onset of circulation, did not exceed 10 min. The total ischemia time counted from the onset of explantation did not exceed 30 min. Meanwhile, the hearts were stored in cold (+4°C) saline solution (NaCl, 0.9%). Rejection was defined as loss of regular palpable contractions and visual activity.

Graft hearts were irradiated by positioning the end of the cable 5 mm from the hearts and slightly moving the fibre cable end over the entire heart surface 10 min before the onset of reperfusion. The saline injected during explantation of the hearts was irradiated by gently moving the fibre cable end for 10 min in the little beaker used to store the saline. Irradiation was also done each day for 10 min through the skin before the MRS acquisition.

Human monocyte model – Paper IV

In paper IV the human monocytes were used to investigate the effect of irradiation on nitric oxide, intracellular ROS, and ATP. Mononuclear cells were obtained from buffy coats obtained from the blood bank at Sahlgrenska University Hospital, Göteborg, Sweden, and were isolated using the Ficoll-Paque procedure (89). The buffy coat blood is layered on the Ficoll Paque and centrifuged. Differential migration during centrifugation results in the formation of layers containing different cell types. The bottom layer contains erytrocytes which have been aggregated by the Ficoll-Paque, and granolocytes which have a density great enough to migrate through the Ficoll-Paque. Because of their lower density, monocytes together with lymphocytes and platelets are found at the interface between the plasma and the Ficoll-Paque. The mononuclear cells were recovered from the interface and subjected to a washing step to remove plasma, Ficoll-Paque and platelets. By seeding the cells to culture plastic the monocytes adhere and the lymphocytes may be washed away.

Cell culture methods are used extensively in the investigation of anti-inflammatory and inflammatory products as well as antioxidants and studies of oxidative stress. Compared to *in vivo* experiments they offer the advantages of relative reproducibility, reduced cost, and simplified interpretation of results. Moreover, cell culture methods can be applied without major ethical concern. The disadvantage of cell culture techniques is the need to re-check *in vivo* the medical relevance of all data obtained. Human monocytes were irradiated by moving the fiber optic cable close to the surface of the cell suspension. This cellular model system has proven to be sensitive to irradiation in previous studies (48). The cells of all experimental groups, including the control group, were exposed to the same environmental conditions.

Prevention of ischemia and reperfusion injury

Preservation solution – Paper I

In paper I Perfadex was used as a preservation method in an *in vitro* model of rat rectus femoris muscle during 5 h of ischemia. The preservation solution Perfadex (Vitrolife, Kungsbacka, Sweden) was stored at 8°C. It was adjusted with Addex-THAM

(trihydroxymethylaminomethane) (0.24 mol/L; Pharmacia AB, Sweden) to a pH of 7.40. During the preservation period (ischemia) the preservation solution was at room temperature (22°). The composition of Perfadex is seen in Table 4. Physiologic saline solution 0.9% NaCl was used as control.

Table 4. The composition of Perfadex with the intracellular and the extracellular electrolyte distribution as comparison.

Component	Perfadex	Intracellular	Extracellular
Na^+ (mmol/l)	138	10	142
K^+ (mmol/l)	6	140	4
Mg^{2+} (mmol/l)	0.8	58	3
Ca^{2+} (mmol/l)	0	<1	5
Cl^- (mmol/l)	142	4	103
HCO_3^-	0	10	28
Phosphate (mmol/l)	0.8	75	4
Sulphate (mmol/l)	0.8	2	1
THAM-buffer (ml/l)	0.24		
Dextran 40 (g/l)	50		
Glucose (mmol/l)	5		
Osmolarity (mOsm/l)	325		
pH	7.40	7.0	7.40

An optimal preservation solution for skeletal muscle should facilitate maintenance of the high energy phosphates, and consequently prolong the cell viability during ischemia (90). Perfadex is extracellular alike preservation solution (Table 4). Perfadex is designed mainly for the storage of lung transplants (43-45), but preserves contractility of rat vascular smooth muscle (36) and porcine pancreatic function (46) after cold ischemia. No previous studies have explored the potential of Perfadex to preserve skeletal muscle.

Irradiation – Papers I, II, III & IV

In papers I, II, and III, irradiation at 634 nm was used as treatment during ischemia and reperfusion. In paper IV irradiation was used to study the effects on NO, intracellular ROS and ATP.

Singlet oxygen can be produced photochemically by energy transfer from an excited photosensitizer (91, 92). Singlet oxygen O_2 ($^1\Delta_g$) was produced photochemically by

energy transfer from an excited photosensitizer using the Oxylight® equipment (GaiaCare AS, Norway) (92, 93). Although singlet oxygen is a free radical that causes oxidative stress mediated injury, the emitted light energy might be beneficial. The energy emitted from singlet oxygen upon relaxation to its triplet ground state O_2 ($^3\sum^-_g$) is captured as photons at 634 nm and referred to as singlet oxygen energy (SOE), Figure 6. Irradiation with SOE photons via a fiber optic cable is here referred to as irradiation.

In the equipment, singlet oxygen was generated through a photosensitization process (93). The photosensitizer used was zinc(II) phthalocyanine, a bluish-red dye, which is one of the few sensitizers able to perform in a gaseous atmosphere. Furthermore, it has good heat and light resistance and can be applied on metal surfaces. As light source, 6 light emitting diodes (LED cluster) were used. The LED is made with Gallium Aluminum Arsenide (GaAlAs) 52 mm LED cluster (model BL0307-50-44, Kingbright) with dominate wavelength 640 nm, viewing angle 40°, power output 2.5 W, beam spot 0.07 cm^2, corresponding to power density 35.7 W/cm^2. The irradiation was via a fibre optic cable, cable length 142 cm, diameter of the end of the cable 3 mm.

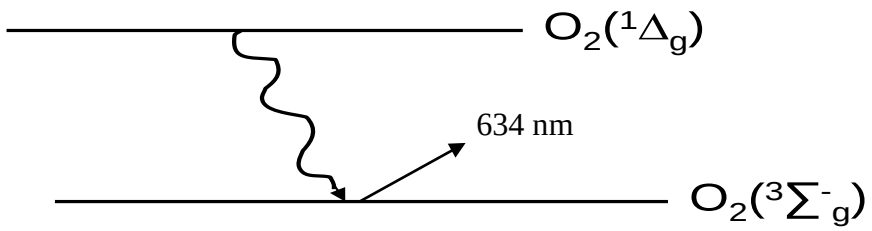

Figure 6. The principles of SOE light. Excited singlet oxygen O_2 ($^1\Delta_g$) is relaxed to its ground state triplet oxygen, O_2 ($^3\Sigma^-_g$) during the emission SOE light, captured as photons at 634 nm.

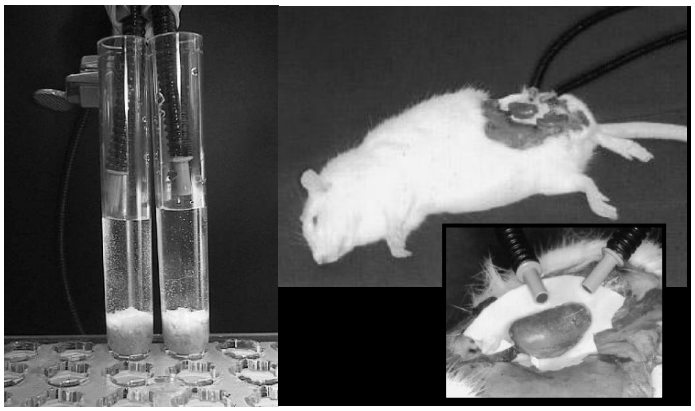

Figure 7. Irradiation of rectus femoris muscle in preservation solution as in paper I, and irradiation *in vivo* as in paper II, and the close up.

Analysis

^{31}P Magnetic Resonance Spectroscopy –Papers 1, II & III

Nuclear Magnetic Resonance (NMR) is a powerful tool in biomedical research, and several Nobel prizes have been awarded in this field. In 1952, Felix Bloch and Edward Mills Purcell (USA) became laureates for their development of new methods for nuclear magnetic measurements. In 1991, Richard Ernst (Switzerland) received the prize for his contributions to the development of the methodology (Fourier Transform algorithm) of high resolution NMR spectroscopy. In 2002, Kurt Wüthrich (Switzerland) received the prize for his development of NMR for determination of the 3-D structure of biological macromolecules in solution. In 2003, Paul Lauterbur (USA) and Peter Mansfield (England) became laureates for their development of gradients in a magnetic filed.

In application of NMR, the term MR (magnetic resonance) is commonly used. MR is divided into two techniques called magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS). Both techniques are performed with conventional MR equipment (94). For clarity and simplicity the term MRS is hereinafter used, according the general convention for medical application of the spectroscopy technique. All experiments in this thesis were performed with the equipments at our MR-lab. Our group established the first *in vivo* MRS-lab in Sweden in 1986. Since then, several methods were developed for a number of various biomedical applications (9, 39, 86, 95).

Atomic nuclei sensitive to MRS, such as ^{1}H, ^{13}C, ^{23}Na and ^{31}P possess an intrinsic property called spin. Spin can be regarded as a rotating motion of the nucleus around its axis. The combination of the positive electrical charge and this spinning motion result in an electrical current which, in turn gives rise to a magnetic field. Thus the nucleus behaves like a tiny bar magnet.

