

Diploma thesis

**Oxidative stress during
Radiofrequency Ablation**

submitted by

Lucas Berger

Mat. Nr.: 0202840

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**Doktor der gesamten Heilkunde
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Medical University of Graz

conducted at the

Department of Transplantation Surgery

supervised by

Ass. Dr. Philipp Stiegler

Univ.-Prof. Dr. Florian Iberer

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Signature

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.....to my beloved family

Abstract

Background: Oxidative stress is defined as imbalance between prooxidants and antioxidants in favour of prooxidants. It is associated with a variety of pathological processes and diseases. As pigs feature rather similar biological conditions to humans they are of major importance for the investigation of new therapy options and the improvement of already existing therapy modalities like radiofrequency ablation (RFA). RFA is a minimal invasive electrosurgical technique for the treatment of focal malignant disease.

Aim of the study: The target of this work was to perform a computed tomography (CT) -guided RFA in the liver of five pigs and examine whether this therapy option leads to an increased production of markers for oxidative stress. This fact would enhance the risk for a retarded or abnormal healing process and the occurrence of other pathological diseases such as infections, cirrhosis of the liver, viral hepatitis and cardiovascular diseases. In order to exhibit the extent of oxidative stress induced cell damage as effectively as possible, appropriate markers were selected.

Methods: RFA treatment was performed in five domestic pigs under the same conditions as this therapy is accomplished in humans. Before, during and after RFA procedures and also before scarification, serum was collected for detection of carbonyl proteins (CP) and nitrotyrosine using an enzyme linked immunosorbent assay (ELISA). Furthermore the liver was removed and the hepatic tissue was investigated immunohistochemically for heat shock protein 70 (HSP70) and nitrotyrosine.

Results: On the basis of the accomplished determinations a significant enhancement of CP and nitrotyrosine was not verifiable. Immunohistochemistry for nitrotyrosine shows positive results in all five pigs whereas the staining for HSP70 is only positive in three pigs.

Discussion: For a more accurate statistical evaluation and a meaningful hypothesis it would be necessary to include more pigs in the study. A further reason why a significant enhancement of oxidative stress markers is not measurable may be that the pigs are exposed to an enormous stress during the narcotization.

Zusammenfassung

Hintergrund: Oxidativer Stress wird als Störung des Gleichgewichts zwischen Prooxidantien und Antioxidantien zu Gunsten der Prooxidantien definiert. Er wird mit dem Auftreten vieler pathologischer Prozesse und Erkrankungen in Zusammenhang gebracht. Da Schweine dem Menschen aufgrund ihrer biologischen Voraussetzungen sehr ähneln, sind sie sowohl für die Erforschung von neuen Therapiemöglichkeiten als auch für die Optimierung bereits existierender therapeutischen Methoden, wie beispielsweise die Radiofrequenzablation (RFA) von großer Bedeutung. RFA ist eine minimal invasive elektrochirurgische Methode zur Behandlung von fokalen bösartigen Erkrankungen.

Zielsetzung dieser Studie: Das Ziel dieser Arbeit war die Untersuchung, ob computertomographisch (CT) unterstützte RFAs in den Lebern von fünf verschiedenen Schweinen zu einer Erhöhung von oxidativem Stress führen. Diese Tatsache würde das Risiko für einen verzögerten bzw. gestörten Heilungsprozess und das Auftreten anderer Erkrankungen wie Infektionen, Leberzirrhose, virale Hepatiden und kardiovaskuläre Erkrankungen erhöhen. Um das Ausmaß des durch oxidativen Stress induzierten zellulären Schadens so effizient wie möglich untersuchen zu können, wurden geeignete Marker ausgewählt.

Methoden: Die RFA wurde unter denselben Bedingungen wie beim Menschen an fünf Hausschweinen durchgeführt. Sowohl vor, während und nach der RFA als auch vor der Sakrifizierung wurde eine Blutabnahme für die Ermittlung von Carbonylproteinen (CP) und Nitrotyrosin im Serum mittels enzymatisch-immunologischem Nachweisverfahren (ELISA) vorgenommen. Des Weiteren wurde die Leber entnommen und immunhistochemisch auf Hitzeschockprotein 70 (HSP70) und Nitrotyrosin untersucht.

Ergebnisse: Anhand der ermittelten Ergebnisse konnte keine signifikante Erhöhung von CP und Nitrotyrosin festgestellt werden. Die Immunhistochemie zeigte jedoch positive Ergebnisse für Nitrotyrosin in allen fünf Schweinen, wobei HSP70-positive Färbungen nur bei drei Schweinen zu finden waren.

Diskussion: Für eine genauere statistische Auswertung und eine aussagekräftige Hypothese wäre es notwendig mehr Schweine in die Studie einzubeziehen. Ein weiterer Grund für die nicht feststellbare Erhöhung der oxidativen Stressmarker könnte der enorme Stress sein, dem die Schweine während der Narkose ausgesetzt waren.

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Table of abbreviations

$^1\text{O}_2$	singlet oxygen
4-HNE	4-hydroxynonenal
8-oxo-dG	8-hydroxy-2'-deoxyguanosine
ATP	adenosintriphosphate
CAT	catalase
CO₂	carbon dioxide
CP	carbonyl protein
CT	computed tomography
DHA	dehydro ascorbic acid
DNA	deoxyribonucleic acid
DNPH	dinitrophenylhydrazine
ELISA	enzyme linked immunosorbent assay
FaPy	(2,6-diamino-4hydroxy-5-formamidpyrimidine
Fe	iron
GPx	glutathione peroxidase
GSH	glutathione
GSR	glutathione reductase
GSSG	oxidized glutathione
H₂O₂	hydrogen peroxide
HE	haemalaun/eosin
HO₂•	hydroperoxyl radical
HPLC	high performance liquid chromatography
HSP	heat shock protein
L•	lipid radical
LDL	low density lipoprotein
LOO•	lipid peroxyl radical
LOOH	lipid hydroperoxide
LPO	lipid peroxidation
MDA	malondialdehyde
MDHA	semihydro ascorbic acid
MRT	magnetic resonance tomography

MsrA	methionine sulfoxide reductase
MT	metallothionein
NAD⁺	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitrogen monoxide
NO⁻	nitroxyl anions
NO⁺	nitrosonium cations
NOS	NO-synthetases
NOX	NADPH-oxidase
O₂^{•-}	superoxide
O₂²⁻	peroxide
O₂	oxygen
OH[•]	hydroxyl radical
ONOO⁻	peroxynitrite
PAC	Port-a-Cath
PARP	poly-ADP-ribose-polymerase
RFA	radiofrequency ablation
RNS	reactive nitrogen species
ROI	region of interest
RO[•]	alkoxyl radical
RO₂[•]	peroxyl radical
ROOH	hydroperoxide
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances
TRX	thioredoxin

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1 INTRODUCTION

1.1 Oxidative stress

1.1.1 Definition

Oxidative stress is defined as imbalance between prooxidants and antioxidants in favour of prooxidants. (1)

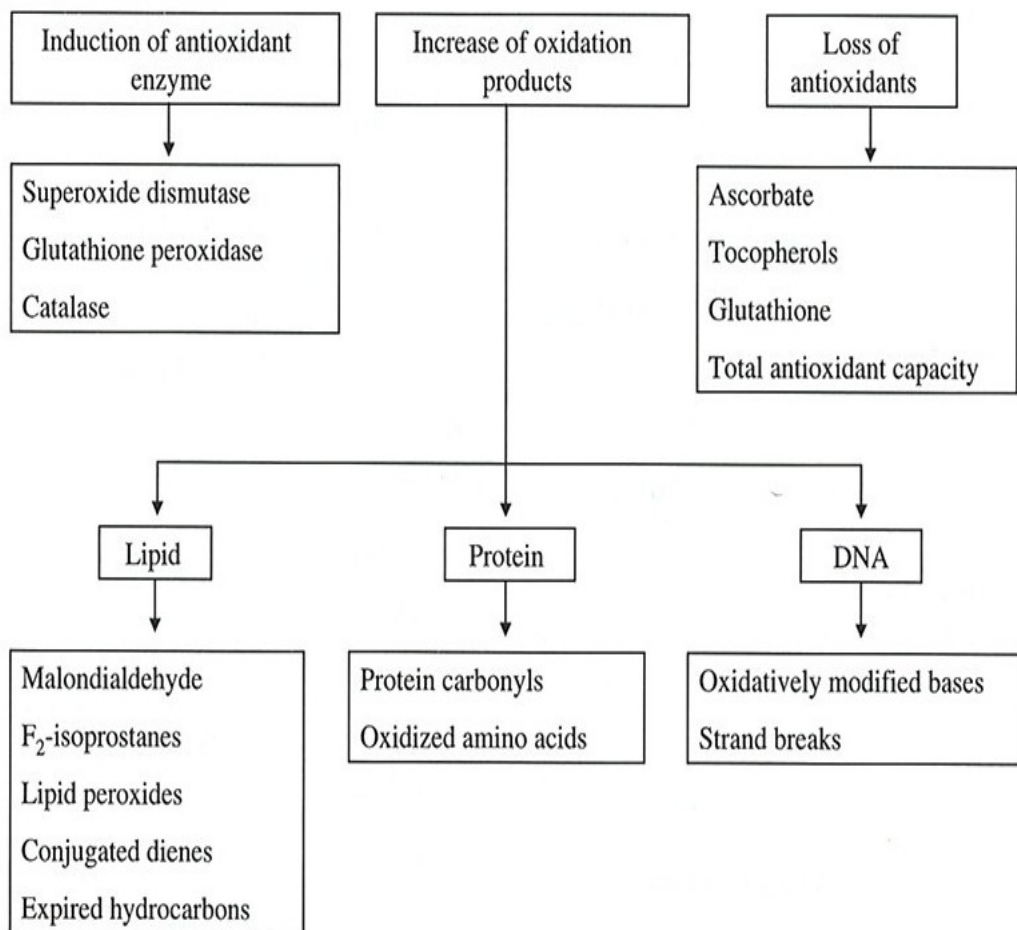


Figure 1.1: Oxidative stress classified as functionality of products of oxidation and antioxidants. (2)

1.1.2 General considerations

Oxidative stress is associated with a variety of pathological processes and diseases, among others including the following:

- | | |
|--------------------------|---------------------------|
| • aging | • atherosclerosis |
| • diabetes | • inflammations |
| • skin diseases | • cardiovascular diseases |
| • ischemia | • infections |
| • ischemic diseases | • cataract |
| • cancer | • cirrhosis of the liver |
| • alzheimer's disease | • crohn's disease |
| • parkinson's disease | • rheumatism |
| • septic shock | • viral hepatitis |
| • immunological diseases | • cystic fibrosis |

Table 1.1: A variety of pathological processes and diseases associated with oxidative stress. (3)

In the course of different intracellular processes reactive oxygen- and nitrogen metabolites, so called prooxidants, are formed. These molecules are able to damage cellular components. Mostly they are free radicals characterized by a high reactivity and an extremely short half-life. They are defined as molecules with one or more unpaired electrons in their ultimate orbital. (3-5)

For instance reactive oxygen metabolites may be formed in the course of mitochondrial respiration, where by means of electron and proton uptake oxygen is reduced to water. Hydroperoxide (ROOH), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH•) occur as intermediate products and may partially escape during this reaction. That means that approximately 5% of the inhaled oxygen are not completely reduced to water and are capable to contribute to the development of free radicals. (6) Free radicals can also occur during the activation of the unspecific immune system, the oxidative desamination of catecholamines in the brain, during ischemia and in the course of traumas as well.

Under physiological circumstances organisms try to compensate oxidative stress with strong antioxidant defenses. If prooxidative processes prevail the body's de-

fense mechanisms, direct and indirect cellular components like proteins, lipids, carbohydrates and deoxyribonucleic acid (DNA), can be damaged. (1;3;7)

For normal cellular functioning, including viability, cell signalling, activation and proliferation, it is essential that the intracellular reduction/oxidation (redox) equilibrium is constantly maintained. The damaging impact of oxidative stress is compensated by an extensive antioxidant system. This system involves low molecular weight antioxidants. Among others, glutathione (GSH), thioredoxin (TRX), ascorbic acid and tocopherols, as well as enzymes including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) are the most important antioxidants in the cellular system. (3;7)

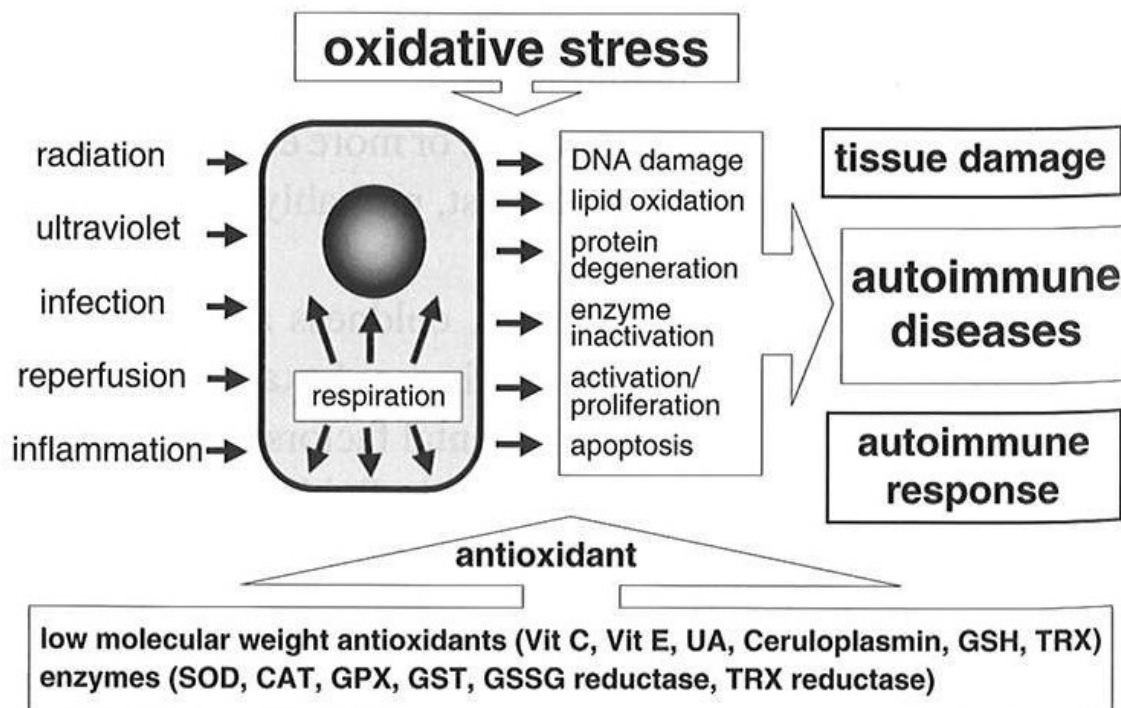


Figure 1.2.: An inadequate antioxidant or even oxidative stress answer are considered to be from essential importance during the development of autoimmune diseases. Reasons may be the aggravation of the inflammatory occurrences by inducing apoptosis, converting autoantigens and reducing the immunological tolerance. (2)

1.1.3 Reactive Oxygen Species/ Reactive Nitrogen Species (ROS/RNS)

1.1.3.1 ROS: Formation, function and pathological consequences

The family of ROS includes a variety of radical and non radical high-reactive oxygen metabolites. $\text{OH}\cdot$, superoxide ($\text{O}_2^{\cdot-}$), hydroperoxyl radicals ($\text{HO}_2\cdot$), alkoxy radicals ($\text{RO}\cdot$) and peroxy radicals ($\text{RO}_2\cdot$) are radical metabolites, whereas singlet oxygen ($^1\text{O}_2$), ROOH and H_2O_2 are denominated as their non radical derivatives.