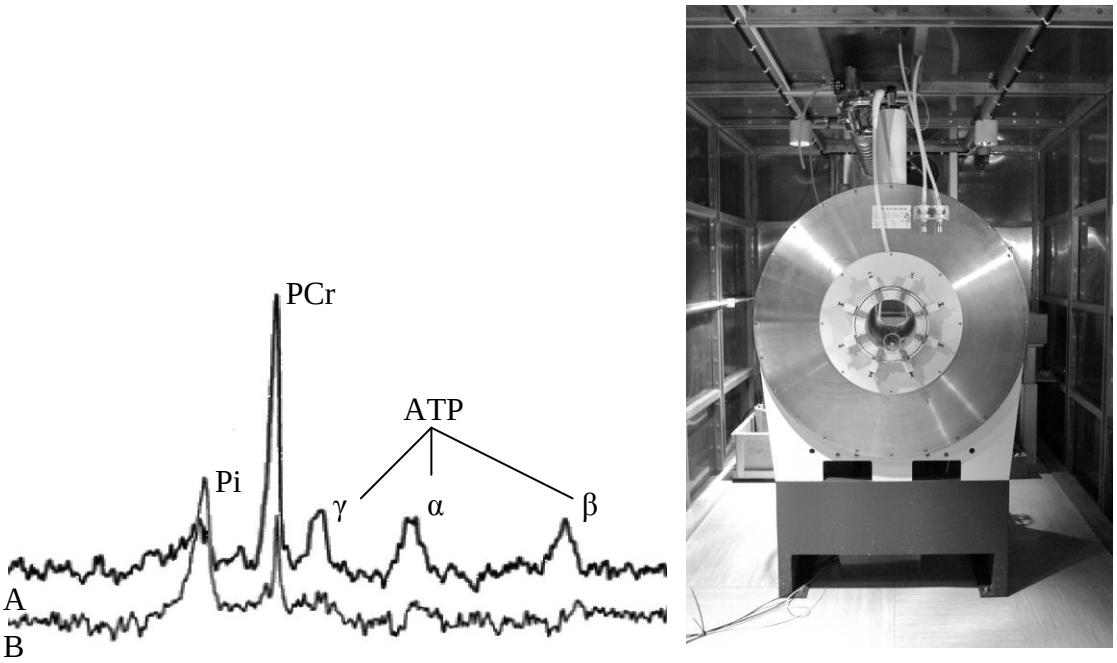

Figure 8. Left: Representative *in vivo* MRS of rectus femoris muscle, analyzed in a 2.35 T MRS magnet. The spectra show Pi, PCr and the three peaks of ATP. A irradiated, B non-irradiated. Right: the *in vivo* MRS magnet.

In the presence of a strong and uniform magnetic field, the nuclei are forced to align either in a parallel or an antiparallel orientation. The energy difference between these two states is proportional to the strength of the magnetic field. The parallel and antiparallel orientations are not equally populated. There is a small excess of spinning nuclei in the parallel orientation. The nuclei not only align in parallel or antiparallel orientation, they also move around the axis of the magnetic field in a movement called precession. When placed in a strong magnetic field, the nucleus precesses at the Larmor frequency which, for proton (^{1}H), is around 100 MHz in magnetic field strength of 2.35 Tesla. Owing to the population differences, the sum of the individual preccessing spins results in a net magnetization aligned to the magnetic field.

A transition can be induced between the two orientations with a radio frequency (RF) pulse. The RF pulse sent at the Larmor frequency swings the net magnetization away from the magnetic field toward the transverse plane. After the RF pulse is switched off, the magnetization returns to its equilibrium position, and its transverse component is registered by a receiver coil during this relaxation process. The signal is called the free induction decay (FID). The FID is converted by a Fourier transform (mathematical algorithm) to a spectrum. A more detailed description of the theory of MRS is well described by Salabi and Brown (96).

An *in vitro* MRS of a muscle extract is presented in Figure 9. There are three peaks from ATP, since the three phosphorus nuclei in the ATP molecule have different chemical environments. The difference in the chemical environment will give rise to a difference in resonance frequency, termed chemical shift. The chemical shift is the most frequently used parameter for identification of an unknown substance in MRS.

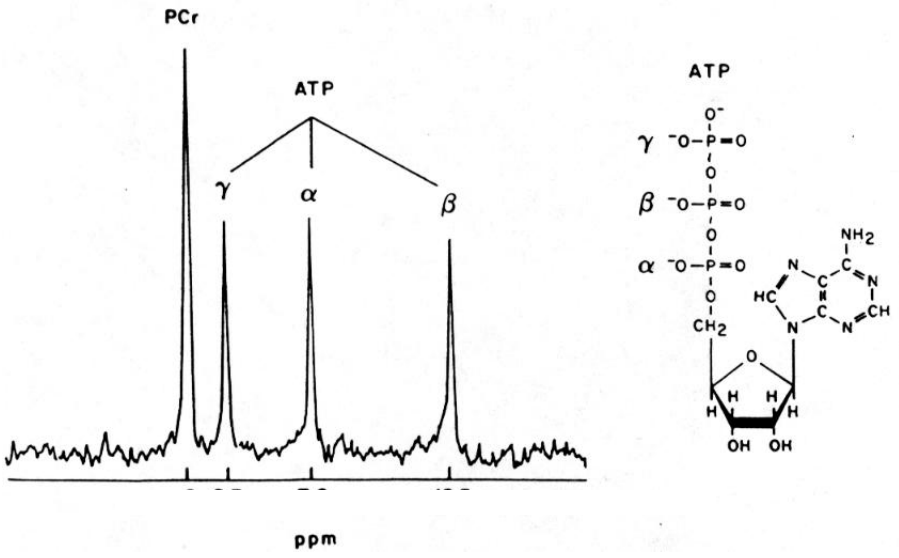

Figure 9. A typical *in vitro* MRS from a muscle, showing the peak of PCr and three peaks of ATP.

Interpretation of MRS consists of identification of the peaks, calculation of the relative or absolute metabolite concentrations and determination of peak shifts for calculation of intracellular pH.

The chemical shift is measured with a cursor giving the position in parts per million (ppm). When the PCr peak is set as 0 ppm, the three peaks of ATP occur approximately at -2.7 (γ), -7.8 (α) and -16.5 (β) ppm (97). The peak of Pi occurs approximately at 5.2 ppm at pH 7.40. Peaks at 6.5-7.5 ppm are assigned to phosphomonoesters (PME) including various sugar phosphates, AMP and IMP (9). The peak at ~16 ppm originates from phenylphosphonic acid, which can be added as an internal standard in *in vitro* MRS.

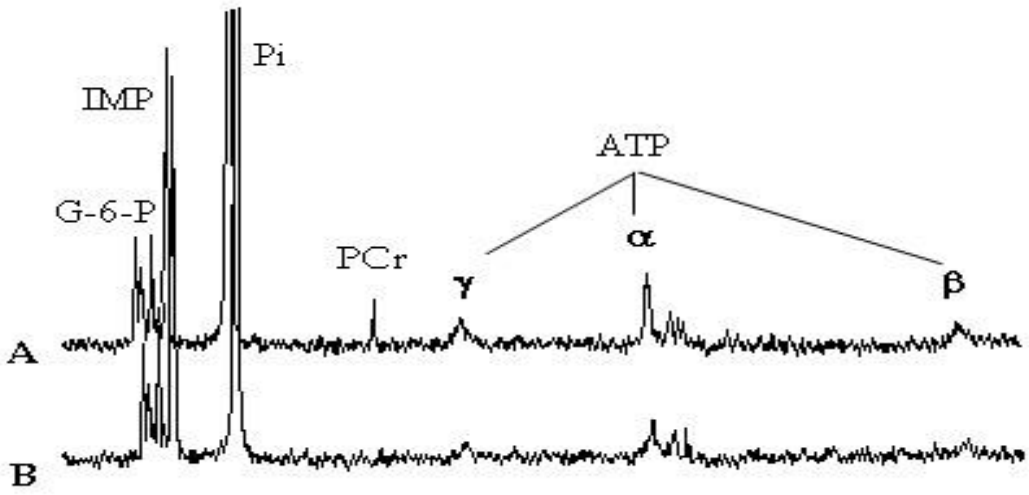

Figure 10. Representative *in vitro* MRS of rectus femoris muscle. The spectra show G-6-P, IMP, Pi, PCr and ATP. A irradiated, B non-irradiated.

Only the β-ATP peak is generally measured (as in paper I-III) as it contains only ATP. The γ-ATP consist of contributions from β-ATP, and the α-ATP contains α-ADP and nicotinamide adenine dinucleotide (NAD) (97).

Calculation of the relative or absolute metabolite concentrations is performed by computing the peak area. The peak is defined by selecting the frequency range with the help of a cursor. Absolute values in *in vitro* MRS can be calculated. The sample of interest is calibrated against a standard of known size and composition. The standard is subjected to the same measurement conditions, which allow direct comparison of the spectral peak areas and calculation of the unknown concentration of the sample from the known concentration of the standard. In paper I, phenylphosphonic acid was used as standard. *In vivo* MRS does not provide absolute values (owing to the absence of a

standard), but the information can be obtained by calculating relative concentrations, such as the ratio of PCr/ATP or PCr/Pi (97, 98).

The cursor is also used when calculating intracellular pH from the chemical shift (σ) of Pi relative to the PCr peak. PCr is used as an internal standard since its chemical shift is constant in the pH region of biological interest 6-8 (9), while Pi is sensitive to H^+. The equation for pH is: $pH = 6.75 + \log [(\sigma-3.27) / (5.69- \sigma)]$ (12).

In vivo and *in vitro* MRS resemble each other but do not provide the exactly the same information. The resolution is higher in *in vitro* spectra owing to the higher magnetic field and to less movement of the object. The spectra show the peaks from ATP, PCr, Pi, IMP and G-6-P. Generally, *in vivo* MRS has a lower magnetic field and consequently lower resolution. The lower resolution makes it more difficult to detect IMP and G-6-P in *in vivo* spectra. Only cytosolic ATP is detectable using *in vivo* MRS (9). *In vivo* MRS gives higher PCr (> 30%) and lower Pi concentrations than other biochemical analysis since the breakdown of PCr in *in vitro* analysis cannot be prevented owing to its high instability. Therefore, no exact comparison can be made with other kinds of biochemical analysis.

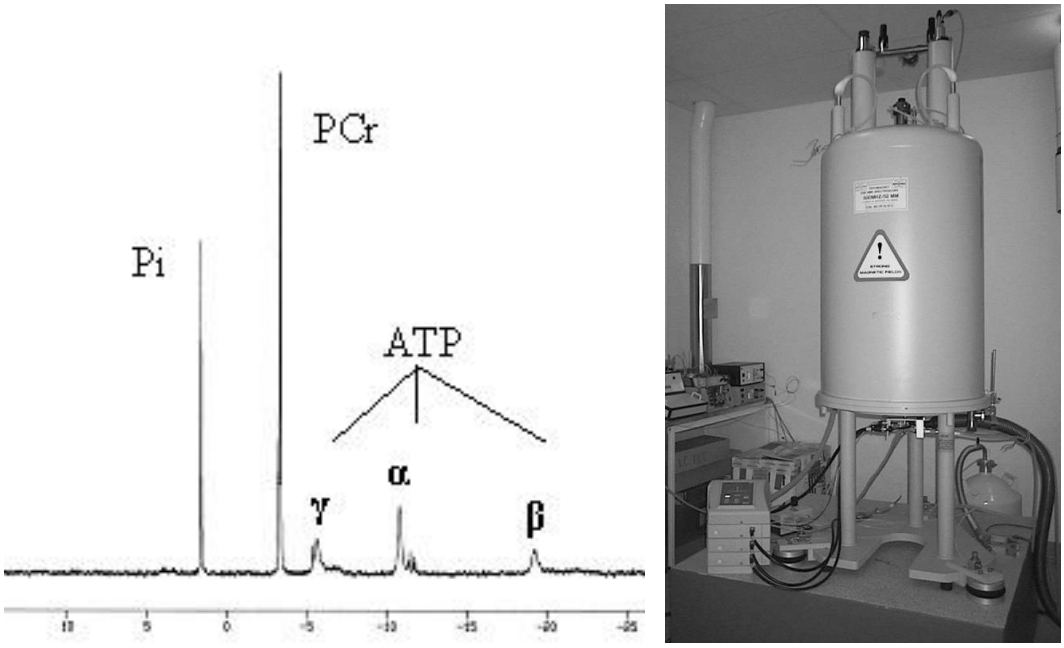

Figure 11. Left: representative *in vitro* MR spectrum of rectus femoris muscle, analyzed in an 11.75 T MRS magnet. The spectra show Pi, PCr and the three peaks of ATP. Right: The *in vitro* MRS magnet.

ATP and PCr can be followed noninvasively and continuously using *in vivo* MRS (99). The pattern of recovery of ATP and PCr reflect the mitochondrial oxidative phosphorylation activity. This activity is very useful in estimating mitochondrial viability. This makes *in vivo* MRS suitable for ischemia/reperfusion studies, where intracellular metabolic processes can be followed noninvasively and continuously, as illustrated in Figure 12.

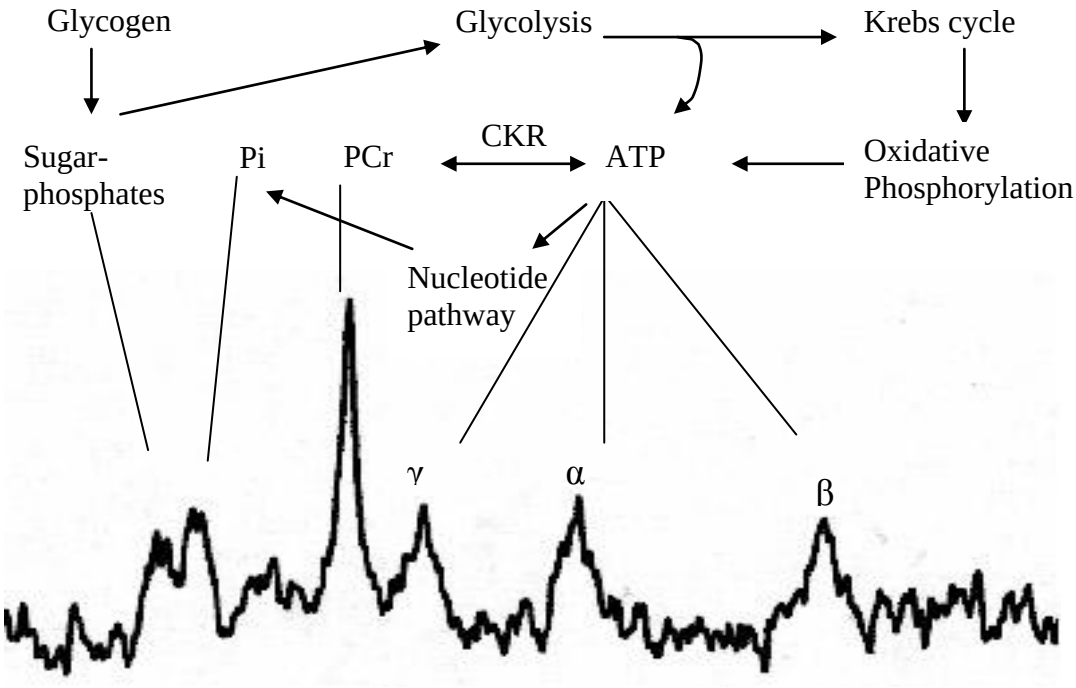

Figure 12. Visualization of the metabolic pathways in skeletal muscle related to an *in vivo* MRS spectrum. ATP is produced by oxidative phosphorylation, glycolysis and the creatine kinase reaction (CKR). After prolonged ischemia, ATP is degraded in the purine nucleotide pathway during formation of Pi. The peak to the left of Pi is assigned to phosphomonoesters resonances (various sugar phosphates, AMP and IMP).

Laser Doppler flowmetry –Paper II

Laser Doppler flowmetry (LDF) was used to evaluate the degree of blood perfusion during ischemia and reperfusion. The theory of LDF is that the tissue is irradiated with laser light (100). If the laser light hits a moving blood cell the laser light changes wavelength (Doppler shift). The laser light is backscattered to the instrument and detected by a photo detector. The perfusion is calculated from the sum of moving blood cells and mean velocity of the cells. LDF measures the total local blood perfusion non-invasive.

There are limitation using LDF due to sensitivity to movements, variations in capillary density in the local tissue and the low depth of penetration. Therefore the LDF measurements were only used as an indication of the success of total ischemia, and only as an indication of success of reperfusion in the muscle.

The measurements were expressed in arbitrary perfusion units (PU). The low power diode laser used 780 nm with a maximum power output of 1 mW (Periflux System 5001, Perimed, Sweden) and with a microtip of 0.5 mm diameter (Periflux 418-1). The probe was calibrated with a motility standard of 250 PU from Perimed. In paper II, LDF measurements were made on the distal two thirds of the rectus femoris muscle. To minimize variations, five measurements at five different sites were recorded. The final value was the mean of the five measurements. The LDF measurements were only used as an indication of the success of total ischemia, and only as an indication of success of reperfusion in the muscle.

Chemiluminescence –Paper IV

The measurement of intracellular ROS was performed using the sensitive chemiluminescence technique. The technique has been presented, modified and tested under different conditions (48, 101, 102). It is based on the induction of ROS-production from human monocytes by phorbol myristate acetate (PMA). The PMA stimulate the reduced nicotineamid adenine dinucleotide phosphate (NADPH) oxidase, leading to the generation of superoxide anions and hydrogen peroxide (48, 102).

The sensitivity of the cellular system has been validated by the use of well known antioxidants and ROS scavengers such as ascorbate, α–tocopherol, catalase and SOD all resulting in significant reduction of ROS production (103). The chemiluminescence data was obtained using luminol. Luminol can diffuse across cell membranes due to its lipophilic chemical structure (16). Light generation in the presence of luminol reflects the intra and extracellular production of superoxide anion and hydrogen peroxide (16). Therefore it can detect both intracellular and extracellular generated ROS. Luminol can be used to measure intracellular ROS when SOD and catalase are added (101). SOD and catalase are both large molecular proteins that do not readily gain access to intracellular sites, and thus, they inhibit the extracellular part of chemiluminescence (16, 101). SOD

dismutates superoxide radicals ($O_2^{\bullet-}$) to form hydrogen peroxide (H_2O_2) and oxygen (O_2). Catalase catalyses the breakdown of H_2O_2 to form water and O_2 (48).
After the chemiluminescence peak, the response decline. The chemiluminescence was recorded over time until the maximal peak values were reached. The chemiluminescence intensity varied with different blood donors; which is due to individual differences in NADPH-oxidase activity.

The ATP assay is based on the chemiluminescence produced in a firefly luciferase reaction, as previously described by Lundin (104). For the determination of intracellular ATP, the ATP was extracted. For that purpose equal volumes of trichloroacetic acid and cellsuspension are mixed. Tris-EDTA buffer, ATP Reagent and the ATP extract are added together and the light emission was recorded. The internal standard method is used to quantify the ATP concentration. The ATP standard is added and the light emission was recorded again.

The measurement of nitric oxide was performed using chemiluminescence. The detector is based on the principle of the chemiluminescence method. NO in the sample reacts with ozone (O_3), producing a defined amount of energized nitrogen dioxide ($NO_2^{\bullet}$) plus oxygen (105, 106). The excited state of $NO_2^{\bullet}$ returns to its basic energy level by emitting a photon seen as an infrared chemiluminescence. The quantity of light is proportional to the NO concentration.