REACTIVE OXYGEN SPECIES (ROS)

<u>RADICALS</u>	<u>NON - RADICALS</u>
Superoxide, $\text{O}_2^{\cdot-}$	Hydrogen peroxide, H_2O_2
Hydroxyl, $\text{OH}\cdot$	Hypoclorous acid, HOCL
Peroxy, $\text{RO}_2\cdot$	Ozon, O_3
Alkoxy, $\text{RO}\cdot$	Singlet oxygen, $^1\text{O}_2$
Hydroperoxyl, $\text{HO}_2\cdot$	Peroxynitrite, ONOO^-

Table 1.2: A variety of radical and non radical reactive oxygen metabolites. ONOO^- could equally be called RNS. (3)

The high reactivity of these molecules is caused by their electron configuration. During the mitochondrial respiration oxygen is reduced by transmission of one, two, three or even four electrons. If one electron is transmitted $\text{O}_2^{\cdot-}$ will arise. In the case of two electrons, peroxide (O_2^{2-}) is released. $\text{OH}\cdot$ is formed during the transmission of three electrons. In the course of the "classic" mitochondrial respiration, that means that four electrons are transmitted, water and energy in form of adenosintriphosphate (ATP) arise. (1;3;7)

ROS are characterized by an extremely short half-life and arise already during the transport of oxygen electrons in the course of the mitochondrial respiration and different hydroxylation and oxygenation processes. (3;5;7) Reactive oxygen metabolites also should be mentioned in context with protein oxidation, lipid peroxida-

tion and DNA damage. All these processes can initiate a cascade of reactions that may cause cell degeneration and tissue injury. (3;7)

1.1.3.2 The singlet oxygen

$^1\text{O}_2$, formed by photoexcitation, is substantially more active in reactions with molecules in singlet state (e.g. fatty acids) than ground state oxygen. (3;8;9) Furthermore $^1\text{O}_2$ is considerably more reactive than the atmospheric triplet oxygen that is absorbed by respiration. Ground state oxygen is characterized by two unpaired electrons in its ultimate orbital which show parallel spins and is arranged as triplet. (3;7)

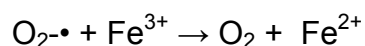
In contrast, $^1\text{O}_2$ carries one of the unpaired electrons in an orbital of higher energy with an inverted spin. Depending on if both electrons are located in the same or in two different orbitals, two forms of $^1\text{O}_2$ can be distinguished. Both of these forms are significantly more reactive than oxygen in its triplet form and could release chain reactions in whose course single electrons are extracted from other molecules. Thus, these molecules become high reactive free radicals themselves. (3;7) During this process a lipid radical is formed by splitting off a hydrogen atom from an unsaturated fatty acid. This atom is able to initiate chain reactions whereas lipid hydroperoxides (LOOH) are released. Furthermore, these molecules are able to react to measurable end products such as malondialdehyde (MDA), ethane and n-pentane. This radical chain reaction is stopped as soon as two radicals react with each other. (3;9)

1.1.3.3 The hydroxyl radical

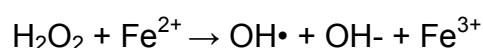
$\text{OH}\cdot$ is the most important and the most reactive form of ROS. It is characterized by an extremely strong oxidizing effect. $\text{OH}\cdot$ is released in the course of many relevant biological systems, for example the Haber-Weiß reaction that includes copper and iron (Fe). Fe^{3+} -ions are reduced by $\text{O}_2\cdot^-$ to Fe^{2+} . The reduced ion reacts with H_2O_2 and so the extremely reactive $\text{OH}\cdot$ is formed. As mentioned above, this molecule is the most important form of reactive oxygen and therefore it is the most considerable source of oxidative damaging of proteins, lipids, carbohydrates and

DNA. In contrast $O_2^{\bullet-}$ is not able to cause cellular impairment due to its instability in such peroxidation chain reactions. (3;7;10;11)

The first step of the Haber-Weiß reaction involves reduction ion to ferrous:



The second step involves the Fenton reaction:



The last step is the net reaction:

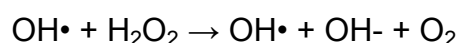


Figure 1.3: The Haber-Weiß reaction (10;11)

Furthermore, $OH^{\bullet}$ is released in the process of homolytic cleavage of H_2O_2 induced by ultraviolet light and decomposition of water caused by ionizing radiation. $OH^{\bullet}$ radicals prefer three types of reactions that play an important role in the autooxidation of lipids: abstraction of hydrogen, addition and electron transfer. (3)

1.1.3.4 The superoxide radical

$O_2^{\bullet-}$ is formed in organisms by a variety of different enzymes that are able to reduce oxygen (O_2) to $O_2^{\bullet-}$. (Table 1.3) It also occurs during various autooxidation reactions.

enzyme	localisation
peroxidases	plants, bacteria, animals (myeloperoxidase in leukocytes)
xanthin-oxidase	bowel, ischemic tissues
tryptophan-dioxygenase	liver
aldehyd-oxidase	liver

Table 1.3: Enzymes that generate $O_2^{\bullet-}$. (3)

The enzyme xanthin-oxidase catalyses the reaction of hypoxanthin to xanthin, where a small share of electrons is transmitted to O_2 instead of nicotinamide adenine dinucleotide (NAD^+). Haemoproteins that release $O_2^{\bullet-}$ after O_2 -binding and transport of electrons from Fe^{2+} to O_2 , as well as electron transport chains like in cytochrome P450 or in mitochondria are considered to be the most important sources of $O_2^{\bullet-}$. Caused by leakage, electrons are directly transmitted to oxygen in this reaction. Approximately 1-3 % of the O_2 exhausted in mitochondria are going to be reduced to $O_2^{\bullet-}$. This is the reason for the increased degradation of mitochondrial DNA.(3)

Furthermore $O_2^{\bullet-}$ is formed in the course of autooxidation reactions of different molecules like flavin, containing reduction equivalents, adrenalin, noradrenalin and dopamine. Chemical substances such as drugs or toxins also generate $O_2^{\bullet-}$ by so called redoxcycling. (Figure 1.4) In this process chinone absorbs an electron from a reductase, which is then transmitted to O_2 , causing the toxicity of many substances. (12)

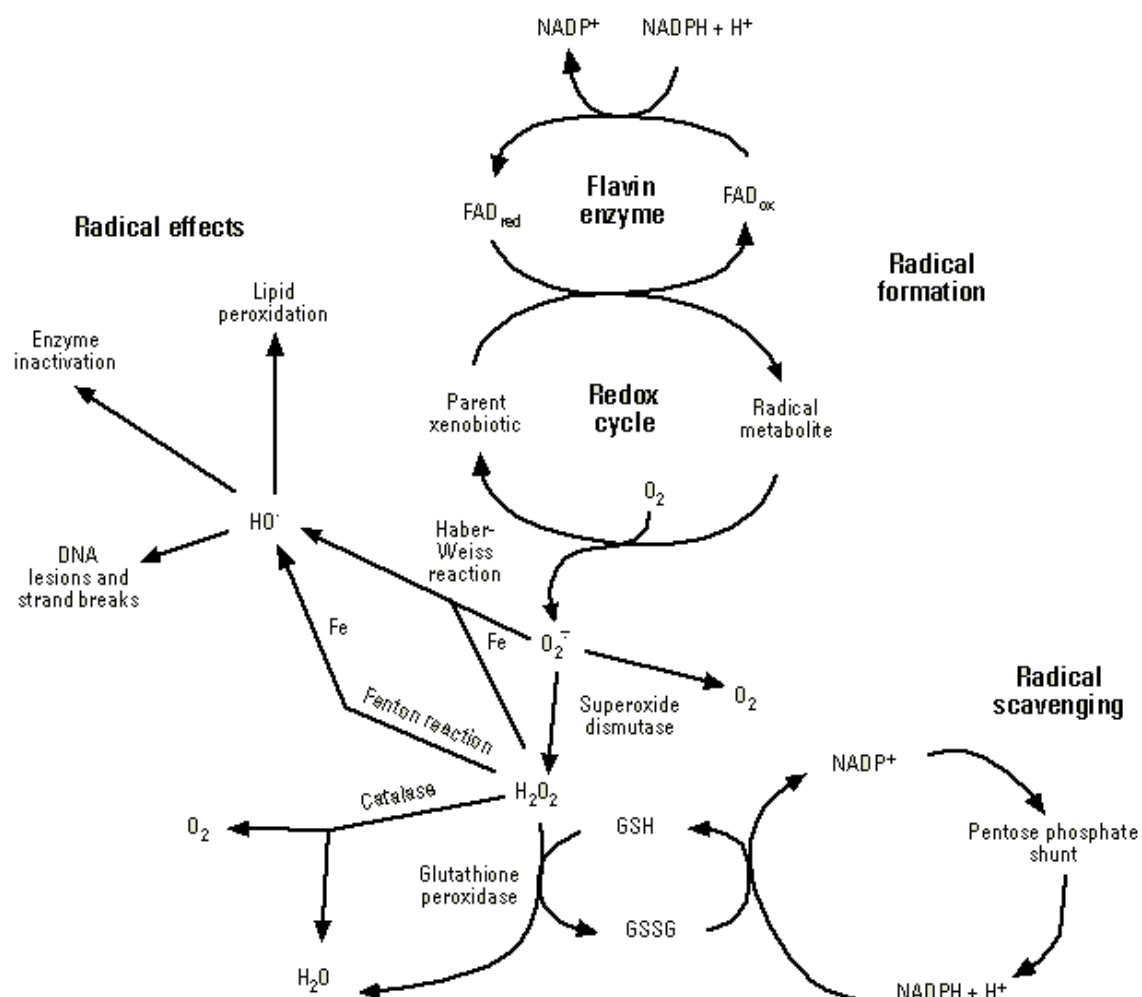


Figure 1.4: Redox cycling which covers a radical formation, scavenging, and effects. (7)

1.1.3.5 The hydrogen peroxide

H_2O_2 is formed by enzyme dependent processes. Guanyl-cyclase, glucose-oxidase, monoamine-oxidase as well as SOD generate H_2O_2 that has a weak oxidizing effect and therefore only shows little reactivity. (3;13) It is not able to oxidize DNA and lipids directly. H_2O_2 can oxidize thiol groups of some proteins that may be inactivated by this reaction. The essential damage caused by H_2O_2 is based on the formation of high reactive $\text{OH}^{\cdot}$. This radical occurs in the course of reactions that are catalyzed by metalions, e.g. the Fenton reaction. (Figure 1.3)

The reactivity of the different ROS is reflected in their half-life period compiled in Table 1.4:

ROS	half-life
OH•	10-9s
alkyl radical	10-8s
¹ O ₂	10-6s
alkoxyl radical	10-6s
alkyl-peroxyl-radical	7s
O ₂ •	spontaneous and enzymatic dismutation
H ₂ O ₂	stable

Table 1.4: Expected half-lives of several ROS. The high reactive OH• shows the shortest half-life whereas H₂O₂ can be used to confect stable solutions. (7)

As ROS are not only hazardous side-products of enzymatic reations they also arise in organisms during the processes mentioned above. For example ROS are formed actively by leukocytes to parry microorganisms. Furthermore they are inevitable for the synthesis of inflammation mediators from arachidonic acid such as prostaglandins, leukotrienes and thromboxans. (3;7)

1.1.3.6 Necrosis and Apoptosis

Necrosis and apoptosis, two different forms of cell degeneration, are considered to be associated with reactive oxygen metabolites. (3) Necrosis is characterized by an early swelling of individual cell organelles and finally by the swelling of the whole cell. The loss of integrity of the different cellular membranes (plasmamem-brane, mitochondria membrane, peroxisomal membranes and lysosomal mem-branes) can lead to a total collapse of the cell. In addition the cell's content is re-leased into the surrounding tissue. The liberated lysosomal enzymes, prooxidants (e.g. iron- and copperions) but even antioxidants like CAT, SOD and GSH have the capacity to impact the adjacent cells.

Apoptosis on the other hand is featured by contraction and in further consequence by condensation of the cell and fragmentation of chromatin. Furthermore, apopto-

sis leads to a breakdown of the cytoskeletal structure and fragmentation of the cell nucleus. Contingently this process may result in a complete decay of the cell in apoptotic fragments without desintegrity of mitochondrial and lysosomal membranes. Apoptotic cell fragmentation does not have any influence on the surrounding cells.

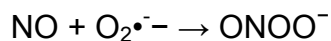
ROS are considered to be directly associated with necrotic and apoptotic degeneration mechanisms. Increased concentrations of ROS and RNS may be able to modify cystein-proteases by oxidizing their sulfur groups.

Apart from cell degeneration ROS and RNS have also a damaging effect during cell proliferation by directly converting receptors of different growth factors and influencing protein kinases and transcription factors. Moreover ROS and RNS can directly cause changes in the redox system of GSH or thiol proteins such as TRX. If transcription factors are impaired, gene expression and cell proliferation will be disturbed. (3)

1.1.3.7 RNS: Formation, function and pathological consequences

Not only reactive oxygen metabolites are involved in the occurrence of oxidative stress but also RNS. The so called nitrosative stress is characterized by an excessive formation of the radical nitrogen monoxide (NO) and its secondary products ONOO⁻, nitrotyrosine and nitrophenyl acetic acid. (3;7)

NO is a radical that is quite stable and is formed in cells by the catalytic effect of NO-synthetases (NOS). The terminal guanido-nitrogen-atom of the amino acid L-arginine is oxidized and furthermore L-arginine reacts with molecular oxygen to NO and L-citrullin. Subsequently the formed NO diffuses to the cell membrane where by means of nicotinamideadeninedinucleotidephosphate (NADPH)-oxidase (NOX) O₂•⁻ is built. In a following reaction, that is limited by diffusion, NO reacts with the formed O₂•⁻ to ONOO⁻. (7;14) This molecule is not a radical but highly reactive. (Equation 1.1)



Equation 1.1: NO reacts with the hyperoxide anion to ONOO⁻ (7;14)

Because of its high redox potential ONOO⁻ is considerably less aggressive than both precursor molecules NO and O₂^{•-}. Furthermore it is featured by a distinctive cytotoxic effect that can cause destruction of endothelium, increased aggregation of thrombocytes and a decreased response of coronar vessels to vasodilators. NO moreover can be converted into reactive nitrosonium cations (NO⁺) or nitroxyl anions (NO⁻). Side products of these reactions are s-nitrocysteine or s-nitroso-glutathione. (15)

All forms of RNS are playing important roles in numerous physiological processes. In particular the induction of apoptosis should be mentioned in this context. ONOO⁻ is able to decrease the intracellular GSH level to initiate apoptosis. (7;16) Furthermore RNS are considered to be involved in a variety of neurological, inflammatory, metabolic and cardiovascular diseases. A relevant marker for nitrosative stress is nitrotyrosine that is formed by the reaction of tyrosine with any reaction product of the nitrogen species. Nitrotyrosine can be detected in vitro with appropriate methods. (17;18)

An example for the pathological effect of ROS and RNS may be Parkinson's disease. The activation of glial cells, in particular the microglia, is hereby from high importance. During this process an increased number of cytokines and glutamate is produced and RNS and ROS metabolites are formed which may have toxic effects on dopaminergic cells. (19;20)

1.1.4 Antioxidants

1.1.4.1 General considerations

As oxygen and ROS are highly reactive molecules, aerobic organisms require efficient antioxidant protective mechanisms. Antioxidants are able to retard or anticipate oxidation of oxidizable substrates like proteins, lipids, carbohydrates and DNA. (3)

Under physiological circumstances the formation and elimination of free radicals is kept balanced by antioxidant defense. (Figure 1.4)

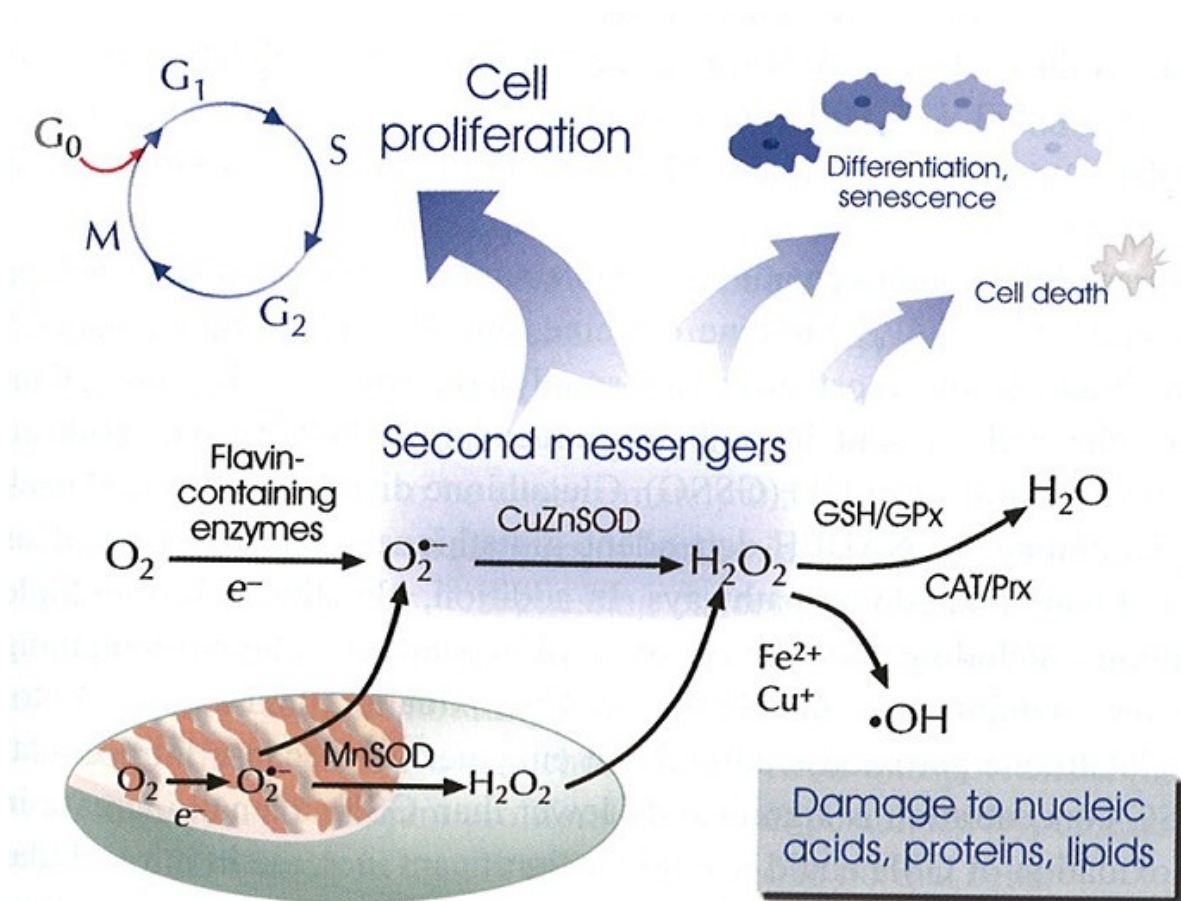


Figure 1.5: A diagram of intracellular ROS generation and their naturalization by antioxidants. ROS can function as a second messenger and regulate cell proliferation, differentiation, senescence and cell death. (2)

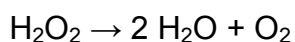
Furthermore, enzymatic and non-enzymatic mechanisms are differentiated. For example SODs are enzymatic antioxidants that catalyze the reaction of $2\text{O}_2^{\bullet-}$ to H_2O_2 and O_2 . The reactive H_2O_2 is subsequently reduced by CAT in the peroxisomes or by cytosolic GPx to water and oxygen. (3)

Beside these mentioned enzymatic mechanisms a variety of non-enzymatic molecules that act as radical interceptors exists: GSH, coeruloplasmine, ubiquinon, melatonin but also vitamin E and C. Vitamin E is liposoluble and integrated in the cell membrane where it acts as an antioxidant. On the other side, vitamin C is water soluble and may act against oxidation reactions in the plasma. In order to avoid any disturbance of the cell's metabolism, molecular repair mechanisms remove modified molecules immediately after their occurrence. (3)

1.1.4.2 Protection by enzymes

1.1.4.2.1 Catalase and Peroxidase

Catalases are able to convert H_2O_2 formed by SOD expeditiously and effectively to water and O_2 (Equation 1.2). They are localized especially in the peroxisomes and contain either manganese or a haem-group in their catalytic centre. (3;7)



Equation 1.2: Catalases are able to convert H_2O_2 educated by SOD expeditiously and effectively to water and oxygen (3;7)