RT PCR –Paper IV

Reverse transcription polymerase chain reaction (RT PCR) was used to analyse iNOS and eNOS mRNA. The total cellular RNA was isolated using an RNeasy Mini Kit. Probes and primers for iNOS and eNOS were TaqMan Gene Expression Assays. First-strand cDNA was synthesized from 100 ng of total RNA and TaqMan Reverse Transcription Reagents. PCR amplification was done with an ABI PRISM 7700 Sequence Detection System (Applied Biosystem). The relative expression of iNOS and eNOS mRNA were normalized to the relative expression of actin in the same cDNA by using the standard curve method. PCR detects the accumulation of amplicon during the reaction. The data is then measured at the exponential phase of the PCR reaction.

Statistical methods

Two-way analysis of variance

The experiment in paper I was regarded as a 2 x 2 factorial experiment with saline/Perfadex and irradiated/non-irradiated as factors. Data were analyzed using two-way analysis of variance with F-tests of as well main effects as of interactions (107). Results are presented as mean ± SEM (standard error of the mean). $p < 0.05$ was considered statistically significant. The SEM and the F-tests for PCr were based on three of the groups, deleting data for non-irradiated 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.

Paired t-test

The statistical analyses in paper II were performed using paired t-test. Results are presented as mean ± SEM (standard error of the mean). $p < 0.05$ was considered statistically significant.

Kolmogorov–Smirnov test

The Kolmogorov–Smirnov test was used in paper III to assess the data for normal distribution. Since the data were normally distributed according to the test, the unpaired students t-test was used to calculate intergroup differences on the respective postoperative days, and the paired t-test, within the groups. The data for graft survival were calculated as mean ± SEM (standard error of the mean). On the basis of the relative ratios for PCr/ATP for each animal on each postoperative day, the means ± SEM for that day were calculated. To compensate for multiple comparisons, $p < 0.01$ was considered statistically significant.

Two-tailed paired t-test

The statistical analyses in paper IV were performed using two-tailed, paired t-test. Results are shown as mean ± standard error of the mean (SEM). $p < 0.05$ was considered statistically significant

RESULTS AND DISCUSSION

This thesis investigates the effects of irradiation at 634 nm on skeletal muscle during ischemia and reperfusion, and on hearts, by measuring the energy status. The preservations solution Perfadex was tested on skeletal muscle during ischemia. Further, it studies the effects of irradiation on monocytes, by measuring NO, intracellular ROS, and iNOS and eNOS mRNA. The results from the different papers are referred to in the text by their Roman numerals, and see the individual papers for more details.

Preservation solution –Paper I

Long periods of surgery push the duration of ischemia to the limits of tissue ischemic tolerance. Therefore, there is a need for a preservation method that does not injure the microvasculature, is easy to handle, decreases the production of ROS, and increases the chances of recovery after surgery. In this study we investigated the effect of the preservation solution Perfadex during 5 h of ischemia on rat skeletal muscle. We showed that Perfadex failed to preserve the high energy phosphates during ischemia. The study showed that ATP was lower in muscles preserved with Perfadex compared with muscles preserved with saline. The results with Perfadex were unexpected and may be attributable to the experimental model used in this study.

Preservation solutions have been successfully used for many organs, such as liver, pancreas, kidneys and hearts (8, 46, 108) The technique of flush perfusion is used to inject the preservation solution into the tissue. The most commonly used preservation method today is flush perfusion of the organ with a cold preservation solution to obtain a quick cooling of the organ and decreased metabolism (8).

For long-term preservation of organs, it is important that the preservation solution reaches all the segments of the tissue (109). In paper I, the muscles were stored in plastic tubes with the solution and not flush perfused. For small tissues, a simple immersion technique has shown to be sufficient for preservation (110). However, this technique is not sufficient in our model probably because the solution cannot penetrate through all of the ischemic tissue. Our experimental model was designed to simulate clinical plastic

microsurgery, where the perfusion-pressure during flushing can damage the microvasculature. A better preservation effect with Perfadex could be obtained by a combination of flush perfusion and hypothermia. Hypothermia has been successfully used during ischemia and reperfusion to prevent injury (38). Hypothermia is often used as an effective method to prolong the ischemic tolerance of a tissue by decreasing the energy metabolism (37, 111).

Irradiation prevents ischemia/reperfusion injury – Papers I and II

Energy status

In these studies, we irradiated rat skeletal muscle during ischemia and reperfusion *in vitro* and *in vivo*. In paper I, we showed that irradiation preserved the viability in rat skeletal muscle *in vitro*, as indicated by higher levels of ATP and PCr compared with control after 5 h of ischemia. In paper II, we showed that irradiation improved the viability *in vivo*, as indicated by increased levels of ATP and PCr/(PCr+Pi) after 4 h of ischemia and 1 h of reperfusion. These results are in agreement with another study, showing that irradiation at 810 nm protects skeletal muscle from ischemia/reperfusion injury (112).

Energetic status is a good marker of viability, where high concentrations of ATP indicate good viability (113). Papers I and II showed that irradiation at 634 improved cell viability during long-term ischemia in rat skeletal muscle. These studies use ischemia and reperfusion, which are often associated with reconstructive surgery and transplantation. Today, it is generally accepted that formation of ROS occurs during ischemia and reperfusion (9, 114-116). ROS cause lipid peroxidation, enhanced membrane permeability and decreased ATP synthesis (117), and thus minimizing ROS generation is of central importance to maintain the viability of the cells.

Irradiation at 634 nm reduced the production of extracellular ROS in isolated human monocytes (48). In paper III, we showed that intracellular production of ROS are also reduced by irradiation. The effect is mainly mediated through the inhibition of NADPH oxidase, the main source of superoxide anions ($\bullet O_2^-$) (48). Thus, it is possible that in

paper I and II, irradiation at 634 nm might achieve its beneficial effect by inhibition of NADPH oxidase, leading to decreased production of ROS.

Photosensitivity occurs in biological systems at various levels, for instance, is cytochrome c oxidase in the mitochondrial respiratory chain sensitive to photoexcitation (62, 91, 118-120). Increased ATP levels using irradiation has been reported both *in vivo* and *in vitro* (70-72, 121). It was been suggested that irradiation increases the electrochemical proton gradient, which induces extra ATP synthesis.

The results in paper I and II are in agreement with these observations of increased energy status upon irradiation. Thus, irradiation at 634 nm might, in addition to reducing the production of ROS, have a stimulatory effect on the respiratory chain resulting in increased ATP synthesis. This warrants further studies of long-term treatment with irradiation.

Blood flow, pH & temperature

In paper II, we investigated the effect of irradiation on the blood flow, pH and temperature during 4 h of ischemia and 1 h of reperfusion in rat skeletal muscle. Irradiation was found to not influence the blood flow during ischemia and reperfusion. The blood flow was measured by laser Doppler flowmetry (LDF). The perfusion value is the perfusion in the tissue volume in front of the probe tip, approximately the radius of the hemisphere of the probe (39, 100). The measured volume represents a small part of the muscle (13). Therefore, repeated measurements were taken. Resting perfusion values of approximately 18 perfusion units (PU) were obtained. During ischemia, values between 5 and 10 were recorded. Reperfusion for 1 h restored the values to the resting perfusion values. Differences in perfusion can be observed when comparing studies (39, 122). The differences between perfusion can be explained by that the area where the measurements were performed were located in the distal two thirds of the rectus femoris muscle.

The temperature of the rectus femoris muscle was obtained using LDF, and the body temperature was obtained using a rectal probe. Irradiation did not increase tissue nor body temperature, as shown in paper II. The pH was calculated by MRS as earlier described, and showed that irradiation did not influence the pH.

Irradiation of transplanted heart – Paper III

In this study we investigated whether irradiation during ischemia could preserve the energy status in an *in vivo* xenotransplantation heart model. Cervical transplantations between rats (recipients) and hamster heart were performed and followed daily over 4 days. Recipients were randomized to two groups: irradiation of the saline and the graft before reperfusion, and irradiation of the saline used during explantation. The cold ischemia (+ 4°C) was standardized to 30 min. The PCr/ATP ratio was higher when irradiation took place before the onset of reperfusion. The difference was seen on postoperative day 1, and the differences became smaller thereafter. Irradiation on subsequent days showed no effect. The rejection process in this model started immediately and irradiation was not effective in delaying the rejection process. Decreasing energy status has been shown to have an over 90% sensitivity, specificity, and predictive value for the subsequent day's biopsy-verified rejection (123). A correlation has also been shown between decreasing energy status and progressive xenografts rejection in concordant mouse-to-rat xenotransplantation heart model (124).

The PCr/ATP ratio is an index of the balance between energy production and utilization (125). The PCR/ATP ratios declined in both groups during the postoperative days due to ischemia/reperfusion injury and the rejection process. Irradiation showed no effect when induced post transplantation, which might be explained because of rejection processes. In paper II irradiation of skeletal muscle showed effect when induced during ischemia and reperfusion, as seen as increased energy status. The difference could be explained by the dose range (48), thus might the dose of irradiation become to high when induced during reperfusion and rejection in a xenotransplantated heart, in contrast to a reperfused skeletal muscle with no immunological incompatibilities. The immunological reactions of transplantation and xenotransplantation have previously been discussed by Lukes (86).

Supportive evidence that irradiation has effect on heart has been provided, showing that irradiation at 660 nm of cold-stored rat hearts has higher ATP levels than in non-irradiated hearts (54). Furthermore it has been documented that myocardial infarct size was reduced by irradiation (80, 81). We believe that the result in paper III is, at least in part, through inhibition of mitochondrial NADPH oxidase, thus decreasing the production of ROS mainly superoxide anions (48), implicating higher membrane integrity and thus

better preservation of ATP. However, this could not delay the rejection process in this model

It remains to be investigated if reduction of early ischemic with irradiation has any impact on chronic rejection. A clinical application of irradiation may be foreseen in transplantation surgery and in other situations of ischemia and reperfusion, as a complement to other treatments.

Effect of irradiation of human monocytes – Paper IV

Reactive oxygen species

In this study we investigated the effect of irradiation at 634 nm on the release of intracellular ROS, release of NO, and activation of inducible nitric oxide synthase (iNOS) and endothelial NOS (eNOS). The data were obtained using chemiluminescence. Reducing chemiluminescence signal can be used to determine the antioxidative capacity of exogenously added compounds. Irradiation decreased *intracellular* ROS in isolated human monocytes. Previous study showed that adding catalase to control cells resulted in a 30% reduction of the PMA-induced response (48). The inhibitory effect was most pronounced for SOD (90%), which indicates that the superoxide radical is of major importance in the chemiluminescence response.

Normally NADPH oxidase is inactive. In this study the NADPH oxidase was stimulated by PMA leading to the generation ROS (48). The main injury to muscle cells occurs at the onset of reperfusion due to formation of ROS (23, 24, 126). ROS formed during ischemia and reperfusion (9, 12, 22-24, 114-116, 127) cause an oxidative breakdown of polyunsaturated fatty acid chains in lipid membranes, i.e. lipid peroxidation (9, 95). Lipid peroxidation of the mitochondrial membranes results in impaired ATP synthesis and diminished activity of energy-dependent processes, leading to mitochondrial injuries and cell death (117, 128). Thus, limiting ROS formation is of importance to reduce ischemia and reperfusion injury. Paper IV showed that irradiated monocytes have decreased production of intracellular ROS, which may result in higher membrane integrity and

better preservation of the high energy phosphates. The results agree with papers I and II where the high energy phosphates are higher in irradiated muscles.

Nitric oxide

Irradiation released NO in human monocytes, which was achieved both without and with PMA pre-activation of the cells. Until recently, an inducible NO pathway was not thought to be present in human monocytes/macrophages (129, 130). However, the expression of iNOS in human monocytes following infection with tuberculosis has been reported (131). NO production has also been found in human monocytes stimulated by vasoactive hormones (132) and by inflammatory activation (133, 134).

Based on these findings, it was of interest to elucidate if irradiation was coupled to activation of iNOS or eNOS. For this purpose, we chose to analyze the mRNA levels of iNOS and eNOS after irradiation. Irradiation did not significantly change the mRNA levels of neither iNOS nor eNOS in monocytes. Aminoguanidine (AG), which is a known selective inhibitor of iNOS (135, 136), was used to further exclude the possible activation of iNOS. Monocytes were incubated with AG and then irradiated, the results demonstrated no significant difference in NO in monocytes incubated with AG and irradiated compared with control cells. The results confirmed that the release of NO upon irradiation was not coupled to increased synthesis of iNOS nor eNOS.

Studies have shown a presence of preformed NO stores that can be released upon irradiation in cardiomyocytes (54). The presence of photoactivable NO in other tissues has also been documented (58, 59, 137). The S-nitrosothiols are often suggested for the photosensitive store of NO (138, 139). *S*-nitrosothiols can decompose photochemically to liberate NO and the corresponding disulfide. The results in Paper IV indicated a presence of preformed NO stores in human monocytes that can be released by irradiation. Further studies are required to determine whether the novel release of NO upon irradiation is coupled to *S*-nitrosothiols, and to investigate the linkage between decreased ROS production altered release of NO.

CONCLUSIONS

In this thesis, the focus was to investigate the effects of irradiation at 634 nm on skeletal muscle during ischemia and reperfusion, and on hearts, by measuring the energy status. The preservation solution Perfadex was tested on skeletal muscle during ischemia. Further, it studied the effects of irradiation on monocytes, by measuring NO, intracellular ROS, and iNOS and eNOS mRNA.

We investigated the effect of the preservation solution Perfadex during 5 h of ischemia on rat skeletal muscle. In our experimental system, the preservation solution Perfadex failed to preserve the high energy phosphates. The ATP level was lower in muscles preserved with Perfadex compared with muscles preserved with saline.

Irradiation of rat skeletal muscle during ischemia and reperfusion were done *in vitro* and *in vivo*. Irradiation of the ischemic skeletal muscle improved the levels of ATP and PCr measured by *in vitro* ^{31}P MRS. Irradiation also improved energetic recovery during reperfusion, as indicated by increased levels of ATP and PCr/(PCr+Pi) measured by *in vivo* ^{31}P MRS. Irradiation did not influence the blood flow, temperature and pH during ischemia and reperfusion.

Irradiation of xenotransplanted heart preserved the energetic status on the first postoperative day. The PCr/ATP ratio was higher when irradiation took place before the onset of reperfusion. The difference was seen on postoperative day 1, and the differences became smaller thereafter. Irradiation on subsequent days showed no effect. The rejection process in this model started immediately and irradiation was not effective in delaying the rejection process.

Irradiation decreased *intracellular* ROS in isolated human monocytes, measured by chemiluminescence. Irradiation released NO in human monocytes, which was achieved both without and with PMA pre-activation of the cells. Additionally, iNOS and eNOS mRNA was not linked to the release of NO upon irradiation. Further studies are required to investigate the linkage between decreased ROS production altered NO release.

The effect of irradiation is mainly mediated through the inhibition of mitochondrial NADPH oxidase, the main source of ROS. Decreasing the production of ROS during ischemia and reperfusion implicates higher membrane integrity and thus improved viability. The salvaging effect of irradiation may have a wide range of clinical applications, for example in complex microsurgery and transplantation surgery. Further research is needed to study the effect of long-term treatment with irradiation.

ACKNOWLEDGEMENTS

This study was carried out in the Bioenergetics Group, Wallenberg Laboratory and Lundberg Laboratory for Bioanalysis, at the Department of Surgery, Göteborg University.

I whish to thank my supervisor, Professor Bassam Soussi for coaching in multidisciplinary and innovative science. For a being an excellent role model in the arts of writing and moral, and a true intellectual character. For always supporting me in the rough university seas, and for listening to my ideas and giving me gentle advice.

I thank Jonas Lundberg, Lillemor Mattsson Hultén, Daniel Lukes, Olivier Rakotonirainy, all co-authors, for your most valuable expertise, and your enthusiasm in novel treatments with irradiation.

Thanks to Lennart Svensson for the nitric oxide analysis made at Astra Zeneca, and thanks to Margareta Filipson for animal operations in paper III.

Thanks are also due to Anna Elander, Elmir Omerovic, Andreas Lundgren, Jakob Wilton, Alex Karlsson Parra, Michael Olausson all co-authors of various papers.

Special thanks to Mihai Oltean for pleasant discussions about preservation solutions and other research-related subjects.

I thank Rosie Perkins for professional linguistic comments.

Thanks to Agneta Särén and Ingela Stave for secretarial assistance during these years and kindness throughout the years.

Special thanks to Laila Abrahamsson for being a wonderful friend throughout many years. It is always a relief to meet you for a cup of coffee or lunch.

Thanks to all persons working at Wallenberg Laboratory for contributing to a nice atmosphere.

To my family members, thanks for your moral support and encouragement throughout the years. After years of questions about when I will finish my thesis, I can finally give an answer.

This work was supported by grants from various sources. I would like to thank all foundations for their generous financial support: Swedish Research Council (grant nr K2005-73X-00536-41A), Inga Britta & Arne Lundberg foundation, Foundation for Scientific Research at the Department of Surgery, Wallenberg foundation., Wilhelm and Martina Lundgrens foundation, Axel Lennart Larsson foundation, Anna Cederbergs foundation, GaiaCare As, HM Sultan Qaboos Research Trust Funds (project nr SR/AGR/FOOD/05/01).

REFERENCES

1. Haapaniemi, T., Sirsjo, A., Nylander, G., Larsson, J. Hyperbaric oxygen treatment attenuates glutathione depletion and improves metabolic restitution in postischemic skeletal muscle. Free Radic Res. 23 (2): 91-101, 1995.

2. Blaisdell, F.W. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. Cardiovasc Surg. 10 (6): 620-30, 2002.

3. Lehninger, A., Nelson, D., Cox, M. Principles of Biochemistry.

4. Marieb, E. Human anatomy and physiology. California: Benjamin/Cummins; 1995.

5. Schlattner, U., Tokarska-Schlattner, M., Wallimann, T. Mitochondrial creatine kinase in human health and disease. Biochim Biophys Acta. 1762 (2): 164-80, 2006.

6. Soussi, B., Idstrom, J.P., Bylund-Fellenius, A.C., Schersten, T. Dynamics of skeletal muscle energetics during ischemia and reperfusion assessed by in vivo 31P NMR. NMR in Biomedicine. 3 (2): 71-7, 1990.

7. Idstrom, J.P., Soussi, B., Elander, A., Bylund-Fellenius, A.C. Purine metabolism after in vivo ischemia and reperfusion in rat skeletal muscle. American Journal of Physiology. 258 (6 Pt 2): H1668-73, 1990.

8. St Peter, S.D., Imber, C.J., Friend, P.J. Liver and kidney preservation by perfusion. Lancet. 359 (9306): 604-13, 2002.

9. Soussi, B. Doctoral Thesis, Göteborg University; 1989.

10. Brunelli, G.A., Brunelli, G.R. Tissue changes at different periods of ischemia. Int. Angiol. 14 (3): 253-63, 1995.

11. Gordon, L., Buncke, H.J., Townsend, J.J. Histological changes in skeletal muscle after temporary independent occlusion of arterial and venous supply. Plast Reconstr Surg. 61 (4): 576-9, 1978.

12. Lagerwall, K., Daneryd, P., Schersten, T., Soussi, B. In vivo 31P nuclear magnetic resonance evidence of the salvage effect of ascorbate on the postischemic reperfused rat skeletal muscle. Life Sci. 56 (6): 389-97, 1995.