GPx reduces ROOH and H_2O_2 . The reduction equivalents are provided by GSH, a non-enzymatic antioxidant. (Equation 1.3) (7;21)



Equation 1.3: GPx reduces ROOH and H_2O_2 . The reduction equivalents are provided by GSH - a non-enzymatic antioxidant. (3;7)

Greatly relevant for the capacity of GPx is the enzyme glutathione reductase (GSR) which reduces oxidized glutathione (GSSG) to GSH again in collaboration with NADPH as an important cofactor. (Figure 1.5)

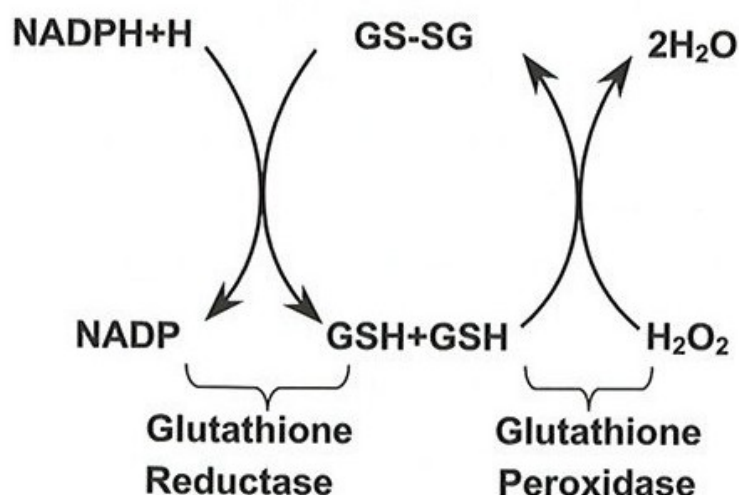
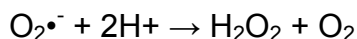


Figure 1.6: This enzyme used intramitochondrial NADPH as a source of electrons for the reduction of GSSG to GSH. (2)

1.1.4.2.2 Superoxide dismutase

In eukaryotes SOD appears in three isoforms: manganese-SOD, copper/zinc-SOD and extracellular SOD. All of these forms have the capacity to dismutate $O_2^{\bullet-}$ to H_2O_2 and O_2 . (Equation 1.4) (7;21)



Equation 1.4: SOD can dismutate $O_2^{\bullet-}$ to H_2O_2 and O_2 . (3;7)

1.1.4.3 Protection by small molecules

These so called “small” molecules can be classified into exogenous molecules that are ingested, and endogenous antioxidants that are produced by the organism itself. In general these molecules act as radical interceptors and are able to form resonance stabilizing radicals that are not capable to abstract hydrogen or to transfer electrons. (3)

1.1.4.3.1 Ascorbic acid

Ascorbic acid (vitamin C) is absorbed by ingestion, is water soluble and able to catch ROS directly. Additionally it works as a cofactor for numerous enzymes (e.g. proline and lysine hydroxylase). If one electron is emitted, a resonance stabilizing

radical anion, semidehydro ascorbic acid (MDHA), will be formed. In the case of the release of two electrons dehydro ascorbic acid (DHA) arises. (Figure 1.6)

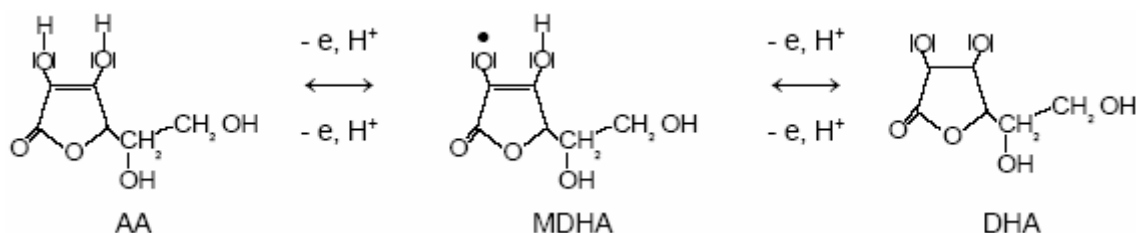


Figure 1.7: MDHA is produced by emitting an electron of ascorbic acid. In the case of two electrons DHA arises. (3)

It has been shown that vitamin C and other antioxidants show prooxidative properties in vitro as they were able to reduce Fe³⁺ to Fe²⁺ which enhanced the formation of OH• from H₂O₂. (3)

1.1.4.3.2 Tocopherol

Vitamin E is the accumulative denomination for α-, β-, γ-, δ-, ε-tocopheroles and tocotrienoles. It is liposoluble and particularly absorbed by fats and oils. The basic structure of vitamin E shows a hydroxylated chromanol ring with a lipophilic phytyl side chain. Due to different saturated side chains several families may be distinguished:

- tocopheroles with one saturated side chain
- tocotrienoles and marine tocopheroles with one monounsaturated side chain
- tocotrienoles with one threefold unsaturated side chain (22)

Besides ascorbic acid vitamin E works as a radical interceptor as well. As it is a lipophilic molecule it protects in particular membrane lipids and low density lipoproteins (LDL) from oxidation.(3;7) It is able to disrupt the lipid peroxidation and to absorb the energy of the ¹O₂'s activated electrons. This energy is emitted radiationless or ¹O₂ is caught by addition. Tocopherol radicals are regenerated by an ascorbate-dependent redox system. Like vitamin C vitamin E shows prooxidative characteristics in vitro due to its ability to reduce metal ions. (3)

Many chromophoric phenols are classified as antioxidants as they form resonance stabilizing radicals after hydrogen abstraction and alloy chelate complexes. Examples for phenols and their occurrence are listed in table 1.6.

phenols	occurrence
flavones	onion, berries, broccoli, apples, cherries, tea, red wine
flavanones	citrus fruits
flavanoles	green and black tea, red wine
catechine	apples, tea
anthocyanidine	red grapes, red wine, cherries, raspberries, strawberries
isoflavones	soya beans, legumes

Table 1.6: Examples for phenols (3)

1.1.4.3.3 Bilirubin

Bilirubin is the end product of the haem degradation and is characterized by its intensive yellow colour. In plasma bilirubin is mostly tied to albumin and has a physiological concentration of about 3-21 μM . In vitro experiments showed that bilirubin is able to catch $\text{RO}_2^\bullet$ and $^1\text{O}_2$ and has so the capacity to protect both albumin and albumin bonded fatty acids. Anyway the impact of bilirubin in vivo still requires further investigations.(3)

1.1.4.3.4 Uric acid

Due to its reduction potential (pH 7) of 0,59 V and its high plasma concentrations uric acid is known as a very effective antioxidant. Uric acid is produced by xanthin-oxidase from hypoxanthin or xanthin. It is the final product of purine degradation in humans and other higher primates. In most other mammals the enzyme uric acid is degraded by the enzyme uricase to generate allantoin. Allatonin furthermore is cleaved into urea and oxalic acid. (23)

1.1.4.3.5 Gluthathione

The thiol group of the tripeptide GSH also features an antioxidative effect. It may reduce different ROS ($^1\text{O}_2$, $\text{OH}^\bullet$, $\text{O}_2^{\bullet-}$) and is in this process reduced to GSSG. The intracellular part of GSH in the entire GSH is approximately 90 %. This ho-

meostasis is supported by GSR. A decrease of the GSH status or a GSH-depletion could be caused by oxidative stress or could cause even that.(3;13)

1.1.4.4 Protection by sequestration of metal ions

1.1.4.4.1 Metallothioneins

Metallothionein (MT) describes a family of cystein-rich proteins with a molecular weight ranging from 3.5 to 14 kDa. MTs have the capacity to bind a wide range of metals such as cadmium, mercury, zinc, copper, arsenic, silver, etc.

Cysteine rests may defang $\text{OH}\cdot$ and $\text{O}_2\cdot^-$ by binding them. In this process cysteine is reduced to cystine and the bonded metals are released. (24;25)

1.1.4.4.2 Transferrin

Transferrin is a glycoprotein that is able to catch free iron ions in plasma to inhibit their redoxactive character. Afterwards the bonded iron ions are integrated into ferritin which is a storage protein. (3)

1.1.4.4.3 Albumin

Copper ions are inactivated by binding to the plasmaprotein albumin and ceruloplasmin which is a specific transport and storage protein. (3)

1.1.4.5 Repair systems

1.1.4.5.1 Methionine sulfoxide reductase

Oxidation of free and protein-bound methionine residues to methionine sulfoxide derivatives by ROS is readily repaired by the action of methionine sulfoxide reductase (MsrA) which catalyzes the TRX-dependent reduction of free and protein-bound methionine sulfoxid to methionine.

MsrA is considered to have at least three important capacities in cellular metabolism:

1. As antioxidant enzyme that eliminates ROS by maintaining the cyclic conversion of methionine residues between their oxidized and reduced forms

2. As repair enzyme by retaining the reduced form of questionable methionine residues
3. As regulator of enzyme activity during the cyclic interconversion mentioned in 1.

The MsrA protein is highly expressed in tissues or cells that are delicate to oxidative damage such as liver, kidney, pigment epithelial cells of the retina, macrophages, cerebellum and brain neurons. (26)

1.1.4.5.2 DNA repair

ROS and RNS may induce multiple lesions in DNA such as single and double strand breaks, damage of abasic sites and modification of pyrimidines and purines. Mainly these damages are repaired by base excision repair but also nucleotide excision repair may occur. A variety of different enzymes are able to repair such lesions. DNA glycosylases can repair oxidized, methylated and deaminated DNA bases. Furthermore a system for the repair of abasic sites induced by spontaneous depurination exists. The enzyme poly-adenosindiphosphate-ribose-polymerase (PARP) is involved in the rejoining of DNA strand breaks. Additionally there exist diverse apurinic and apyrimidinic endonucleases with different specificities for the recognition of the hydrolysis of the base-sugar bond and modified abasic sites. (27)

1.1.4.5.3 Degradation of abnormal proteins

Heat shock proteins (HSP) are molecular chaperons that are expressed as a response to stress. They are part of the cellular defense system such as antioxidative defense mechanisms and the ubiquitin-proteasome-system. The aim of this system is to anticipate the accumulation of protein damages. They are considered to be required for the reparation of wrong folded proteins as well as for the proteasomale degradation of heavily damaged proteins. Carbonyl reductases and other dehydrogenases could be from high importance for the reparation of oxidized proteins and lipids. However these processes are little known so far. Apparently chaperons are responsible for the regeneration of the tertiary structure of oxidized pro-

teins. If these defense and reparation mechanisms fail cells have no other opportunity than degrade damaged proteins. The degradation of oxidized proteins is mainly performed by the proteasome complex. (3;28)

1.1.5 „free radical theory of aging“

The “free radical theory of aging”, advanced by Harman, says that the formation of intracellular oxygen species is the most important limiting factor for life expectancy. Aging is characterized by decreased activity of various enzymes and accumulation of mutations of nuclear and mitochondrial DNA. These properties can be verified in many postmitotic tissues such as the brain. Oxygen radicals are more and more associated with this topic. Comparative studies with animals of different aging rates show a correlation between ROS formed in mitochondria and the detected damages in mitochondrial DNA. These facts in turn are related with life expectancy. Furthermore diets that reduce aging, deplete the formation of mitochondrial DNA proportionally. In contrast fewer findings about the ROS’ relevant cellular contact points and how their oxidative modifications may influence the life expectancy are known so far. As ROS are currently considered to play an important role in aging of the brain a lot of studies concentrate on the relevance of ROS for nerve cells and neural degeneration. (29;30)

1.1.6 Damage of Proteins, Lipids and DNA

1.1.6.1 Lipidperoxidation (LPO)

1.1.6.1.1 General considerations

Lipids are an essential element of cellular membranes where strong impairments could have pathological consequences. As they contain numerous double bonds with high reactive methylene groups mostly polyunsaturated fatty acids are peroxidized. This process is a three-phase chain reaction that consists of initiation, propagation and termination. (Figure 1.7) (20)

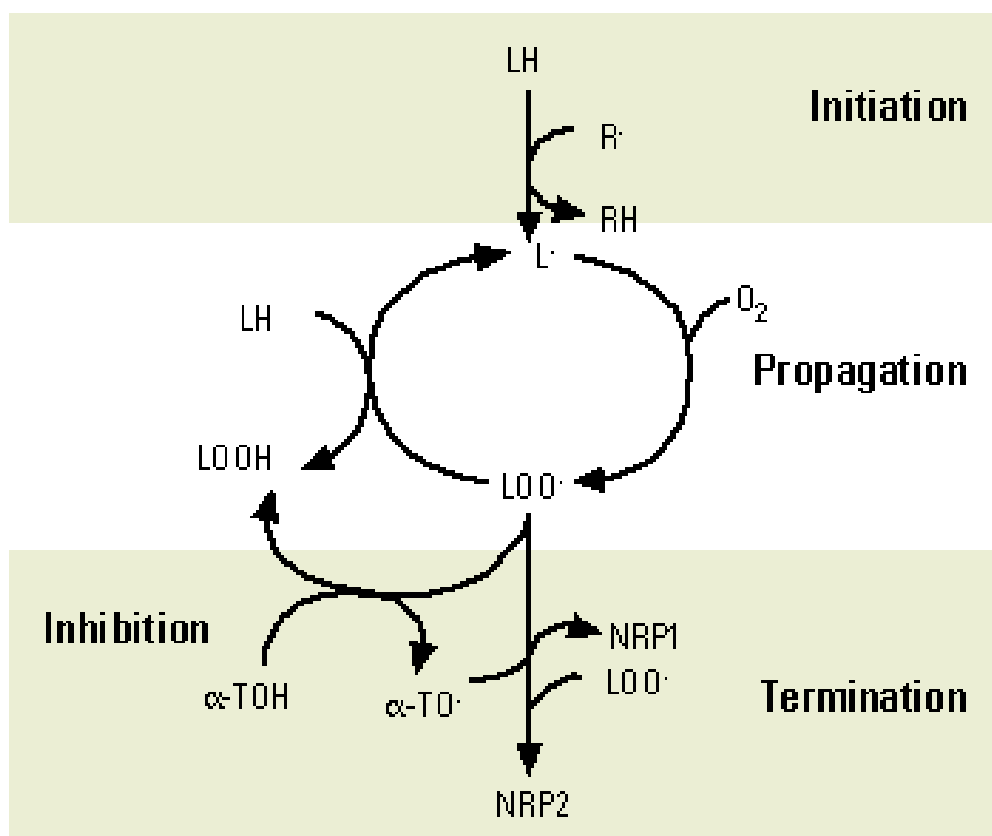


Figure 1.8: A schematic diagram of lipid peroxidation. Abbreviations: NRP: nonradical product; LOOH: lipid hydroperoxide; $\alpha\text{-}TOH$: α -tocopherol; $\alpha\text{-}TO\cdot$: α -TOH radical; LH: lipid substrate; $LOO\cdot$: lipid peroxy radical. (31)

In LPOs without enzymatic involvement a free radical raises enough energy to split off a hydrogen atom from a carbon atom of an unsaturated fatty acid. In this process a lipid radical ($L\cdot$) is released that subsequently reacts with molecular oxygen so that a lipid peroxy radical ($LOO\cdot$) is formed. This radical splits off a hydrogen atom of an unsaturated fatty acid and so a second $L\cdot$ occurs. At the same time the $LOO\cdot$ is converted into LOOH. This molecule has the capacity to release further chain reactions. As soon as two radicals react with each other the chain reaction is stopped. (3;13;20)

In the course of LPO chemically instable LOOHs (Figure 1.8) are formed that dissociate to alkanes, ketones, furans, $RO\cdot$, alcohol, aliphatic and unsaturated aldehydes such as MDA and 4-hydroxynonenal (4-HNE). (2;3)

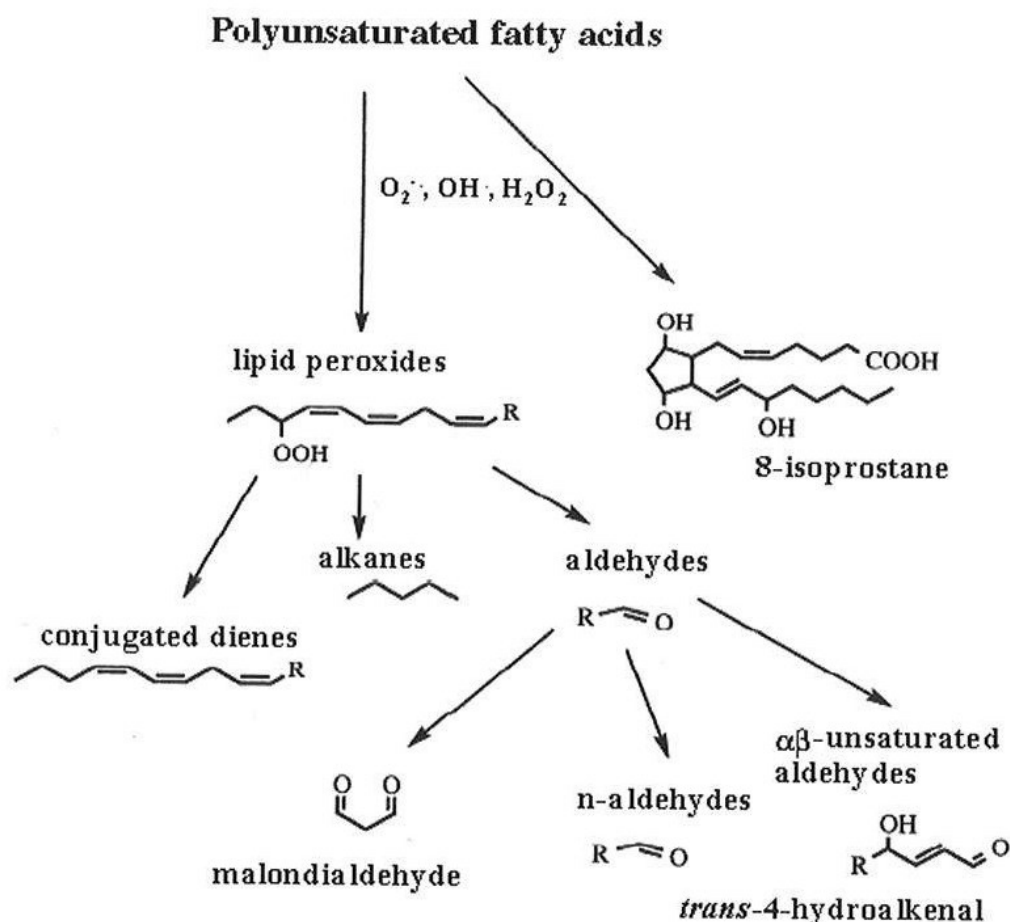


Figure 1.9: Intermediate and end-products resulted from lipid peroxidation (2)