13. Skjeldal, S., Grogaard, B., Reikeras, O., Muller, C., Torvik, A., Svindland, A. Model for skeletal muscle ischemia in rat hindlimb: evaluation of reperfusion and necrosis. Eur Surg Res. 23 (5-6): 355-65, 1991.

14. Lindsay, T.F., Liauw, S., Romaschin, A.D., Walker, P.M. The effect of ischemia/reperfusion on adenine nucleotide metabolism and xanthine oxidase production in skeletal muscle. J Vasc Surg. 12 (1): 8-15, 1990.

15. Labbe, R., Lindsay, T., Walker, P.M. The extent and distribution of skeletal muscle necrosis after graded periods of complete ischemia. J Vasc Surg. 6 (2): 152-7, 1987.

16. Kopprasch, S., Pietzsch, J., Graessler, J. Validation of different chemilumigenic substrates for detecting extracellular generation of reactive oxygen species by phagocytes and endothelial cells. Luminescence. 18 (5): 268-73, 2003.

17. Jaeschke, H. Reactive oxygen and mechanisms of inflammatory liver injury. J Gastroenterol Hepatol. 15 (7): 718-24, 2000.

18. Halliwell, B., Gutteridge, J. Free radicals in biology and medicine. New York: Oxford University Press, inc.; 1999.

19. Andreoli, T.E. Free radicals and oxidative stress. Am J Med. 108 (8): 650-1, 2000.

20. Halliwell, B. Antioxidants in human health and disease. Annu Rev Nutr. 16 33-50, 1996.

21. Powers, S.K., Lennon, S.L. Analysis of cellular responses to free radicals: focus on exercise and skeletal muscle. Proc Nutr Soc. 58 (4): 1025-33, 1999.

22. De Santis, G., Pinelli, M. Microsurgical model of ischemia reperfusion in rat muscle: evidence of free radical formation by spin trapping. Microsurgery. 15 (9): 655-9, 1994.

23. Lagerwall, K., Madhu, B., Daneryd, P., Schersten, T., Soussi, B. Purine nucleotides and phospholipids in ischemic and reperfused rat skeletal muscle: effect of ascorbate. American Journal of Physiology. 272 (1 Pt 2): H83-90, 1997.

24. Nilsson, U.A., Lundgren, O., Haglind, E., Bylund-Fellenius, A.C. Radical production during in vivo intestinal ischemia and reperfusion in the cat. American Journal of Physiology. 257 (3 Pt 1): G409-14, 1989.

25. McCord, J.M. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med. 312 (3): 159-63, 1985.

26. Pagliaro, P. Differential biological effects of products of nitric oxide (NO) synthase: it is not enough to say NO. Life Sci. 73 (17): 2137-49, 2003.

27. Brown, G.C. Cell biology. NO says yes to mitochondria. Science. 299 (5608): 838-9, 2003.

28. Khanna, A., Cowled, P.A., Fitridge, R.A. Nitric oxide and skeletal muscle reperfusion injury: current controversies (research review). J Surg Res. 128 (1): 98-107, 2005.

29. Alderton, W.K., Cooper, C.E., Knowles, R.G. Nitric oxide synthases: structure, function and inhibition. Biochem. J. 357 (3): 593-615, 2001.

30. Barker, J.E., Knight, K.R., Romeo, R., Hurley, J.V., Morrison, W.A., Stewart, A.G. Targeted disruption of the nitric oxide synthase 2 gene protects against ischaemia/reperfusion injury to skeletal muscle. J Pathol. 194 (1): 109-15, 2001.

31. Liu, K., Chen, L.E., Seaber, A.V., Urbaniak, J.R. S-nitroso-N-acetylcysteine protects skeletal muscle against reperfusion injury. Microsurgery. 18 (5): 299-305, 1998.

32. Meldrum, D.G., Stephenson, L.L., Zamboni, W.A. Effects of L-NAME and L-arginine on ischemia-reperfusion injury in rat skeletal muscle. Plast Reconstr Surg. 103 (3): 935-40, 1999.

33. Jugdutt, B.I. Nitric oxide and cardioprotection during ischemia-reperfusion. Heart Fail Rev. 7 (4): 391-405, 2002.

34. Belzer, F.O. Evaluation of preservation of the intra-abdominal organs. Transplant Proc. 25 (4): 2527-30, 1993.

35. Muhlbacher, F., Langer, F., Mittermayer, C. Preservation solutions for transplantation. Transplant Proc. 31 (5): 2069-70, 1999.

36. Ingemansson, R., Sjoberg, T., Massa, G., Steen, S. Long-term preservation of vascular endothelium and smooth muscle. Ann Thorac Surg. 59 (5): 1177-81, 1995.

37. Sapega, A.A., Heppenstall, R.B., Sokolow, D.P., Graham, T.J., Maris, J.M., Ghosh, A.K., Chance, B., Osterman, A.L. The bioenergetics of preservation of limbs before replantation. The rationale for intermediate hypothermia. J Bone Joint Surg [Am]. 70 (10): 1500-13, 1988.

38. Lundberg, J., Elander, A., Soussi, B. Effect of hypothermia on the ischemic and reperfused rat skeletal muscle, monitored by in vivo (31)P-magnetic resonance spectroscopy. Microsurgery. 21 (8): 366-73, 2001.

39. Lundberg, J. Ph.D. Thesis, Göteborg University; 2002.

40. Southard, J. The right solution for organ preservation. Busienss Briefing: North American Pharmacotherapy. (2): 1-4, 2004.

41. Southard, J.H., Belzer, F.O. Organ preservation. Annu Rev Med. 46 235-47, 1995.

42. Ben Abdennebi, H., Elrassi, Z., Scoazec, J.Y., Steghens, J.P., Ramella-Virieux, S., Boillot, O. Evaluation of IGL-1 preservation solution using an orthotopic liver transplantation model. World J Gastroenterol. 12 (33): 5326-30, 2006.

43. Steen, S., Sjoberg, T., Massa, G., Ericsson, L., Lindberg, L. Safe pulmonary preservation for 12 hours with low-potassium-dextran solution. Ann Thorac Surg. 55 (2): 434-40, 1993.

44. Oto, T., Griffiths, A.P., Rosenfeldt, F., Levvey, B.J., Williams, T.J., Snell, G.I. Early outcomes comparing Perfadex, Euro-Collins, and Papworth solutions in lung transplantation. Ann Thorac Surg. 82 (5): 1842-8, 2006.

45. Muller, C., Furst, H., Reichenspurner, H., Briegel, J., Groh, J., Reichart, B. Lung procurement by low-potassium dextran and the effect on preservation injury. Munich Lung Transplant Group. Transplantation. 68 (8): 1139-43, 1999.

46. Montgomery, A., Kallen, R., Falt, K., Haglund, U., Borgstrom, A. Reperfusion of duodenum and pancreas following 24 hr of cold storage in Perfadex or UW solution. Journal of Surgical Research. 55 (6): 615-21, 1993.

47. Jonsson, O., Lindgård, A., Fae, A., Gondalia, J., Aneman, A., Soussi, B. Enhanced post-ischaemic recovery in rabbit kidney after pretreatment with an indeno-indole compound and ascorbate monitored in vivo by 31P magnetic resonance spectroscopy. Scand. J. Urol. Nephrol. 37 (6): 450-5, 2003.

48. Hultén, L.M., Holmstrom, M., Soussi, B. Harmful singlet oxygen can be helpful. Free Radic. Biol. Med. 27 (11-12): 1203-7, 1999.

49. Sorensen, V., Jonsson, O., Pettersson, S., Schersten, T., Soussi, B. In vivo 31P NMR OSIRIS of bioenergetic changes in rabbit kidneys during and after ischaemia: effect of pretreatment with an indeno-indole compound. Acta Physiol Scand. 162 (4): 495-500, 1998.

50. Daniell, M.D., Hill, J.S. A history of photodynamic therapy. Aust. N. Z. J. Surg. . 61 (5): 340-8, 1991.

51. Alora, M.B., Anderson, R.R. Recent developments in cutaneous lasers. Lasers Surg. Med. 26 (2): 108-18, 2000.

52. Whelan, H.T., Smits, R.L., Jr., Buchman, E.V., Whelan, N.T., Turner, S.G., Margolis, D.A., Cevenini, V., Stinson, H., Ignatius, R., Martin, T., Cwiklinski, J., Philippi, A.F., Graf, W.R., Hodgson, B., Gould, L., Kane, M., Chen, G., Caviness, J. Effect of NASA light-emitting diode irradiation on wound healing. Journal of clinical laser medicine & surgery. 19 (6): 305-14., 2001.

53. Lane, N. New light on medicine. Sci. Am. 288 (1): 38-45, 2003.

54. Zhu, Q., Yu, W., Yang, X., Hicks, G.L., Lanzafame, R.J., Wang, T. Photo-irradiation improved functional preservation of the isolated rat heart. Lasers in Surgery and Medicine. 20 (3): 332-9, 1997.

55. Macdonald, I., Dougherty, T. Basic principles of photodynamic therapy. Journal of Porphyrins and Phthalocyanines. 5 (2): 105-129, 2001.

56. Topping, A., Gault, D., Grobbelaar, A., Sanders, R., Green, C., Linge, C. Does low penetration of human skin by the normal mode ruby laser account for poor permanent depilatory success rates? Lasers Med Sci. 16 (3): 224-9, 2001.

57. Vinck, E.M., Cagnie, B.J., Cornelissen, M.J., Declercq, H.A., Cambier, D.C. Increased fibroblast proliferation induced by light emitting diode and low power laser irradiation. Lasers Med. Sci. 18 (2): 95-9, 2003.

58. Ferezin, C.Z., Oliveira, F.S., da Silva, R.S., Simioni, A.R., Tedesco, A.C., Bendhack, L.M. The complex trans-[RuCl([15]aneN4NO]2+ induces rat aorta relaxation by ultraviolet light irradiation. Nitric Oxide. 13 (3): 170-5, 2005.