LOOH reacts with a lot of different molecules. For example it is able to penetrate the cell membrane and to react with nuclear acids. Moreover it may change the chemical and physical properties of the cell membrane so that its barrier function is disturbed. Further, intracellular potassium and enzymes are released. Affection of erythrocytes by LOOH may lead to hemolysis. The released haemoglobin can activate the mentioned three-phase chain reaction again. (3)

1.1.6.1.2 Markers for LPO

Most frequently unsaturated linoleic acid and arachidonic acid are oxidized. Their end products respectively secondary LPO products are 4-HNE (Figure 1.9), MDA, isoprostanes, conjugated diene, pentane and ethane. (32)

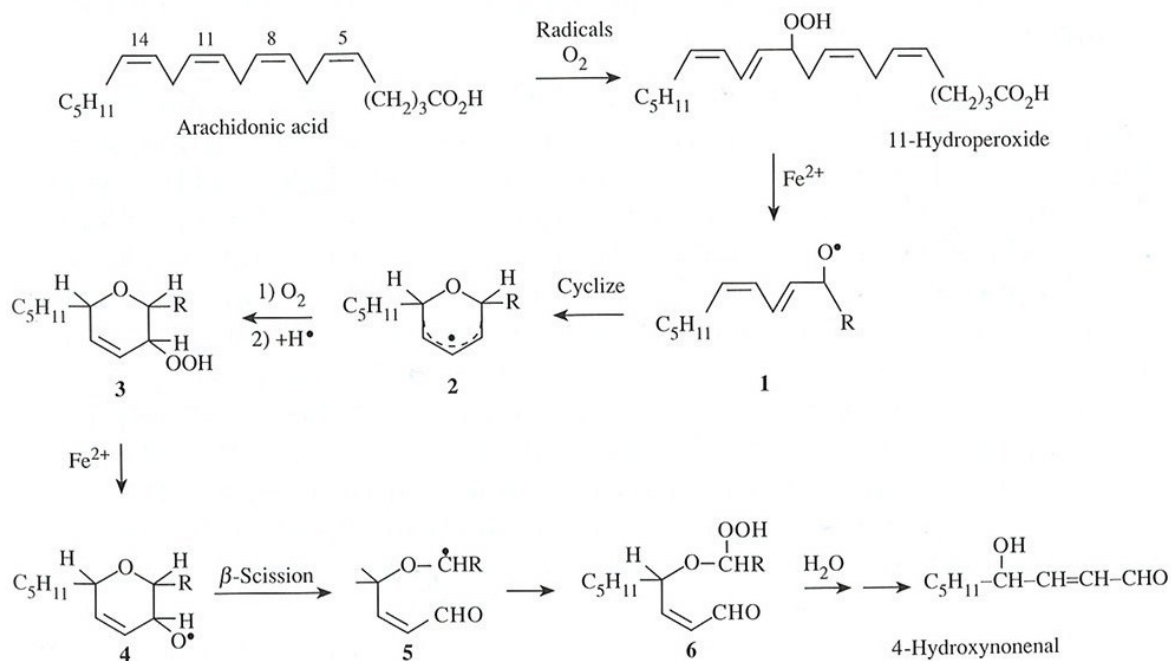


Figure 1.10: A diagram for the production of 4-HNE from the peroxidation of arachidonic acid. The mechanism involves a cyclization to form a six-membered ring hydroperoxide, which undergoes further reactions to produce 4-HNE. (3)

Commonly MDA and other aliphatic aldehydes are used as markers for LPO. They are thiobarbituric acid reactive substances (TBARS) and therefore they can be determined photometrically after reaction with thiobarbituric acid. A specific detection of MDA is possible by chromatographic separation of TBARS by high performance liquid chromatography (HPLC) and adjacent fluorescence detection. (32) Another suitable marker for LPO is the isoprostane 8-epi-PGF $_{2\alpha}$. (Figure 1.10) It is formed during oxidation of arachidonic acid and is stereochemically related to the prostaglandin PGF $_{2\alpha}$. (3)

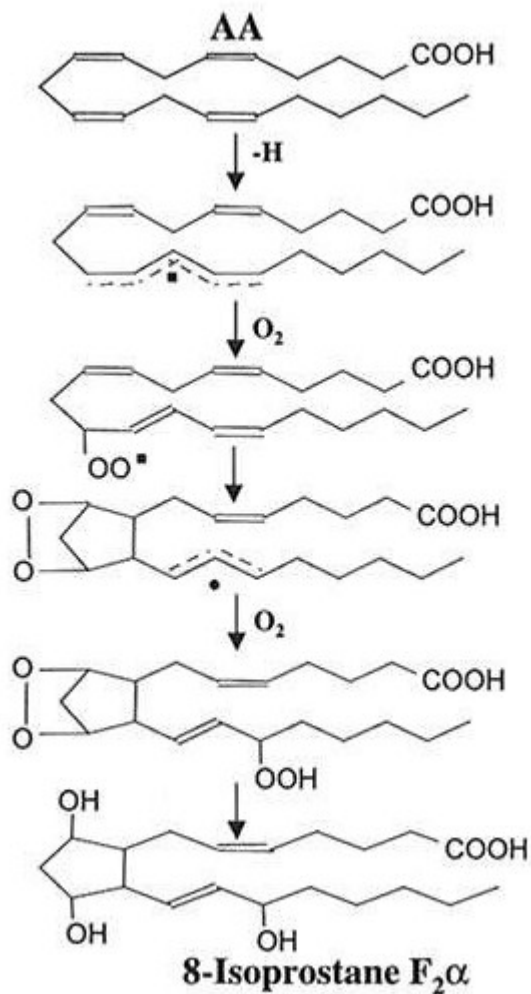


*Figure 1.11: Formation of 8-isoprostane $F_2\alpha$ - Peroxidation of arachidonic acid.
(2)*

1.1.6.2 Protein oxidation

1.1.6.2.1 General considerations

Protein oxidation directly affects the capacity of different receptors, antibodies, enzymes, transport proteins and signal transmitting mechanisms.

A variety of factors may cause an increase of protein oxidation (Table 1.7): (33;34)

activation of neutrophil granulocytes	chronic alcohol consume
increased O ₂ partial pressure	ischemia
magnesium deficiency	nicotine inhalation
ozone pollution	reperfusion
X-ray radiation	overexertion
all too fast correction of hypernatraemia	

Table 1.7: A variety of factors may cause an increase of protein oxidation (33;34)

Damage of DNA reparation enzymes and DNA polymerases can lead to the production of antigens in a following immune response. Thus, they are able to induce an increase of protein oxidation too. The main responsible molecule for protein oxidation is OH•, the most reactive ROS which is able to split peptide bonds. (35;36) Other points of action for ROS are amino acids. Mostly concerned are tyrosine, phenylalanine (Figure 1.11) and tryptophan, but also methionine that is oxidized to methionine sulfoxide, histidine that is oxidized to aspartat and asparagin, and leucin that is oxidized to hydroxy leucin. (36-38)

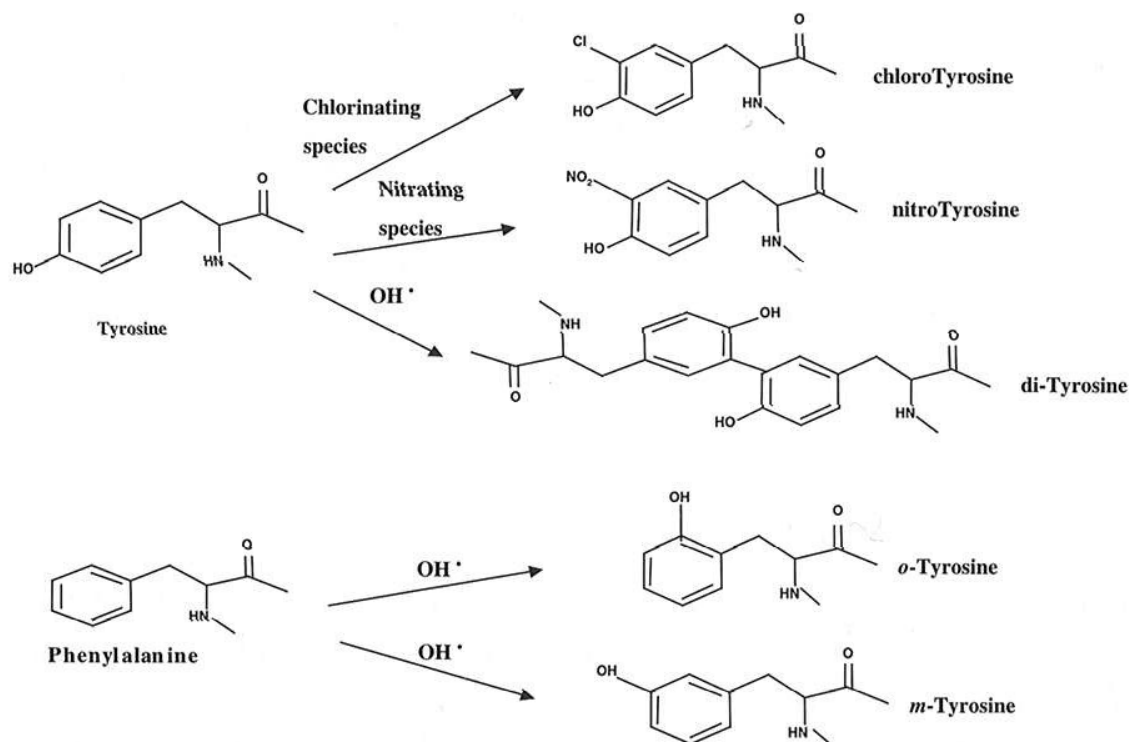


Figure 1.12: Formation of oxidation products from tyrosine and phenylalanine by ROS. (2)

1.1.6.2.2 Markers for Protein oxidation

In the course of the reaction of proteins with ROS protein derivatives and peptide fragments may be formed that feature an extremely reactive carbonyl group. (39) Especially proline, arginine, lysine and histidine are converted to carbonyl derivatives. These carbonylated proteins (CP) can be detected by enzyme linked immunosorbent assay (ELISA). (40;41)

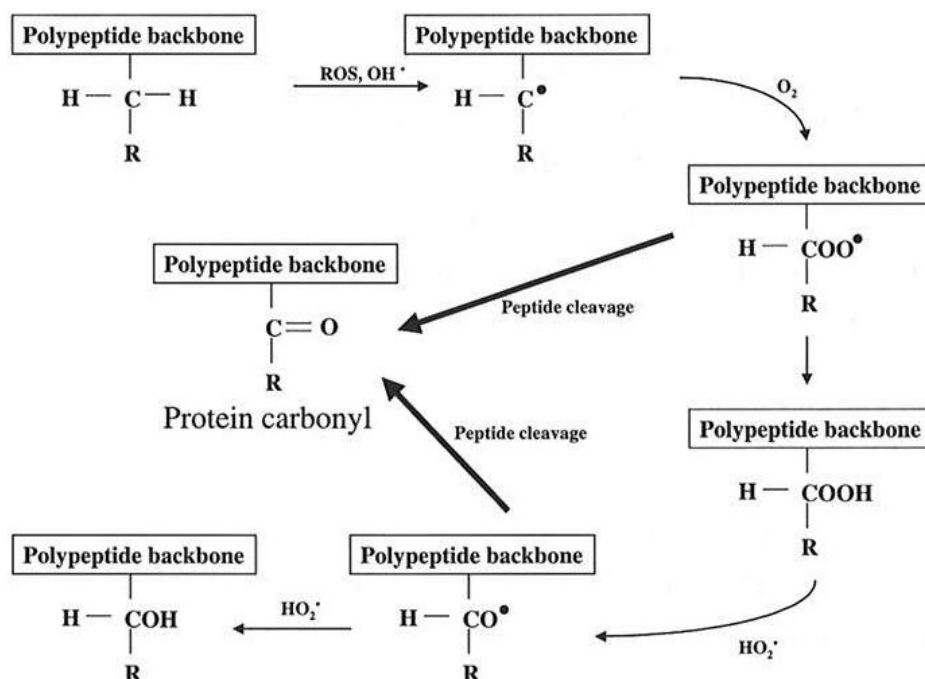


Figure 1.13: Protein carbonyl formation after peptide cleavage and ROS attack. (2)

1.1.6.3 DNA-damage

1.1.6.3.1 General considerations

DNA damages are mainly caused by $\text{OH}^\bullet$. $\text{ROO}^\bullet$ and H_2O_2 on the other hand do not directly react with DNA. $\text{OH}^\bullet$ may affect the bases whereby hydroxylations, ring degradation and fragmentations occur. (42;43) Furthermore the sugar-phosphat-framework could be damaged which leads to DNA strand breaks.

As mentioned above ROS are considered to play important roles in the accrue-ment of DNA damages. (7;44) The oxidation of DNA may be enhanced by in-creased formation of ROS as well as by decreased antioxidant defense and inhibi-tion of mechanisms that repair or replace damaged or changed DNA sections. (3;44)

In the course of oxidative damaging different oxidation products (Figure 1.13a) are formed that can be used as markers for the determination of the extent of oxidative stress. For instance the purine guanine is oxidized to 8-oxo-guanin and further to 2,6-diamino-4hydroxy-5-formamidopyrimidine (FaPy) (Figure 1.13b). Pyrimidines

may be converted to oxidation products as well as to base dimers after being attacked by ROS. (3;13)

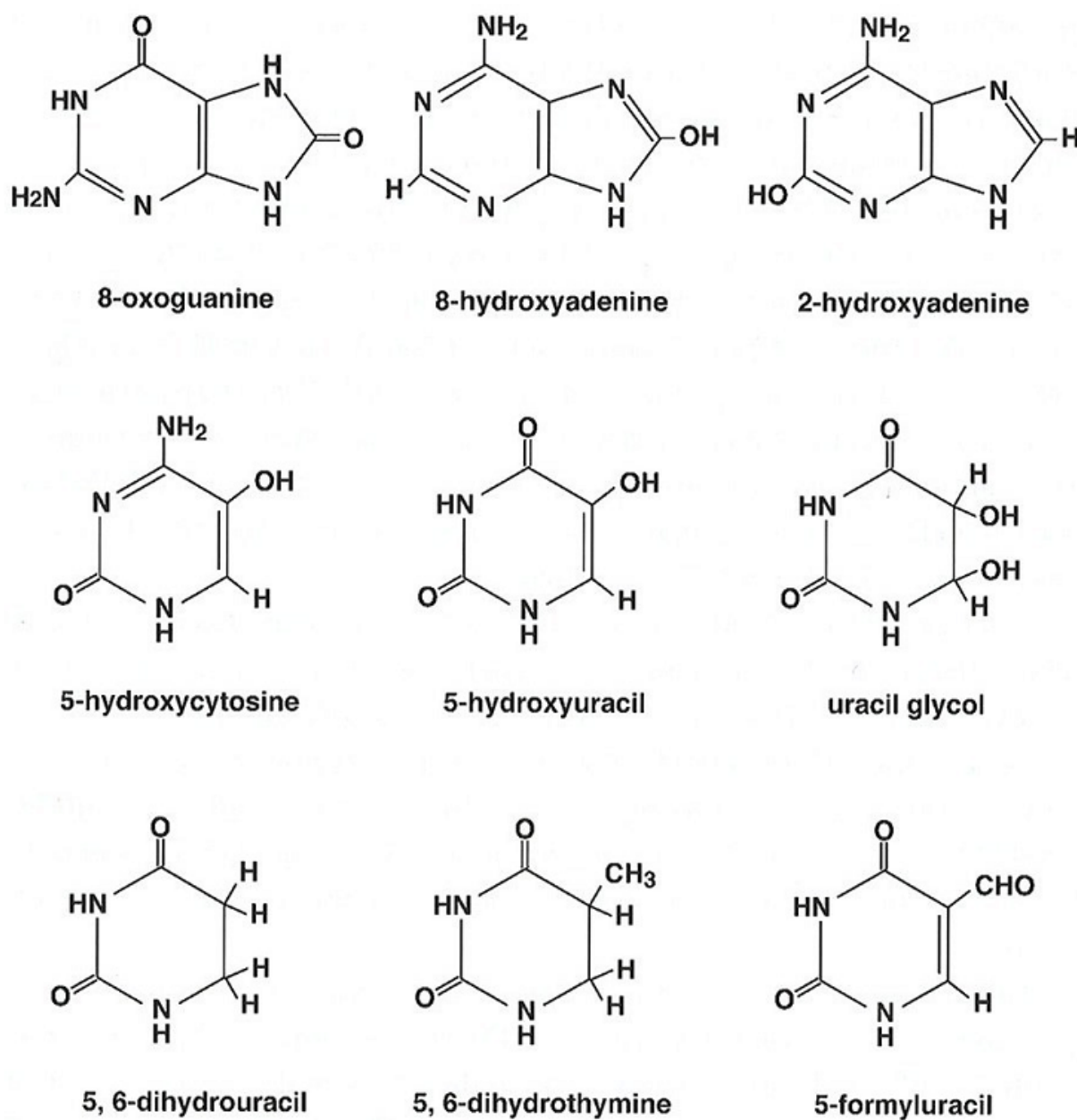


Figure 1.14a: A diagram of oxidized purines and pyrimidines. Deoxyribonucleoside-5'-triphosphate derivatives of these modified bases can be implemented into DNA by DNA polymerases. (2)

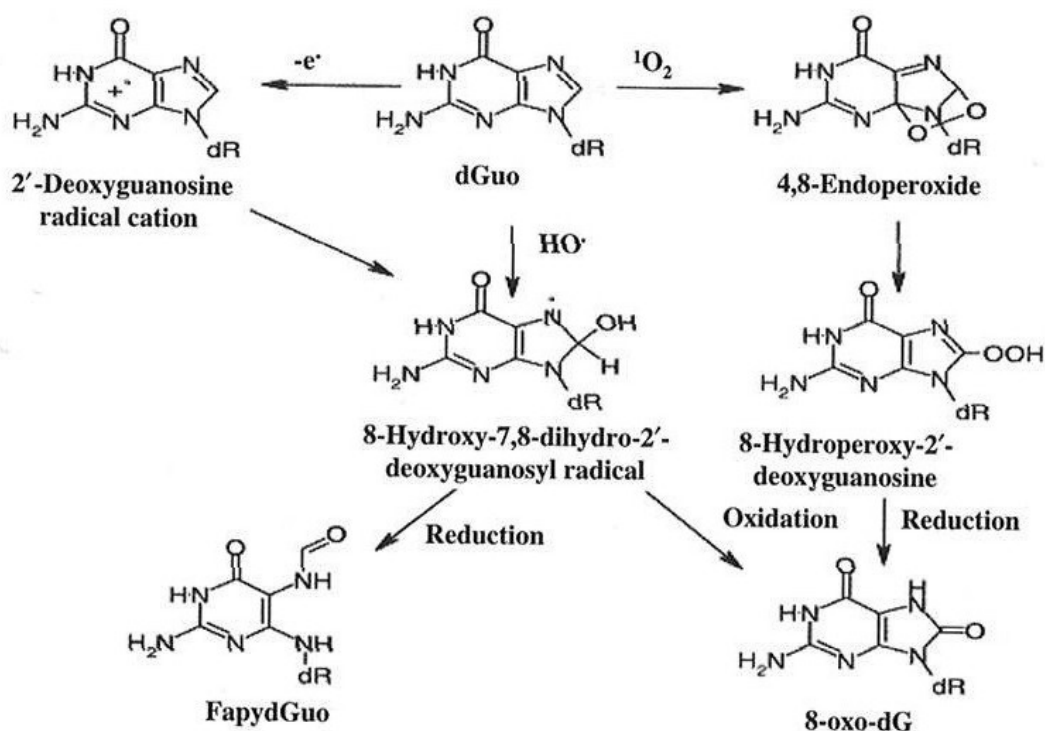


Figure 1.14b: The formation of 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy) by ROS. (2)

All these kinds of DNA degradation initiate the onset of a repair cascade. If the damages are not discovered or repaired in the run of replication, bases may be paired wrong which can lead to further mutations. (3;13)

1.1.6.3.2 Markers for DNA-damage

Changes or damages in DNA are generally measured by the extent of DNA strand breaks, so for example bromdesoxiuridine can be incorporated. Oxidized purines or pyrimidines, such as 8-hydroxy-2'-deoxyguanosine (8-oxo-dG), can be detected by HPLC. (36;45;46))

Furthermore DNA damages in single cells can be determined by the so called Comet Assay. In this method DNA fragments migrate in an electric field, whereas long fragments migrate in a more slowly way than short ones. Intact DNA doesn't migrate anyway. As the DNA fragments migrate differently so called comet forms

are formed that can be detected with a microscope. Because of these special forms, DNA breaks can be detected. (17;45;46)

1.2 Radiofrequency Ablation (RFA)

1.2.1 General Considerations

Radiofrequency ablation (RFA) is a minimal electrosurgical invasive technique for the treatment of focal malignant disease implicitly supported under image guidance such as computed tomography (CT) or ultrasound.(47;48) The first experiments to generate heat in liver, was performed by d'Arsonval in 1868. He applied an alternating current with a frequency of 10 kHz in liver in the absence of creating neuromuscular reactions.(49) RFA of liver tumours in humans was first described by Rossi et al. in 1993.(50) Now it has become clinically accepted as a surgical treatment technique for malignant hepatic tumours.(51;52)

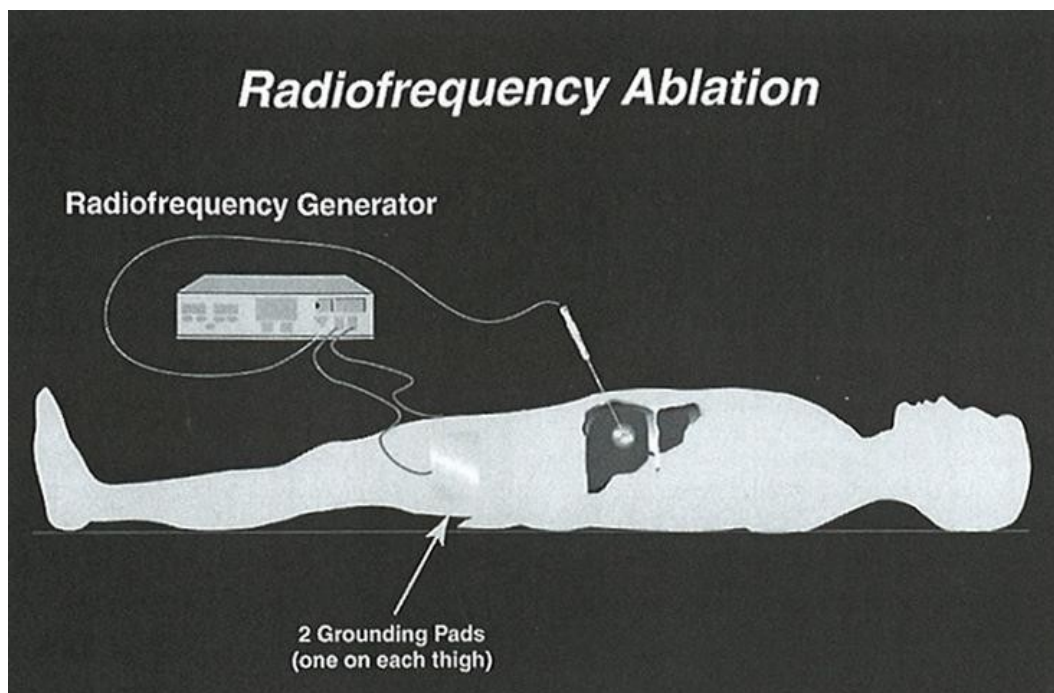


Fig. 1.15: A RFA needle electrode with the multiple array is deployed in a liver lesion. The alternating RFA current moves between the electrode and the grounding pads, which are attached onto the patient. Frictional heating, which is caused by the ionic stimulation, results in coagulative necrosis of the ablated area.(53)

1.2.2 Physical Background

RFA converts radio waves into heat. A high frequency alternating signal (with a frequency range of 100 – 500 kHz) is going through an unisolated needle electrode into the surrounding tissue and induces movement of the ions. These ions

try to follow the fast alternating signal, thereby resulting in frictional heating of the tissue around the electrode. The aim of the arising temperature is denaturing and killing the tissue.(53;54) When the temperature exceeds 45 – 50°C, cell membranes are destroyed and intracellular proteins are denatured through melting and dissolution of lipid bilayers.(55;56)

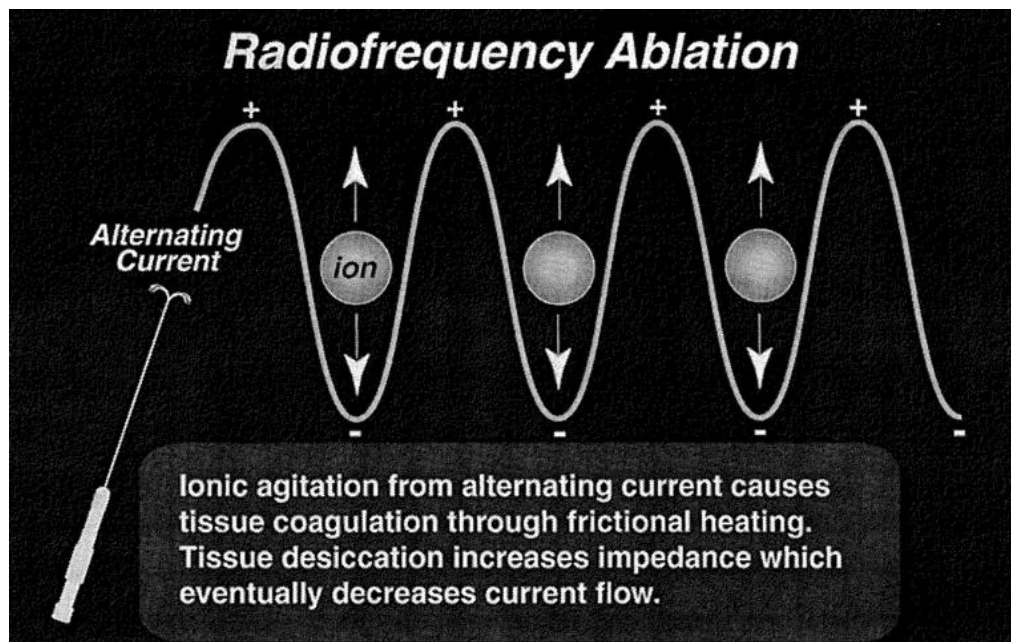


Fig. 1.16: An electrical current moves from the tip of the electrode into surrounding tissue and causes ionic agitation and heating.(53)

The aim is to use temperatures that suffice to denature and destroy the tissue.(53;57) As an additional effect the heating of the cells leads to a discharge of intra- and extracellular water out of the tissue what accelerates necrosis. Further experiments showed that hyperthermia increases the migration of monocytes in the dying tissue. The following secretion of cytokines and shock-proteins may involve apoptosis. (53;54) The extent of the caused necrosis by RFA depends on the reached temperature in the tissue and the duration of the treatment. (47;57)

1.2.3 Indication, planning and implementation

The indication for the implementation of a thermo ablation is similar to that for a surgical intervention. A few authors delimitate the application of RFA to the absence of extrahepatic metastases. If they are located near the portal veins or bile ducts metastases may be problematic due to the risk of damaging these structures

and accrue ment of thromboses. Furthermore a subcapsular localisation leads to an increased pain symptomatology. Another limiting factor is the lesion's dimension. (53;57)

Currently there exist electrodes up to a diameter of 5 cm. In the case of larger lesions the application can be effected overlapping. Primary hepatocellular carcinomas up to a size of 11 cm have been treated under temporal blockade of liver veins. (53)

Ideally the lesion does not excess a size of 3 cm in diameter and is located at least 1 cm subcapsular and 2 cm afar from the bile ducts and portal veins. In addition there should be kept a safety margin of 1-2 cm to the adjacent thrombosis endangered organs (stomach, duodenum, gall bladder, colon and diaphragm). If this is not possible a dissociation of these organs by carbon dioxide (CO₂), air or sodium chloride solution in order to avoid any perforation is recommended. Furthermore general requirements such as an intact coagulation and absence of infections have to be fulfilled. (53;57)

As part of the planning of the RFA a safety margin of 1 cm around the lesion should be considered, what is comparable with the surgical procedure. That means that in the case of a metastasis with a diameter of 3 cm a necrosis with the size of 4 cm should be aspired. The position of the probe may be monitored by ultrasound. This method is the cheaper and easier alternative to a CT that allows a more accurate positioning of the needle and an examination of the success of the treatment immediately after removing the probe.

The electrode is placed in the tumour using the Seldinger-technique and is heated by a generator. (53;58)

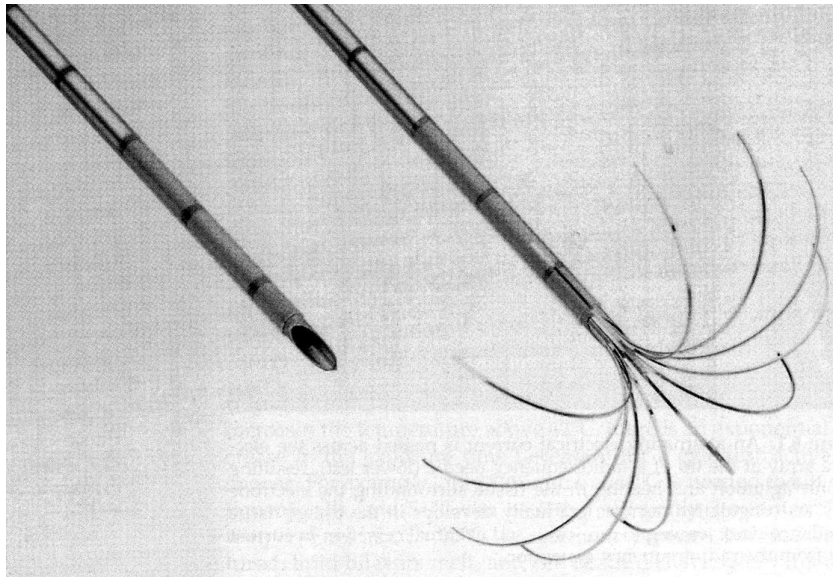


Fig. 1.17: RFA needle electrode (left) pictured with the multiple array retracted into the needle sheath. RFA needle electrode (right) with fully extended multiple array to ablate larger areas.(53)

Ideally temperatures of about 80-90°C should be achieved. From a temperature of approximately 50°C necrosis starts to set in. Limiting factors for the heat's development are particularly adjacent taller vessels. Vessels with a diameter up to 3 mm are coagulated during RFA whereas bigger ones are still integrated. The remaining blood flow is considered to have a cooling effect to the surrounding tissue. So the required temperatures cannot be attained and tumour sections may remain in the tissue. Studies showed that such leavings occur cumulative in the surrounding area of taller vessels. Therefore experiments have been conducted where this cooling effect was decreased by reducing the blood flow by medications (Halothan, Vasopressin, Epinephrin) or by interlocking the afferent vessels. (47;57)

As mentioned above subcapsular ablations may cause considerable pain. But also lesions located close to the portal vein or subphrenic can be extremely painful. Therefore an adequate pain management plays an important role. Interventions that do not require apnoea can be done under analgosedation. This application has to be carried out under permanent monitoring of the vital functions. In the case of more complex interventions, an endotracheal intubation with general anaesthe-

sia should be preferred due to the possibility to regulate the diaphragm's actions. Even hours after the ablation strong pain symptoms may occur what makes an analgetic after-treatment indispensable. (59)

1.2.4 Complications

Although RFA shows various advantages also some disadvantages and complications have to be considered. In particular local pain and fever should be mentioned in this context.

Further RFA associated complications are hemorrhage, biliary stricture or leak, subcapsular hematoma, liver dysfunction, abscess formation, pleural effusion, fever, pain, subcutaneous hematoma and ventricular fibrillation.

A further specific problem is the correct positioning of the needle what requires several experiences and a sensitive image-guided method for real-time documentation. A result of a wrong positioned needle may be a relapse in the adjacent tissue. In contrast to primary tumours the portal venous blood flow in the liver parenchyma may additionally complicate the heating of the tissue. (53;59;60)

1.2.5 Follow-up

In order to exclude complications (abscesses, thromboses, haematoma, etc.) and to approve the treatment's success a magnetic resonance tomography (MRT) or a CT should be performed within one week after RFA. A control via ultrasound is rather not recommended.

Furthermore an examination within the first 48 hours after the treatment as well as after three, six, nine and twelve months will be advised. Unless there does not occur any relapse within this time further controls every six months are considered to be sufficient. Moreover the tumour markers in serum should be checked at regular intervals. (59;60)

2 AIM OF THE STUDY

The aim of this study was to perform a CT-guided RFA on the liver of five pigs and ascertain whether this therapy option leads to an increased production of markers for oxidative stress induced cell damage. As pigs feature rather similar biological conditions to humans they represent adequate human scale models. For the investigation of new therapy options and the improvement of already existing therapy forms like RFA, animal models are of major importance. RFA is a minimally invasive form of cancer treatment that does not require open surgery.

Two of the five pigs were sacrificed immediately after RFA, whereby for the three remaining pigs different survival times were determined. One of them survived for two weeks, one for two months and the last one for three months after the treatment. The different survival times were chosen to find out whether these periods influence the occurrence of oxidative stress induced cell damage or not.

In order to exhibit the extent of oxidative damage as effectively as possible appropriate markers were selected. Therefore immunohistochemical stainings of the liver tissue for HSP70 and nitrotyrosine as well as an investigation of the porcine serum for nitrotyrosine and CPs were performed.

3 MATERIALS AND METHODS

3.1 Overview

The study is part of the IMPPACT project and has received funding from the European Community's Seventh Framework Programme under grant agreement no.223877. The European research project IMPPACT is taking place from September 2008 to August 2011 and develops an intervention planning system for RFA of liver tumours. The experiment is accomplished according to the guidelines of the Austrian animal protection law. All experiments, procedures and clinical research have been performed at the Medical University of Graz. All Pigs have been kept in stables at the Institute for Biomedical Research and were transported to the operation room of the department for surgical research for vena-cava-implantation or for the final sacrifice. Pigs were also transported to the paediatric clinic for CT-guided RFA.

3.2 Pigs

For the study five domestic pigs were used that were acquired from Styrian pig breeders. They have been kept in single or double boxes depending on their size. Twice a day pigs were fed with the usual diet and continuous access to water was provided. When the experiment started, animals had an average weight of 42 kg. Two pigs were sacrificed immediately after RFA, whereby for the three remaining pigs different survival times were determined. One of them survived for two weeks, one for two months and the last one for three months after the treatment. The livers were removed for histological staining. Tissues were examined by immunohistochemistry for HSP70 and nitrotyrosine.