59. Ergun, Y., Ogulener, N. Selective modifiers of glutathione prevent restoration of photorelaxations in mouse gastric fundus. Fundam. Clin. Pharmacol. 19 (4): 503-9, 2005.

60. Vladimirov, Y., Borisenko, G., Boriskina, N., Kazarinov, K., Osipov, A. NO-hemoglobin may be a light-sensitive source of nitric oxide both in solution and in red blood cells. J. Photochem. Photobiol. B. 59 (1-3): 115-22, 2000.

61. Mendez, F., Penner, R. Near-visible ultraviolet light induces a novel ubiquitous calcium- permeable cation current in mammalian cell lines. J Physiol (Lond). 507 (Pt 2): 365-77, 1998.

62. Orii, Y. Immediate reduction of cytochrome c by photoexcited NADH: reaction mechanism as revealed by flow-flash and rapid-scan studies. Biochemistry. 32 (44): 11910-4, 1993.

63. Morimoto, Y., Arai, T., Kikuchi, M., Nakajima, S., Nakamura, H. Effect of low-intensity argon laser irradiation on mitochondrial respiration. Lasers Surg Med. 15 (2): 191-9, 1994.

64. Lapotko, D., Romanovskaya, T., Gordiyko, E. Photothermal monitoring of redox state of respiratory chain in single live cells. Photochem Photobiol. 75 (5): 519-26, 2002.

65. Kipshidze, N., Keelan, M.H., Petersen, J.R., Bachutashvili, I., Vossoughi, J., Karanian, J., Ghosh, C., Iversen, P., Roubin, G.S., Leon, M.B., Moses, J.W. Photoactivation of vascular iNOS and elevation of cGMP in vivo: possible mechanism for photovasorelaxation and inhibition of restenosis in an atherosclerotic rabbit models. Photochem Photobiol. 72 (4): 579-82, 2000.

66. Kipshidze, N., Sahota, H., Komorowski, R., Nikolaychik, V., Keelan, M.H., Jr. Photoremodeling of arterial wall reduces restenosis after balloon angioplasty in an atherosclerotic rabbit model. J Am Coll Cardiol. 31 (5): 1152-7, 1998.

67. El Batanouny, M., Korraa, S., Fekry, O. Mitogenic potential inducible by He:Ne laser in human lymphocytes in vitro. J Photochem Photobiol B. 68 (1): 1-7, 2002.

68. Manteifel, V., Bakeeva, L., Karu, T. Ultrastructural changes in chondriome of human lymphocytes after irradiation with He-Ne laser: appearance of giant mitochondria. J Photochem Photobiol B. 38 (1): 25-30, 1997.

69. Shefer, G., Barash, I., Oron, U., Halevy, O. Low-energy laser irradiation enhances de novo protein synthesis via its effects on translation-regulatory proteins in skeletal muscle myoblasts. Biochimica et Biophysica Acta. 1593 131-139, 2003.

70. Passarella, S., Ostuni, A., Atlante, A., Quagliariello, E. Increase in the ADP/ATP exchange in rat liver mitochondria irradiated in vitro by helium-neon laser. Biochem. Biophys. Res. Commun. 156 (2): 978-86, 1988.

71. Karu, T., Pyatibrat, L., Kalendo, G. Irradiation with He-Ne laser increases ATP level in cells cultivated in vitro. J. Photochem. Photobiol. B. 27 (3): 219-23, 1995.

72. Passarella, S., Casamassima, E., Molinari, S., Pastore, D., Quagliariello, E., Catalano, I.M., Cingolani, A. Increase of proton electrochemical potential and ATP synthesis in rat liver mitochondria irradiated in vitro by helium-neon laser. FEBS Lett. 175 (1): 95-9, 1984.

73. Greco, M., Vacca, R.A., Moro, L., Perlino, E., Petragallo, V.A., Marra, E., Passarella, S. Helium-Neon laser irradiation of hepatocytes can trigger increase of the mitochondrial membrane potential and can stimulate c-fos expression in a Ca2+-dependent manner. Lasers Surg Med. 29 (5): 433-41, 2001.

74. Shefer, G., Partridge, T.A., Heslop, L., Gross, J.G., Oron, U., Halevy, O. Low-energy laser irradiation promotes the survival and cell cycle entry of skeletal muscle satellite cells. J Cell Sci. 115 (Pt 7): 1461-9, 2002.

75. Vacca, R.A., Marra, E., Passarella, S., Petragallo, V.A., Greco, M. Increase in cytosolic and mitochondrial protein synthesis in rat hepatocytes irradiated in vitro by He-Ne laser. J Photochem Photobiol B. 34 (2-3): 197-202, 1996.

76. Breitbart, H., Levinshal, T., Cohen, N., Friedmann, H., Lubart, R. Changes in calcium transport in mammalian sperm mitochondria and plasma membrane irradiated at 633 nm (HeNe laser). J Photochem Photobiol B. 34 (2-3): 117-21, 1996.

77. Yu, W., Naim, J.O., McGowan, M., Ippolito, K., Lanzafame, R.J. Photomodulation of oxidative metabolism and electron chain enzymes in rat liver mitochondria. Photochem Photobiol. 66 (6): 866-71, 1997.

78. Wong-Riley, M.T., Bai, X., Buchmann, E., Whelan, H.T. Light-emitting diode treatment reverses the effect of TTX on cytochrome oxidase in neurons. Neuroreport. 12 (14): 3033-7, 2001.

79. Morrone, G., Guzzardella, G.A., Orienti, L., Giavaresi, G., Fini, M., Rocca, M., Torricelli, P., Martini, L., Giardino, R. Muscular Trauma Treated with a Ga-Al-As Diode Laser: In Vivo Experimentally Study. Lasers in Medical Science. 13 293-298, 1998.

80. Oron, U., Yaakobi, T., Oron, A., Mordechovitz, D., Shofti, R., Hayam, G., Dror, U., Gepstein, L., Wolf, T., Haudenschild, C., Haim, S.B. Low-Energy Laser Irradiation Reduces Formation of Scar Tissue After Myocardial Infarction in Rats and Dogs. Circulation. 103 (2): 296-301, 2001.

81. Oron, U., Yaakobi, T., Oron, A., Hayam, G., Gepstein, L., Rubin, O., Wolf, T., Ben Haim, S. Attenuation of infarct size in rats and dogs after myocardial infarction by low-energy laser irradiation. Lasers Surg Med. 28 (3): 204-11, 2001.

82. Karu, T.I., Pyatibrat, L.V., Kalendo, G.S. Donors of NO and pulsed radiation at lambda = 820 nm exert effects on cell attachment to extracellular matrices. Toxicol. Lett. 121 (1): 57-61, 2001.

83. Kipshidze, N., Nikolaychik, V., Muckerheidi, M., Keelan, M.H., Chekanov, V., Maternowski, M., Chawla, P., Hernandez, I., Iyer, S., Dangas, G., Sahota, H., Leon, M.B., Roubin, G., Moses, J.W. Effect of short pulsed nonablative infrared laser irradiation on vascular cells in vitro and neointimal hyperplasia in a rabbit balloon injury model. Circulation. 104 (15): 1850-5, 2001.

84. Diaz-Herrera, P., Garcia-Castellano, J.M., Torres, A., Morcuende, J.A., Calbet, J.A., Sarrat, R. Effect of high-intensity running in rectus femoris muscle fiber in rats. J Orthop Res. 19 (2): 229-32, 2001.

85. Vega-Alvarez, J.A., Pena, C., Bengoechea, M.P., Perez-Casas, A. Histochemical study, morphometric analysis and systematic distribution of fiber types in the rectus femoris muscle of the albino rats. Bull Assoc Anat (Nancy). 73 (221): 29-33, 1989.

86. Lukes, D. Ph.D. Thesis, Göteborg University; 2003.

87. Goddard, M., Large, S.R. Preservation, reperfusion, and rejection in transgenic xenograft organs. J Card Surg. 15 (2): 136-40, 2000.

88. Olausson, M., Mjornstedt, L., Lindholm, L., Brynger, H. Non-suture organ grafting to the neck vessels in rats. Acta Chir Scand. 150 (6): 463-7, 1984.

89. Boyum, A. Isolation of lymphocytes, granulocytes and macrophages. Scand. J. Immunol. Suppl 5 9-15, 1976.

90. McLaren, A.J., Friend, P.J. Trends in organ preservation. Transpl Int. 16 (10): 701-8, 2003.

91. Karu, T. Primary and secondary mechanisms of action of visible to near-IR radiation on cells. J. Photochem. Photobiol. B. 49 (1): 1-17, 1999.

92. Frimer, A. Physical - chemical aspects. in Rayton, B. eds. Singlet O2, volume 1. Florida: CRC Press Inc; 2000: 8-18.

93. Wilkinson, F., Helman, W.P., Ross, A.B. Quantum yields for the photosensitized formation of the lowest electronically excited singlet state of molecular oxygen in solution. J. Phys. Chem. Ref. Data. 22 113-262, 1993.

94. Ballerini, L., Högberg, A., Borgefors, G., A-C., B., Lindgård, A., Lundström, K., Rakotonirainy, O., Soussi, B. A Segmentation Technique to Determine Fat Content in NMR Images of Beef Meat. IEEE Transaction on Nuclear Science. 49 (1): 195-199, 2002.

95. Lagerwall, K. Doctoral Thesis, Göteborg University; 1997.

96. Salibi, N., Brown, M.A. Clinical MR spectroscopy. First principles. New York: John Wiley & Sons; 1988.

97. Ingwall, J.S. Phosphorus nuclear magnetic resonance spectroscopy of cardiac and skeletal muscles. Am J Physiol. 242 (5): H729-44, 1982.