3.3 RFA under computer-assisted tomography

3.3.1 Sedation for RFA

Pigs were transferred to the operation room of the facility for surgical research for premedication and intubation. Animals were premedicated with 1 mg/kg midazolam (ERWO Pharma GmbH, Brunn am Gebirge, Austria), 10 mg/kg ketamin (Ketasol®, aniMedica GmbH, Senden – Bösensell, Germany) and 2 mg/kg azaperon (Stresnil®, Janssen – Cilag Pharma, Vienna, Austria). To maintain a continuous general anaesthesia and to infuse saline solution for managing the fluid balance an i.v. cannula (TriCath®, CODAN Medizinische Geräte GmbH, Lensahn, Germany) was installed into an ear vein. Animals were intubated with a 8,0 spiral tubus (Willy Rüschi GmbH, Kernen, Germany) and ventilated with an mobile ventilator (Oxylog® 2000, Dräger Medical, Best, Netherlands). A continuous sedation during the whole procedure was maintained by a sustainable application of 5 – 10 mg/kg/h propofol (Diprivan®, Astra Zeneca, Vienna, Austria) through a syringe pump (Perfusor® F, Braun Melsungen AG, Melsungen, Germany).

3.3.2 Vena-cava-catheter – Implantation for RFA

After sedation and intubation a vena-cava-catheter was implanted to simplify blood sampling. Further, pigs were placed on the back onto the operating table. To ensure an adequate analgesia during the procedure 30 µg/kg fentanyl (Janssen – Cilag Pharma, Vienna, Austria) per hour was applied through the ear vein by a syringe pump (Perfusor® F, Braun Melsungen AG, Melsungen, Germany). After sterile ablation of the operation area and sterile covering of adjoining areas an incision at the ventral side of the neck was made. Afterwards the vena jugularis dextra was explored to insert the vena-cava-catheter. Finally the operation wound was treated.

3.3.3 Implementation of RFA

The RFA was accomplished at the pediatric clinic of the Medical University Graz which provided a dynamic volume CT scanner (Aquilion ONE ®, Toshiba Medical Systems, Japan).

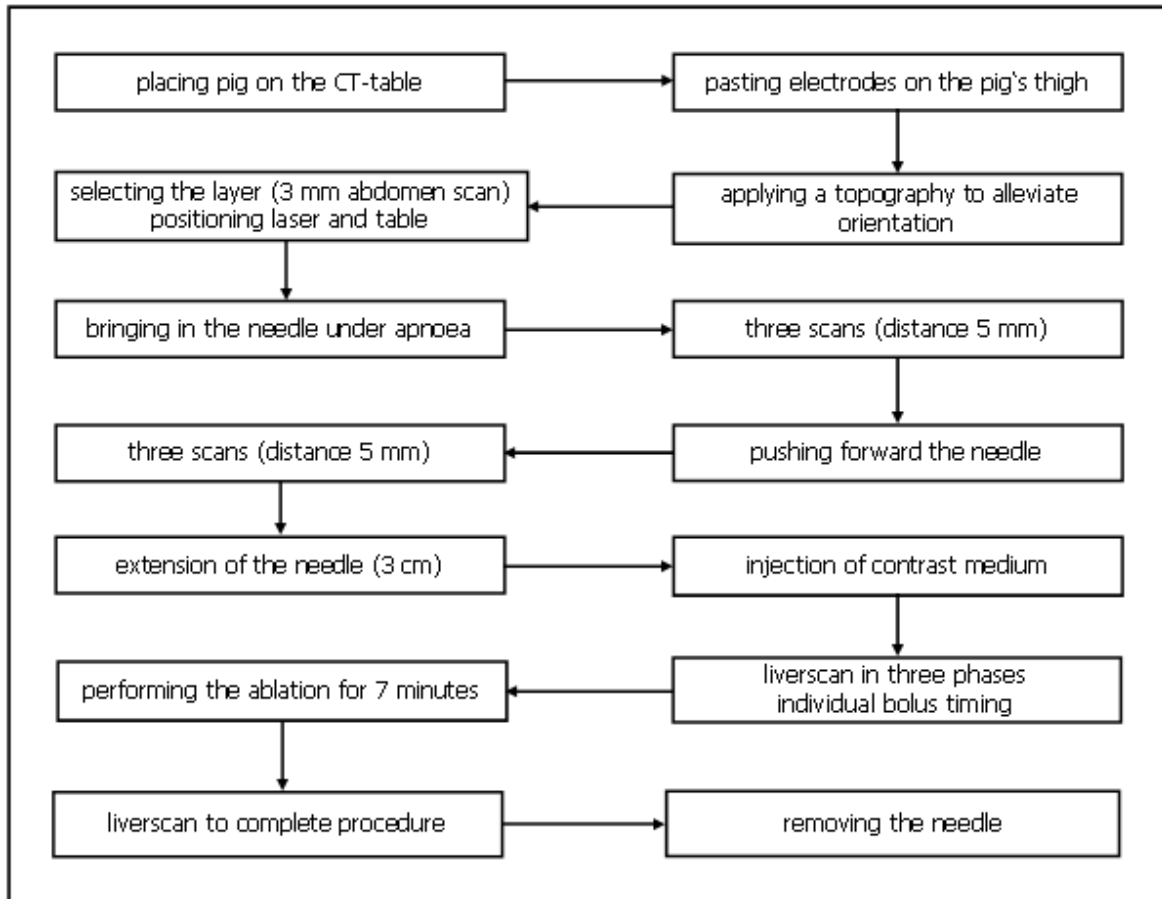


Table 3.1: Procedure of computed tomography-guided RFA.

After placing the anesthetized pig on the CT-table two electrodes (Rita Dispersive Electrode, AngioDynamics, Latham, USA) were pasted on the shaved thigh of the pig. To get an overview and to simplify orientation a topographic CT scan was performed. For selecting the layer a 3 mm abdomen scan was made and the laser and table were positioned to this selected layer before the pig's skin was scarified with a scalpel. Afterwards apnoea was induced by switching of the ventilator and the needle (Rita StarBurst XL, AngioDynamics, Latham, USA) was slightly inserted into the liver through the skin. Subsequently three scans with a distance of 5 mm around the selected position were made (-5 mm, 0 mm, +5 mm) under apnoea. Then the needle was pushed deeper into the liver. To examine the position three

scans with a distance of 5 mm were made again. When the aspired position was achieved the needle was extended 3 cm. After injection of 2,5 ml/kg of the contrast medium Iopamidol (Jopamiro® 300mg, Bracco Austria, Vienna, Austria) a scan of the liver in three phases was performed under apnoea from the selected region of interest (ROI) around the aorta. These three phases were the arterial, portal venous and hepatic venous phase. The bolus timing was individual (about 5-15-50). Then ablation was performed for 7 minutes via Rita ModelX RF Generator whereby the ablation countdown started as soon as the target temperature of 100 °C was achieved. After finishing the procedure the needle was removed and if required a second ablation was performed. Finally a liver scan was made to complete the treatment. For editing and interpreting the CT-scans a 3D workstation (Vitrea®, Toshiba Medical Systems, Japan) was used.

After RFA one group of pigs was sacrificed through an intravenous injection of 100 mg/kg phenobarbital (Nembutal®, Sanofi Ceva, Düsseldorf, Germany) by a professional veterinarian, the remaining pigs survived for a determined time.

3.4 Blood sampling

3.4.1 Blood sampling via vena-cava-catheter

In this study pigs were equipped with a vena-cava-catheter for collecting blood and for administration of sedative substances during RFA. The vena-cava-catheter was connected to a Luer adapter (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria), which was screwed into a standard tube holder (Vacuette®, Greiner Bio-One GmbH, Kremsmünster, Austria). Furthermore, required Vacuette® tubes were put into the tube holder to fill them with blood. Blood sampling was carried out for 3 times:

1. before the first RFA
2. during the first and the second RFA
3. after the second RFA

3.4.2 Blood sampling via vena jugularis

The blood collection on pigs that were not implemented with a vena-cava-catheter was performed by direct puncture of the jugular vein. Therefore the pigs were sedated and placed on their back on the CT-table. To perpetuate an appropriate position, the front legs were localized in a caudal direction. Before puncturing, the decided area was accurately disinfected.

An imagined line between the manubrium sterni and a shoulder articulation helps to find the suitable spot for puncture. The procedure was performed with a single use syringe (Braun, Melsungen AG, Melsungen, Germany), attached to a Strauss cannula (Dispomed Witt oHG, Gelnhausen, Germany), containing 5 ml 9 % saline solution to simplify continuous aspiration. The cannula was implemented medio-caudal. After puncturing the vena jugularis the syringe was substituted by a Luer adapter (Vacuette ®, Greiner Bio-One GmbH, Kremsmünster, Austria) plugged to a standard tube holder (Vacuette ®, Greiner Bio-One GmbH, Kremsmünster, Austria). Subsequently the blood tubes were filled with blood by attaching them into the standard tube holder. After finishing the blood collection the cannula was dislodged and to stop hemorrhage the vessel was compressed for a few minutes.

3.5 Detecting methods for oxidative stress

3.5.1 CP ELISA

Determination of CPs was performed with a CP ELISA (Immundiagnostik AG, Bensheim, Germany). The ELISA Kit contains the following items:

- 96-Well-Plate (= 12 x 8-Well-Strips)
- Wash buffer concentrate 10x
- Standard stock solution
- Control, positive
- Conjugate, peroxidase-labeled
- Antibody
- Antibody dilution buffer

- Derivatization reagent
- Assay buffer
- TMB substrate
- Stop solution

3.5.1.1 Test procedure

All reagents and samples were adapted to room temperature (18-26 °C). The analysis of standards, controls and samples was performed in duplicates.

First of all the samples were derivatized with dinitrophenylhydrazine (DNPH) that reacts with the contained proteins. Therefore 4 µl of each standard, control, assay buffer (blank) and samples were added in a corresponding centrifugal filtration tube (Sartorius Stedim Biotech GmbH, Göttingen, Germany) containing 80 µl of DNPH and were incubated for 45 min at room temperature. In a following centrifugation (15 min at 11000 x g) in a table centrifuge (Heraeus Biofuge pico, Kendor Laboratory Products, Hanau, Germany) unconjugated non-protein constituents were separated. After centrifugation 60 µl of Assay buffer were added into the tube to wash bounded proteins before centrifugation was repeated. All in all, this step was performed four times. Afterwards 60 µl of Assay buffer were added again and standard, control and samples were diluted three times in assay buffer as following: 1:4, 1:20 and 1:100. The second dilution (1:20) was used for the determination of the total protein content. The standard was diluted four times in 1:2 steps after the first dilution to achieve five standards altogether which were diluted then 1:20 and 1:100. Afterwards 200 µl of each diluted standard, control, blank and samples were added into the corresponding well of the microtiter plate and incubated for 3 hours at 37 °C or over night at 2-8 °C. After washing the plate five times with 250 µl of wash buffer, 200 µl of the antibody (anti-DNPH-antibody) diluted 1:100 in antibody dilution buffer were applied to each well of the plate followed by an incubation of 20 min at room temperature. Then the plate was washed again and 200 µl of conjugate were added in all wells. After incubation for 20 min at room temperature another washing cycle followed. Finally 200 µl of TMB-substrate were added and after reacting for 5-10 min at room temperature in the dark the reaction was stopped by adding 50 µl of stop solution. The determination was done imme-

diately at 450 nm against 620 nm with a microtiter plate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA).

The CP content has to be related to the total protein content of the sample. Therefore a parallel determination of the protein content was necessary, which was performed with the BCA Protein Assay Kit according to Pierce (Thermo Scientific, Rockford, IL, USA) as following: The working solution was prepared by diluting solution B 1+50 with solution A. Afterwards preparation of the standards followed. Therefore the included BSA standard (2 mg/ml) was diluted 1:20 in PBS to a concentration of 100 µg/ml (standard 1). This standard then was diluted in 1:2 steps to achieve five standards altogether. Subsequently 50 µl of standards and samples were pipetted into the corresponding well of an empty microtiter plate and 200 µl working solution were added. After an incubation of 2 hours at 37 °C the extinction was determined at 562 nm.

The CP content was then calculated according to the formula mentioned below:

$$\text{CP}_{\text{Sample}} [\text{pmol/mg}] \text{ standardized} = \frac{\text{CP}_{\text{Sample}} [\text{pmol/mg}] \times \text{Proteins}_{\text{Standard}} [\text{mg/ml}]}{\text{Proteins}_{\text{Sample}} [\text{mg/ml}]}$$

3.5.2 Nitrotyrosine ELISA

For determination of nitrotyrosine the nitrotyrosine ELISA (Immundiagnostik, Bensheim, Germany) was used. The ELISA Kit contains:

- Microtiter plate, precoated
- ELISA wash concentrate 10x
- HRP-antibody (concentrate)
- Assay buffer
- Standards, lyophilized (reconstitute with 500 µl of Assay buffer)
- Positive control (reconstitute with 500 µl of Assay buffer)

- Negative control (reconstitute with 500 µl of Assay buffer)
- TMB substrate (Tetramethylbenzidine)
- Stop solution

3.5.2.1 Test procedure

All reagents and samples were adapted to room temperature (18-26 °C). Samples were diluted 1:5 with Assay buffer. The analysis of standards, controls and samples was performed in duplicates.

The microtiter plate was washed five times with 250 µl of diluted wash buffer (1:10 in aqua bidest). Afterwards 100 µl of standards, controls and diluted samples were pipetted in the corresponding well of the microtiter plate and were incubated 2,5 hours at room temperature on a horizontal shaker (Heidolph Titramax 100, Heidolph Instruments GmbH & CoKG, Schwabach, Germany). Then the plate was washed again five times with 250 µl wash buffer before 100 µl of HRP-Antibody diluted 1:100 in wash buffer were added into all wells. After an incubation of 1 hour at room temperature on the horizontal shaker another washing step followed and 100 µl of TMB-substrate were added into all wells. The reaction was stopped after incubation for 10-20 min at room temperature in the dark by adding 100 µl stop solution. Determination was done immediately at 450 nm against 620 nm with a microtiter plate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). The nitrotyrosine concentration of the samples was calculated by using a 4-parameter-algorithm. Finally the obtained value was multiplied by a factor of 5.

3.5.3 Immunohistochemistry against HSP70 and nitrotyrosine

Tissues were fixed in 4% formalin solution (Sigma Aldrich, Vienna, Austria) over night. For the following dehydration of the tissue blocks a Tissue – Tek® VIP tissue processor (Sakura Finetek Germany GmbH, Staufen, Germany) was used. Afterwards dehydrated tissue blocks were embedded in paraffin (melting temperature 60 °C) applying an embedding console system (Tissue - Tek® tec 5, Sakura Finetek Germany GmbH, Staufen, Germany). With a motorized rotary microtome HM 360 (Microm GmbH, Wallendorf, Germany) the compounded paraffin tissue blocks then were sliced to sections with a thickness of 1,5 µm. Subsequently these

sections were stretched in a water bath and transferred onto microscope slides (26x76x1 Menzel GmbH & Co KG, Braunschweig, Germany).

The produced sections were subjected to immunohistochemical staining for nitrotyrosine and HSP70. Therefore they were preheated at 65°C for 30 minutes, deparaffinized in xylol, rehydrated in a descending series of alcohol solutions (100 %, 90 %, 70 %, 50 %), and washed in distilled water. For HSP70 staining the antigen retrieval was accomplished by microwaving at 150 W for 15 minutes in 0.1 M citratebuffer, pH 6.0. For detection of nitrotyrosine the sections were pretreated in a pressure cooker at 120 °C for 3 minutes in the same citratebuffer. Endogenous peroxidase was blocked by incubation in 10 %, (HSP70) respectively 3 % (nitrotyrosine) H₂O₂ dissolved in methanol for 15 min at room temperature. Before incubation with the primary antibody, tissue sections were treated with UV-Block (Thermo Fisher Scientific, Fremont, CA, USA) for 10 min. After that the primary antibody was applied. Therefore the following antibodies, dilutions and incubation times were used: Rabbit polyclonal to HSP70 (Abcam plc, Cambridge, UK) diluted 1:100 in DAKO REALTM Antibody Diluent (DAKO, Glostrup, Denmark) for 60 min at room temperature, and Anti-Nitrotyrosine, Rabbit Fraction (Molecular Probes Inc., Eugene, OR, USA) diluted 1:200 DAKO REALTM Antibody Diluent for 30 min at room temperature. After a following incubation with a Primary Antibody Enhancer (Thermo Fisher Scientific, Fremont, CA, USA) for 10 min at room temperature tissue sections were incubated with a HRP-Polymer (Thermo Fisher Scientific, Fremont, CA, USA) for 30 min at room temperature in the dark. Furthermore, horseradish peroxidase was visualized using a substrate: DAB (DAKO, Glostrup, Denmark) for tissue sections incubated with HSP70 antibody and AEC (DAKO, Glostrup, Denmark) for those that were incubated with nitrotyrosine antibody. Additionally all sections were counterstained with hematoxylin. AEC treated sections were covered immediately with a water soluble medium and DAB treated sections were dehydrated in an ascending series of alcohol solutions, inserted in xylol and covered with an adequate mounting medium (GLCTM Mounting Medium, Skura Finetek Europe B.V., Zoeterwoude, The Netherlands).

4 RESULTS

4.1 Overview

Five pigs were used for analyses. Pig 1 and Pig 2 were sacrificed immediately after RFA, Pig 3 survived two weeks, Pig 4 two months and Pig 5 three months after the treatment. Serum was collected for detection of CP and nitrotyrosine by ELISA after narcotization (S1), before the first (S2) during the first and the second (S3) RFA and after the ablation (S4) as well as before sacrifice (S5). For histological stainings liver tissue was adequately prepared.

4.2 CP ELISA

Pig	Sample	Carbonyl proteins (pmol/mg protein)
Pig 1	S1	2368,7
	S2	2649,0
	S3	2971,1
	S4	3132,0
Pig 2	S1	2356,6
	S2	2515,7
	S3	2376,7
	S4	2030,7
Pig 3	S1	7639,0
	S2	3318,9
	S3	2451,3
	S4	2354,6
	S5 (2 w.)	2991,7
Pig 4	S1	2843,1
	S2	2952,4
	S3	3054,4
	S4	4375,3
	S5 (2 mo.)	2827,6
Pig 5	S1	2731,7
	S2	2581,4
	S3	3201,1
	S4	2973,0
	S5 (3 mo.)	3481,1

Table 4.1: Results of carbonyl protein ELISA; S1=after narcotization, S2=before first RFA, S3=during first and second RFA, S4=after second RFA, S5=before scarification;

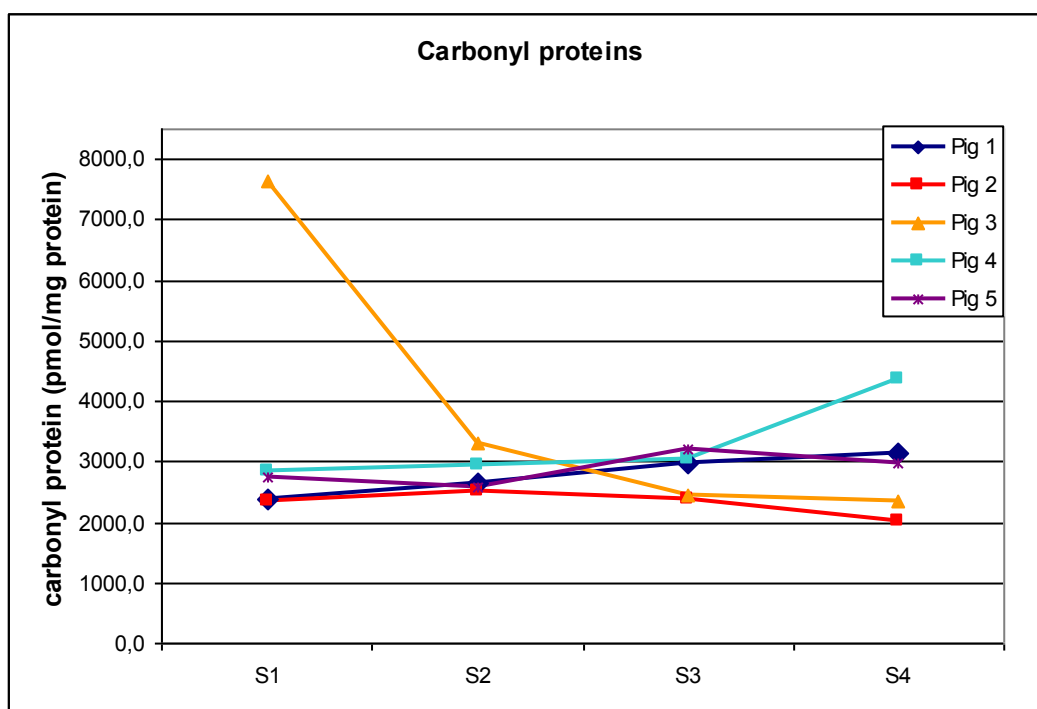


Figure 4.1a: Changes of carbonyl protein levels during RFA; S1=after narcotization, S2=before first RFA, S3=during first and second RFA, S4=after second RFA;

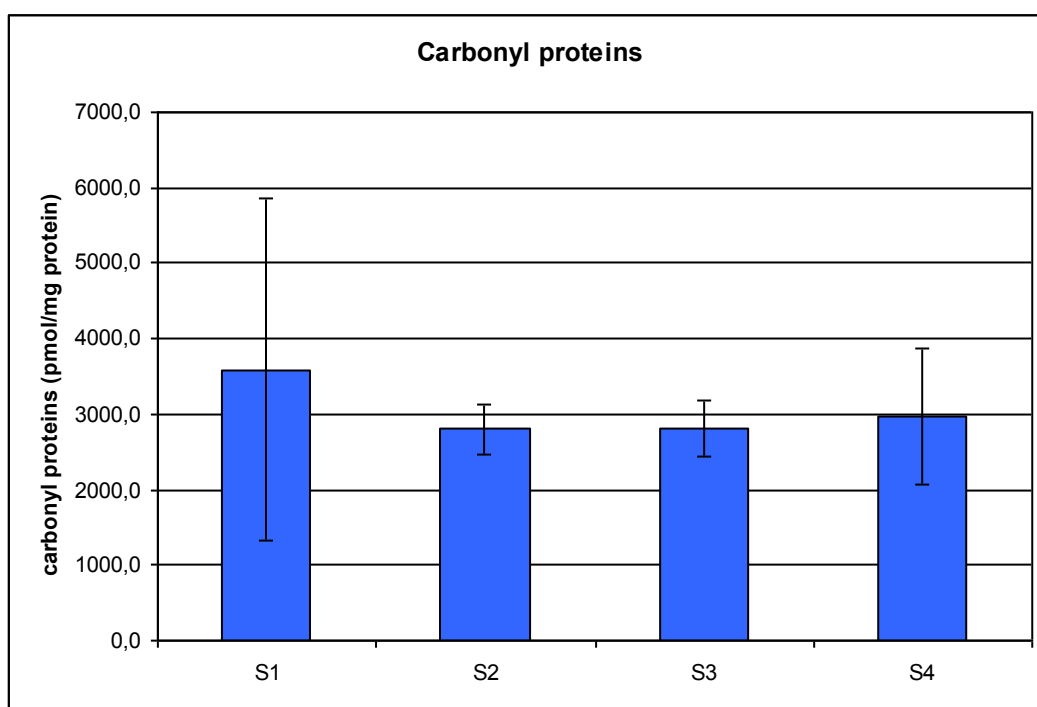


Figure 4.1b: Changes of carbonyl protein levels during RFA; S1=after narcotization, S2=before first RFA, S3=during first and second RFA, S4=after second RFA;

4.3 Nitrotyrosine ELISA

Pig	sample	nitrotyrosine (nM)
Pig 1	S1	20,5
	S2	47,5
	S3	0,0
	S4	51,5
Pig 2	S1	145,0
	S2	141,5
	S3	112,0
	S4	74,5
Pig 3	S1	244,0
	S2	183,5
	S3	0,0
	S4	0,0
	S5 (2 w.)	252,0
Pig 4	S1	33,0
	S2	61,0
	S3	43,5
	S4	25,0
	S5 (2 mo.)	149,5
Pig 5	S1	105,0
	S2	105,5
	S3	92,0
	S4	0,0
	S5 (3 mo.)	165,5

Table 4.2: Results of nitrotyrosine ELISA; S1=after narcotization, S2=before first RFA, S3=during first and second RFA, S4=after second RFA, S5=before scarification;

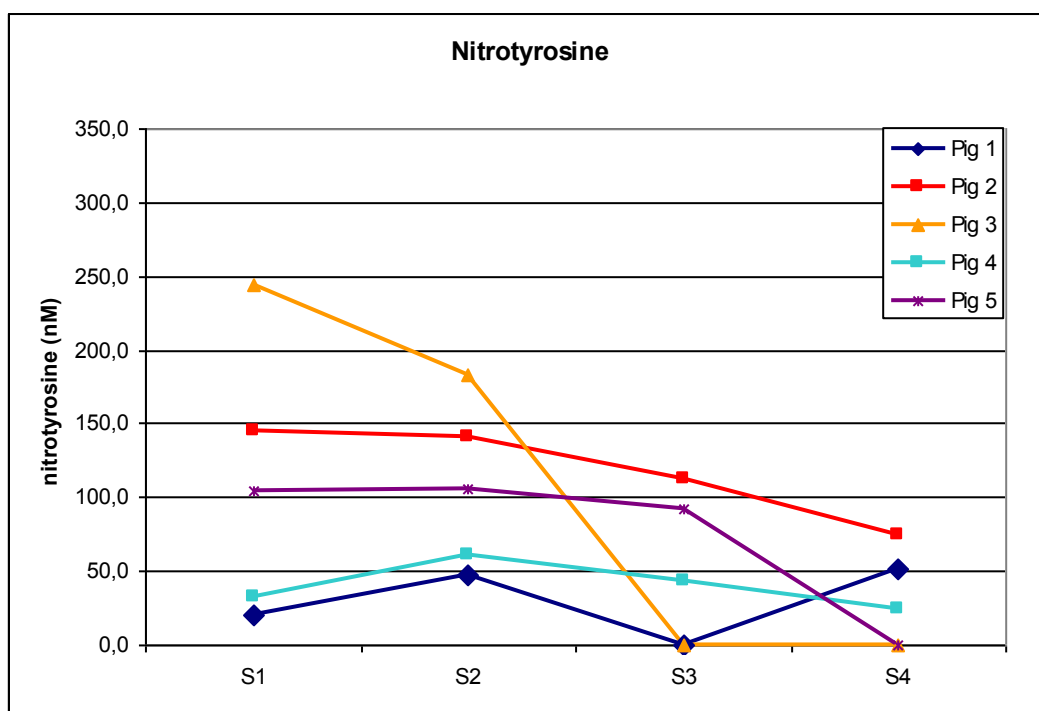


Figure 4.2a: Changes of nitrotyrosine levels during RFA; S1=after narcotization, S2=before first RFA, S3=during first and second RFA, S4=after second RFA;

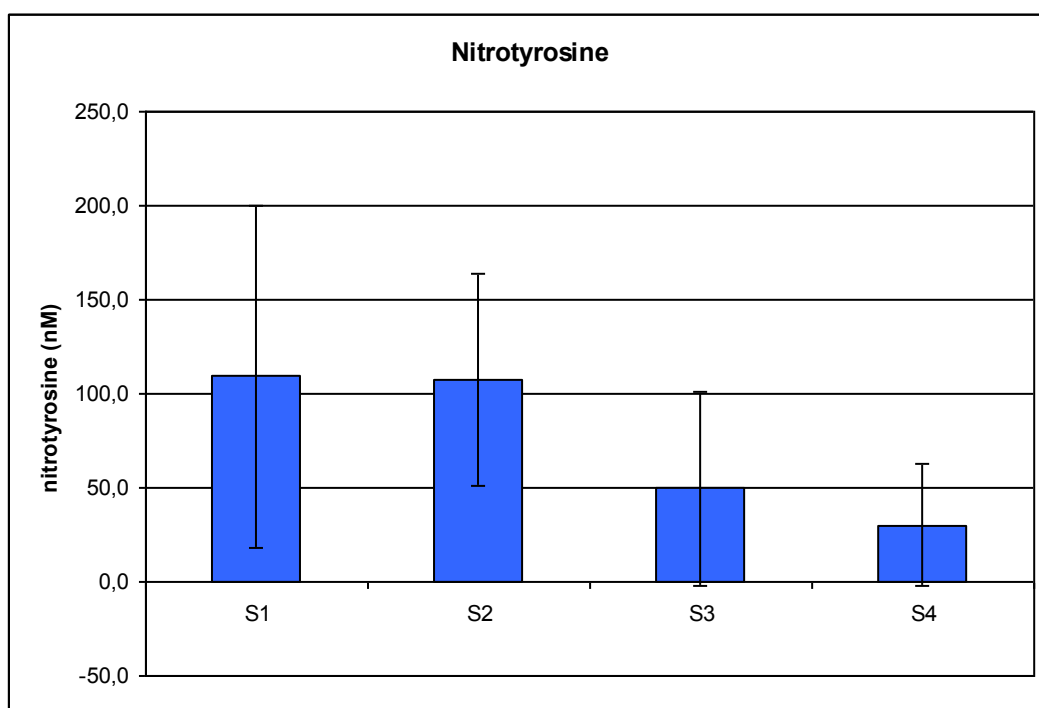


Figure 4.2b: Changes of nitrotyrosine levels during RFA; S1=after narcotization, S2=before first RFA, S3=during first and second RFA, S4=after second RFA;

4.4 Histological stainings

Sections of the extracted liver were stained with haemalaun/eosin (HE) and immunohistochemically for HSP70 and nitrotyrosine.

4.4.1 Pig 1

Pig 1 was sacrificed immediately after RFA. The HE stain is shown in figure 4.3a and the immunostaining is demonstrated in figure 4.3b (HSP70) and 4.3c (nitrotyrosine). In contrast to staining for HSP70, where only a few positive cells are found, an intensive positive staining for nitrotyrosine is observable.

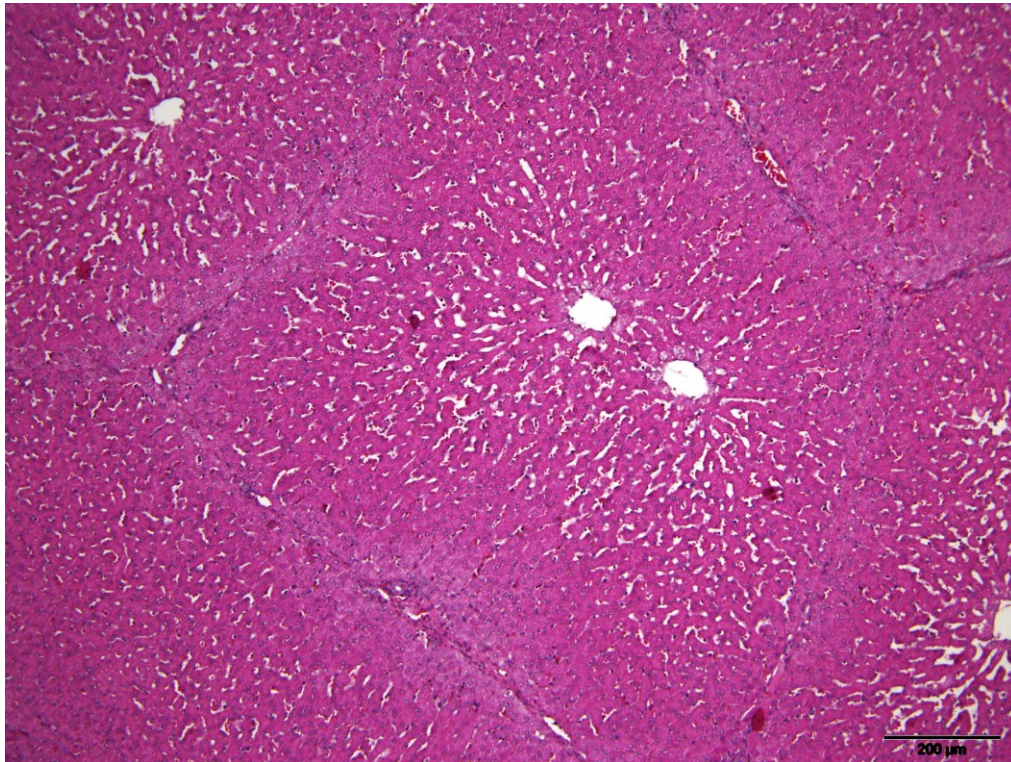


Figure 4.3a: Histological image of a radiofrequency ablated porcine liver (Pig 1). The image shows the lesion's surrounding undamaged tissue in HE-staining, magnification is 10X.

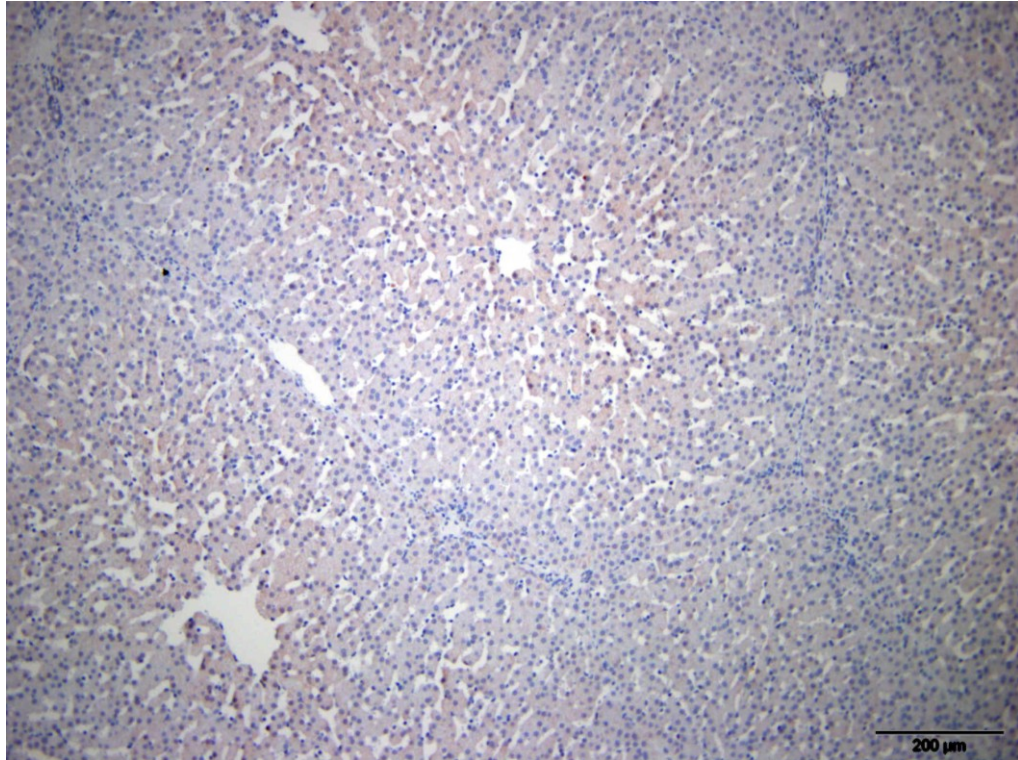


Figure 4.3b: Histological picture of a radiofrequency ablated porcine liver (Pig 1) immunohistochemically stained for HSP70, magnification is 10X.