98. Chance, B., Leigh, J.S., Jr., Clark, B.J., Maris, J., Kent, J., Nioka, S., Smith, D. Control of oxidative metabolism and oxygen delivery in human skeletal muscle: a steady-state analysis of the work/energy cost transfer function. Proc Natl Acad Sci U S A. 82 (24): 8384-8, 1985.

99. Tamraz, J., Outin, C., Forjaz Secca, M., Soussi, B. MRI Principles of the Head, Skull Base and Spine. A clinical approach. Springer Verlag; 2002.

100. Leahy, M.J., de Mul, F.F., Nilsson, G.E., Maniewski, R. Principles and practice of the laser-Doppler perfusion technique. Technol Health Care. 7 (2-3): 143-62, 1999.

101. Johansson, A., Dahlgren, C. Characterization of the luminol-amplified light-generating reaction induced in human monocytes. J. Leukoc. Biol. 45 (5): 444-51, 1989.

102. Lundqvist, H., Dahlgren, C. Isoluminol-enhanced chemiluminescence: a sensitive method to study the release of superoxide anion from human neutrophils. Free Radic Biol Med. 20 (6): 785-92, 1996.

103. Gunnarsson, G., Undeland, I., Sannaveerappa, T., Sandberg, A.S., Lindgard, A., Mattsson-Hulten, L., Soussi, B. Inhibitory effect of known antioxidants and of press juice from herring (Clupea harengus) light muscle on the generation of free radicals in human monocytes. J Agric Food Chem. 54 (21): 8212-21, 2006.

104. Lundin, A. Use of firefly luciferase in ATP-related assays of biomass, enzymes, and metabolites. Methods Enzymol. . 305 346-70, 2000.

105. Kharitonov, S., Alving, K., Barnes, P.J. Exhaled and nasal nitric oxide measurements: recommendations. The European Respiratory Society Task Force. Eur. Respir. J. 10 (7): 1683-93, 1997.

106. Archer, S. Measurement of nitric oxide in biological models. FASEB J. 7 (2): 349-60, 1993.

107. Hinkelmann, K., Kempthorne, O. Design and Analysis of Experiments. in. eds. Introduction to Experimental Design, first ed., volume 1. New York: Wiley, Cop.; 1994: 349-359.

108. Michel, P., Hadour, G., Rodriguez, C., Chiari, P., Ferrera, R. Evaluation of a new preservative solution for cardiac graft during hypothermia. J Heart Lung Transplant. 19 (11): 1089-97, 2000.

109. Steen, S., Kimblad, P.O., Sjoberg, T., Lindberg, L., Ingemansson, R., Massa, G. Safe lung preservation for twenty-four hours with Perfadex [see comments]. The Annals of Thoracic Surgery. 57 (2): 450-7, 1994.

110. Tsuchida, T., Kato, T., Yamaga, M., Ikebe, K., Oniki, Y., Irie, H., Takagi, K. Effect of perfusion during ischemia on skeletal muscle. J Surg Res. 101 (2): 238-41, 2001.

111. Yoshioka, T., Shires, G.T., Fantini, G.A. Hypothermia relieves oxidative stress in reperfused skeletal muscle following partial ischemia. J Surg Res. 53 (4): 408-16, 1992.

112. Avni, D., Levkovitz, S., Maltz, L., Oron, U. Protection of skeletal muscles from ischemic injury: low-level laser therapy increases antioxidant activity. Photomed Laser Surg. 23 (3): 273-7, 2005.

113. Eckert, P., Schnackerz, K. Ischemic tolerance of human skeletal muscle. Annals of Plastic Surgery. 26 (1): 77-84, 1991.

114. Becker, L.B., vanden Hoek, T.L., Shao, Z.H., Li, C.Q., Schumacker, P.T. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. American Journal of Physiology. 277 (6 Pt 2): H2240-6., 1999.

115. Vanden Hoek, T.L., Shao, Z., Li, C., Schumacker, P.T., Becker, L.B. Mitochondrial electron transport can become a significant source of oxidative injury in cardiomyocytes. J Mol Cell Cardiol. 29 (9): 2441-50, 1997.

116. Soussi, B., Idstrom, J.P., Schersten, T., Bylund-Fellenius, A.C. Cytochrome c oxidase and cardiolipin alterations in response to skeletal muscle ischaemia and reperfusion. Acta Physiologica Scandinavica. 138 (2): 107-14, 1990.

117. Lee, C.P. Biochemical studies of isolated mitochondria from normal and diseased tissues. Biochim. Biophys. Acta. 1271 (1): 21-8, 1995.

118. Cooper, C.E., Springett, R. Measurement of cytochrome oxidase and mitochondrial energetics by near- infrared spectroscopy. Philosophical Transactions: Biological Sciences. 352 (1354): 669-76., 1997.

119. Beauvoit, B., Kitai, T., Chance, B. Contribution of the mitochondrial compartment to the optical properties of the rat liver: a theoretical and practical approach. Biophysical Journal. 67 (6): 2501-10., 1994.

120. Chernomorsky, S., Wong, C., Poretz, R.D. Pheophorbide a-induced photo-oxidation of cytochrome c: implication for photodynamic therapy. Photochem. Photobiol. 55 (2): 205-11, 1992.

121. Vekshin, N.L. Light-dependent ATP synthesis in mitochondria. Biochem Int. 25 (4): 603-11, 1991.

122. Rocca, M., Giavaresi, G., Fini, M., Orienti, L., Giardino, R. Laser Doppler evaluation of microcirculation behaviour during an ischaemia-reperfusion injury. Eur Surg Res. 30 (2): 108-14, 1998.

123. Fraser, C.D., Jr., Chacko, V.P., Jacobus, W.E., Mueller, P., Soulen, R.L., Hutchins, G.M., Reitz, B.A., Baumgartner, W.A. Early phosphorus 31 nuclear magnetic resonance bioenergetic changes potentially predict rejection in heterotopic cardiac allografts. J Heart Transplant. 9 (3 Pt 1): 197-204, 1990.

124. Lukes, D.J., Madhu, B., Kjellstrom, C., Gustavsson, M.L., Mjornstedt, L., Olausson, M., Soussi, B. Decreasing ratios of phosphocreatine to beta-ATP correlates to progressive acute rejection in a concordant mouse heart to rat xenotransplantation model. Scand J Immunol. 53 (2): 171-5, 2001.

125. Schwartz, G.G., Greyson, C.R., Wisneski, J.A., Garcia, J., Steinman, S. Relation among regional O2 consumption, high-energy phosphates, and substrate uptake in porcine right ventricle. Am J Physiol. 266 (2 Pt 2): H521-30, 1994.

126. Simpson, P.J., Lucchesi, B.R. Free radicals and myocardial ischemia and reperfusion injury. Journal of Laboratory and Clinical Medicine. 110 (1): 13-30, 1987.

127. Cadenas, E., Davies, K.J. Mitochondrial free radical generation, oxidative stress, and aging. Free Radic Biol Med. 29 (3-4): 222-30, 2000.

128. Malis, C.D., Bonventre, J.V. Mechanism of calcium potentiation of oxygen free radical injury to renal mitochondria. A model for post-ischemic and toxic mitochondrial damage. J Biol Chem. 261 (30): 14201-8, 1986.

129. Schneemann, M., Schoedon, G., Hofer, S., Blau, N., Guerrero, L., Schaffner, A. Nitric oxide synthase is not a constituent of the antimicrobial armature of human mononuclear phagocytes. J. Infect. Dis. 167 (6): 1358-63, 1993.

130. Denis, M. Human monocytes/macrophages: NO or no NO? J. Leukoc. Biol. . 55 (5): 682-4, 1994.

131. Wang, C.H., Lin, H.C., Liu, C.Y., Huang, K.H., Huang, T.T., Yu, C.T., Kuo, H.P. Upregulation of inducible nitric oxide synthase and cytokine secretion in peripheral blood monocytes from pulmonary tuberculosis patients. Int. J. Tuberc. Lung Dis. 5 (3): 283-91, 2001.

132. Schena, M., Mulatero, P., Schiavone, D., Mengozzi, G., Tesio, L., Chiandussi, L., Veglio, F. Vasoactive hormones induce nitric oxide synthase mRNA expression and nitric oxide production in human endothelial cells and monocytes. Am. J. Hypertens. 12 (4 Pt 1): 388-97, 1999.

133. Fang, F.C. Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J. Clin. Invest. 99 (12): 2818-25, 1997.

134. MacMicking, J., Xie, Q.W., Nathan, C. Nitric oxide and macrophage function. Annu. Rev. Immunol. 15 323-50, 1997.

135. Ruetten, H., Thiemermann, C. Prevention of the expression of inducible nitric oxide synthase by aminoguanidine or aminoethyl-isothiourea in macrophages and in the rat. Biochem. Biophys. Res. Commun. 225 (2): 525-30, 1996.

136. Corbett, J.A., Tilton, R.G., Chang, K., Hasan, K.S., Ido, Y., Wang, J.L., Sweetland, M.A., Lancaster, J.R., Jr., Williamson, J.R., McDaniel, M.L. Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. Diabetes. 41 (4): 552-6, 1992.

137. Matsunaga, K., Furchgott, R.F. Interactions of light and sodium nitrite in producing relaxation of rabbit aorta. J. Pharmacol. Exp. Ther. 248 (2): 687-95, 1989.

138. Lovren, F., Triggle, C.R. Involvement of nitrosothiols, nitric oxide and voltage-gated K+ channels in photorelaxation of vascular smooth muscle. Eur. J. Pharmacol. 347 (2-3): 215-21, 1998.

139. Singh, R.J., Hogg, N., Joseph, J., Kalyanaraman, B. Mechanism of nitric oxide release from S-nitrosothiols. J. Biol. Chem. 271 (31): 18596-603, 1996.