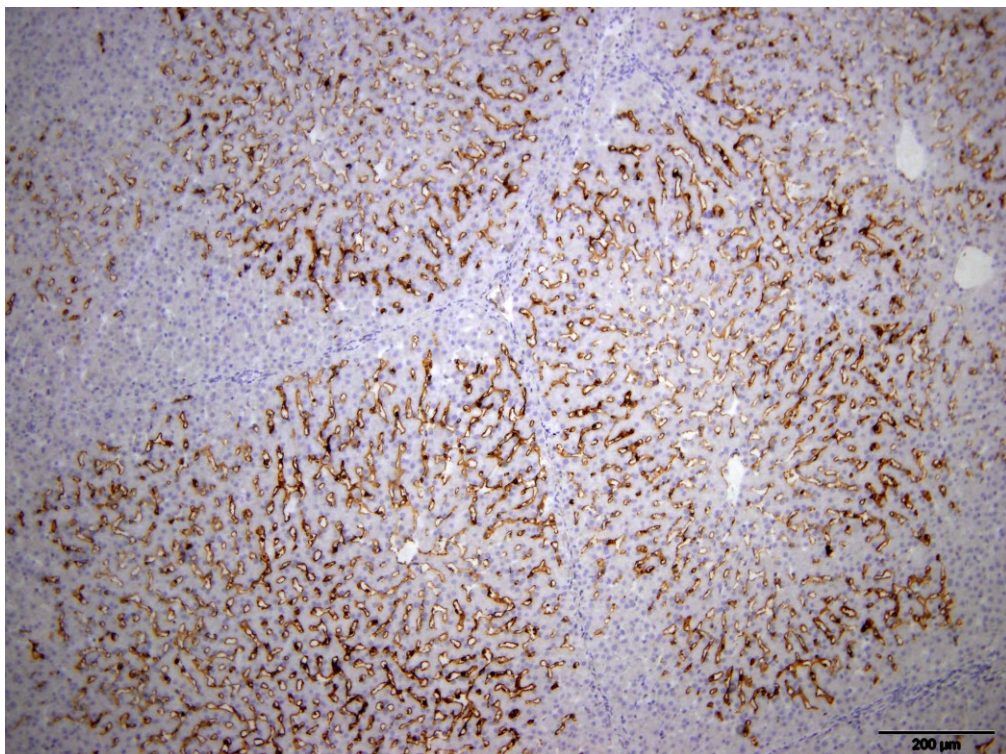


Figure 4.3c: Image of a histological section of a radiofrequency ablated porcine liver (Pig 1) immunohistochemically stained for nitrotyrosine, magnification is 10X.

4.4.2 Pig 2

Pig 2 was sacrificed immediately after RFA. The HE stain is shown in figure 4.4a and the immunostaining is demonstrated in figure 4.4b (HSP70) and 4.4c (nitrotyrosine). Comparable with Pig1 an obvious positive staining for nitrotyrosine is recognisable.

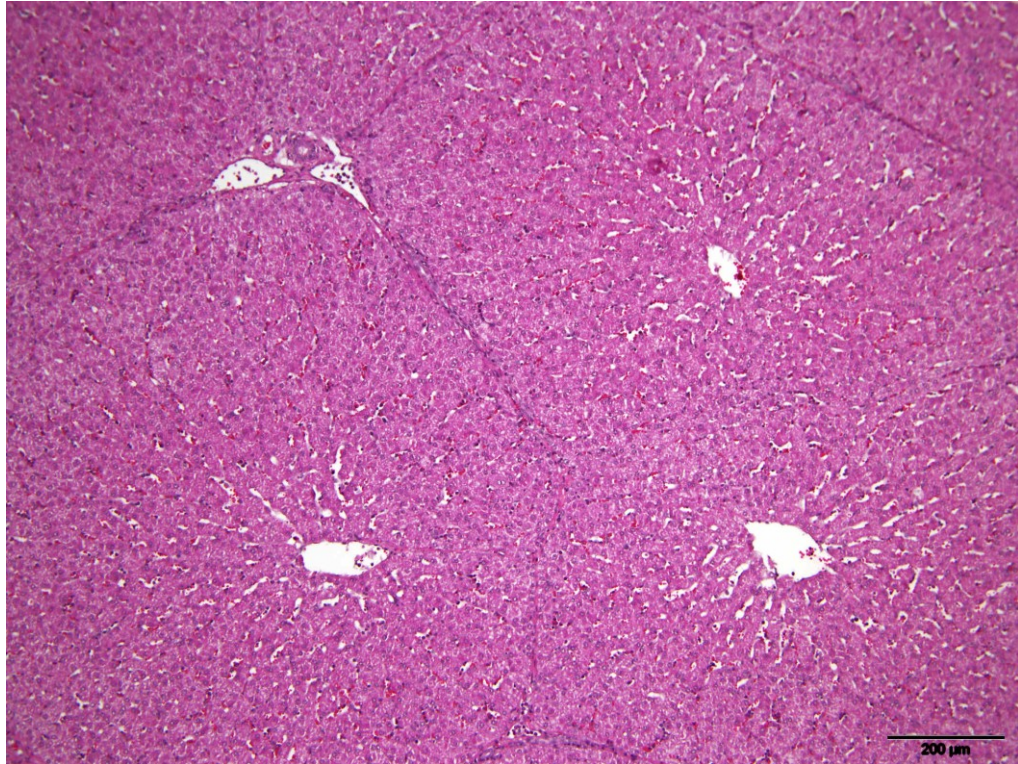


Figure 4.4a Histological section of a radiofrequency ablated porcine liver (Pig 2). The image shows the lesion's surrounding undamaged tissue in HE-staining, magnification is 10X.

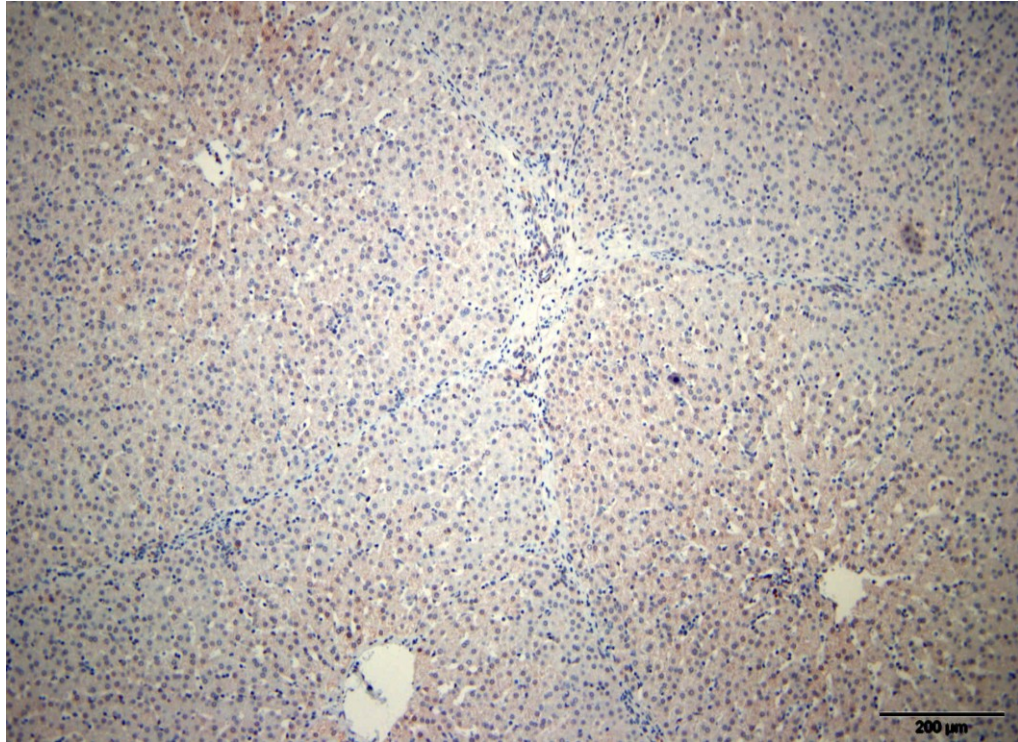


Figure 4.4b: Histological image of a radiofrequency ablated porcine liver (Fig 2) immunohistochemically stained for HSP70, magnification is 10X.

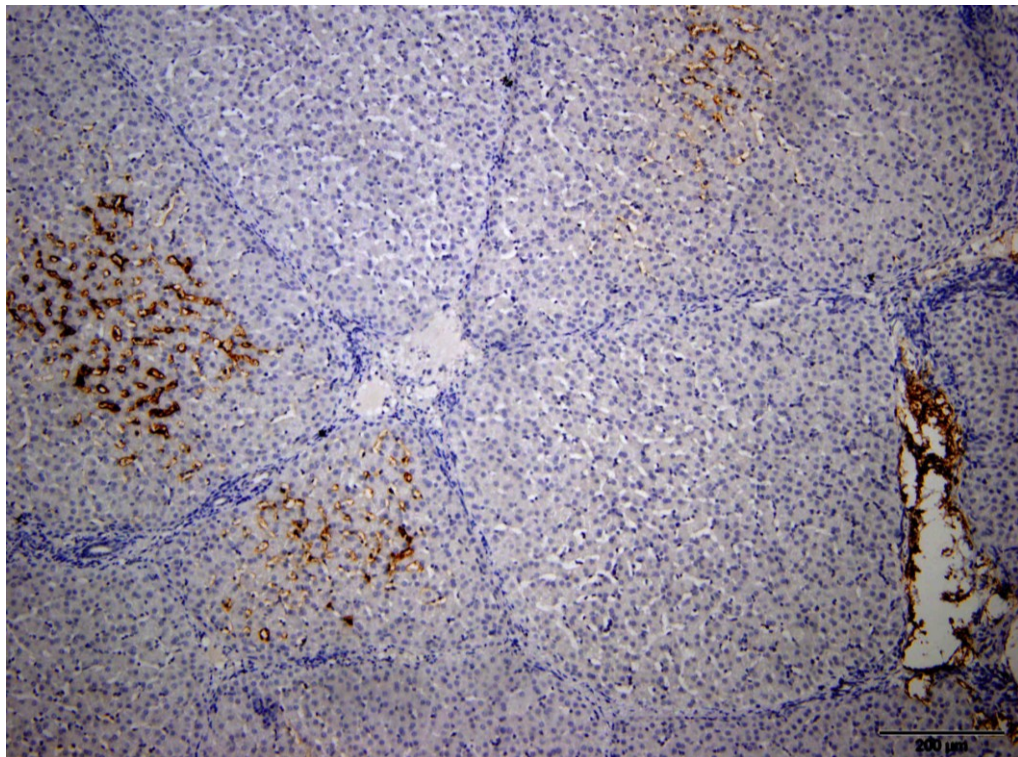


Figure 4.4c: This image shows a histological image of a radiofrequency ablated porcine liver (Fig 2) immunohistochemically stained for nitrotyrosine, magnification is 10X.

4.4.3 Pig 3

Pig 3 survived two weeks after RFA treatment. For demonstration histological pictures were chosen that show distinctly the transition zone between normal and ablated tissue.

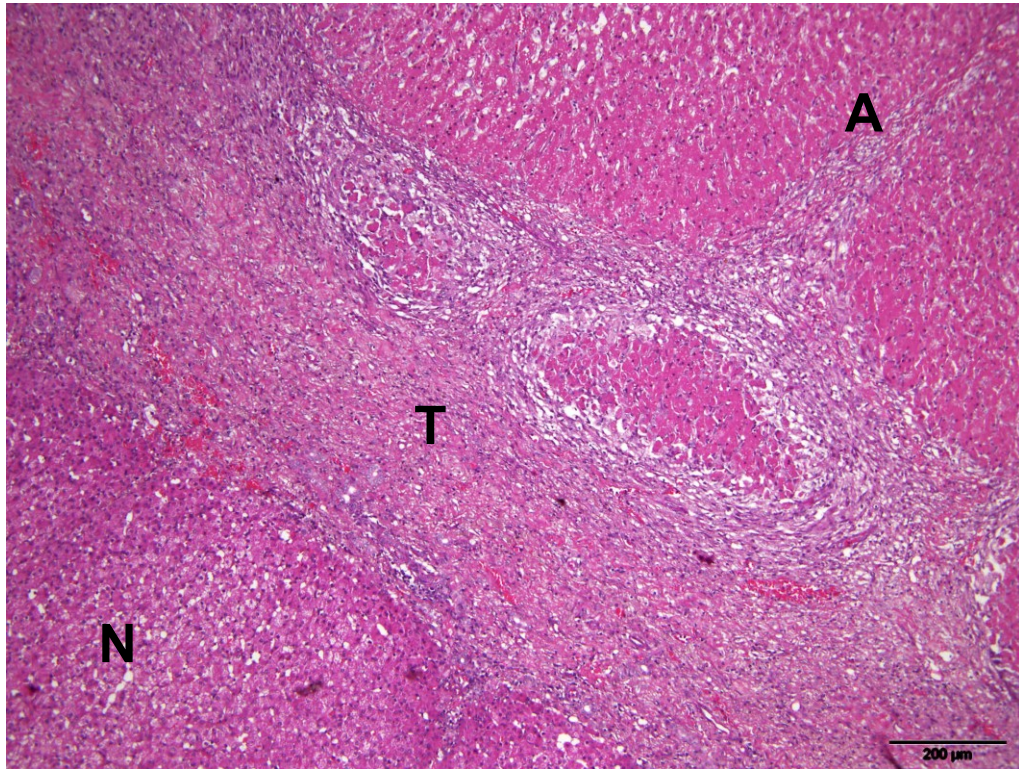


Figure 4.5a: Histological image of a radiofrequency ablated porcine liver (Pig 3) demonstrating the transition zone (T) between normal tissue (N) and the ablated region (A) in HE-staining, magnification is 10X.

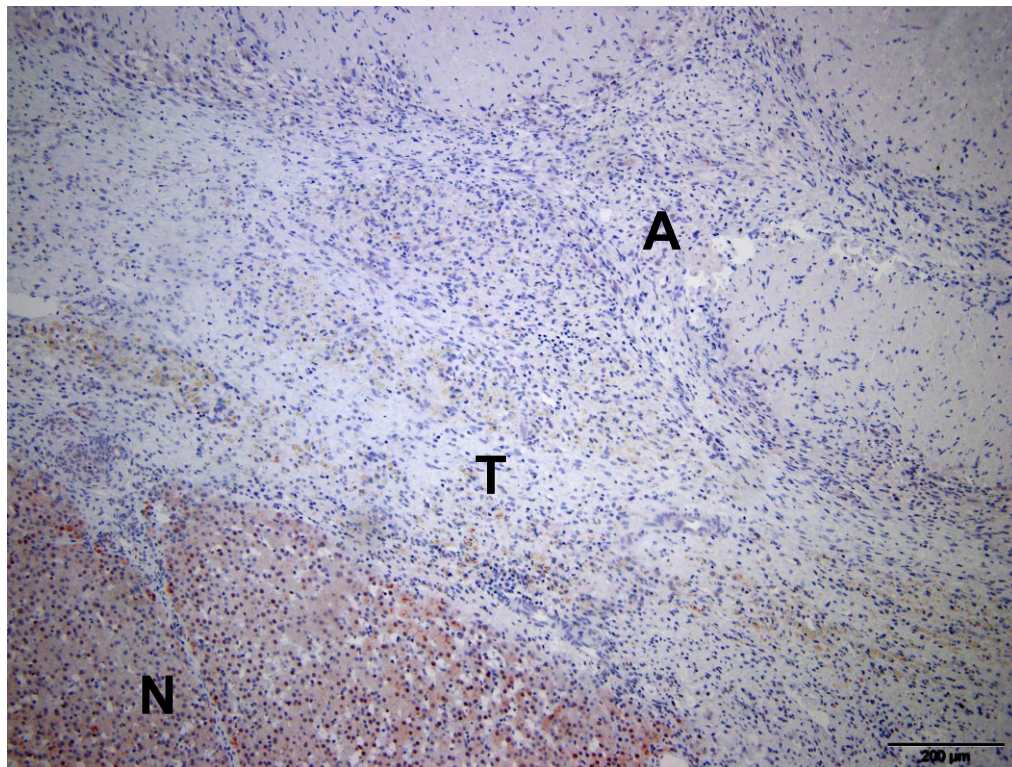


Figure 4.5b: Histological picture of a radiofrequency ablated porcine liver (Pig 3) demonstrating the transition zone (T) between normal tissue (N) and the ablated region (A) and immunohistochemical staining for HSP70, magnification is 10X.

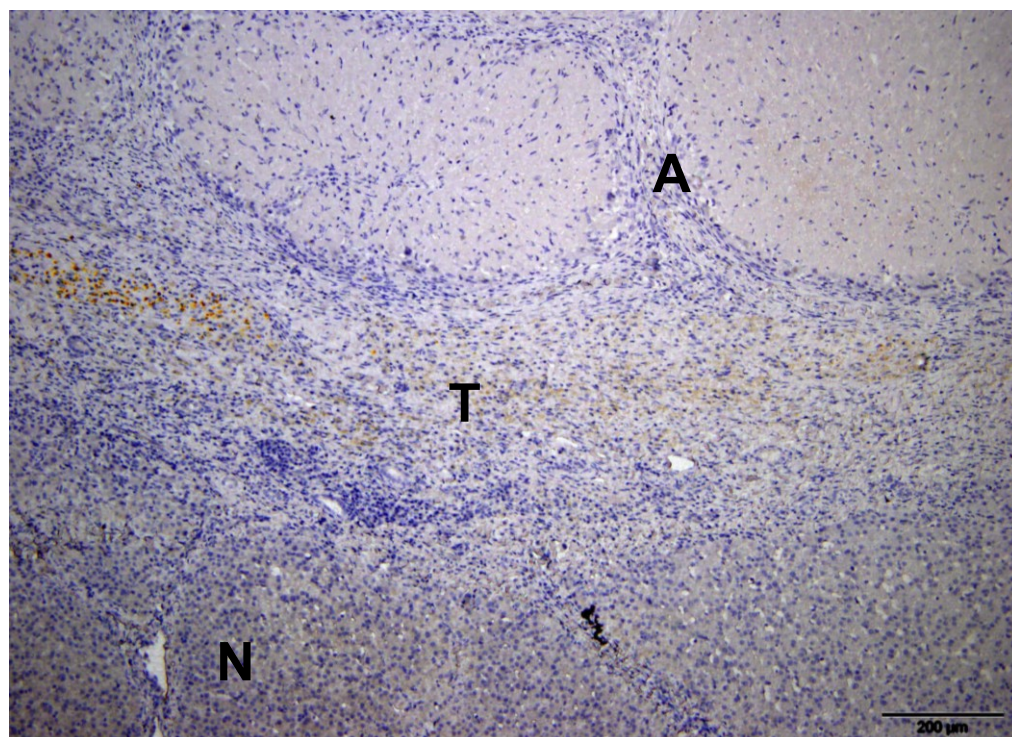


Figure 4.5c: Histological image of a radiofrequency ablated porcine liver (Pig 3) immunohistochemically stained for nitrotyrosine. The image demonstrates the transition zone (T) between normal liver tissue (N) and the ablated region (A) , magnification is 10X.

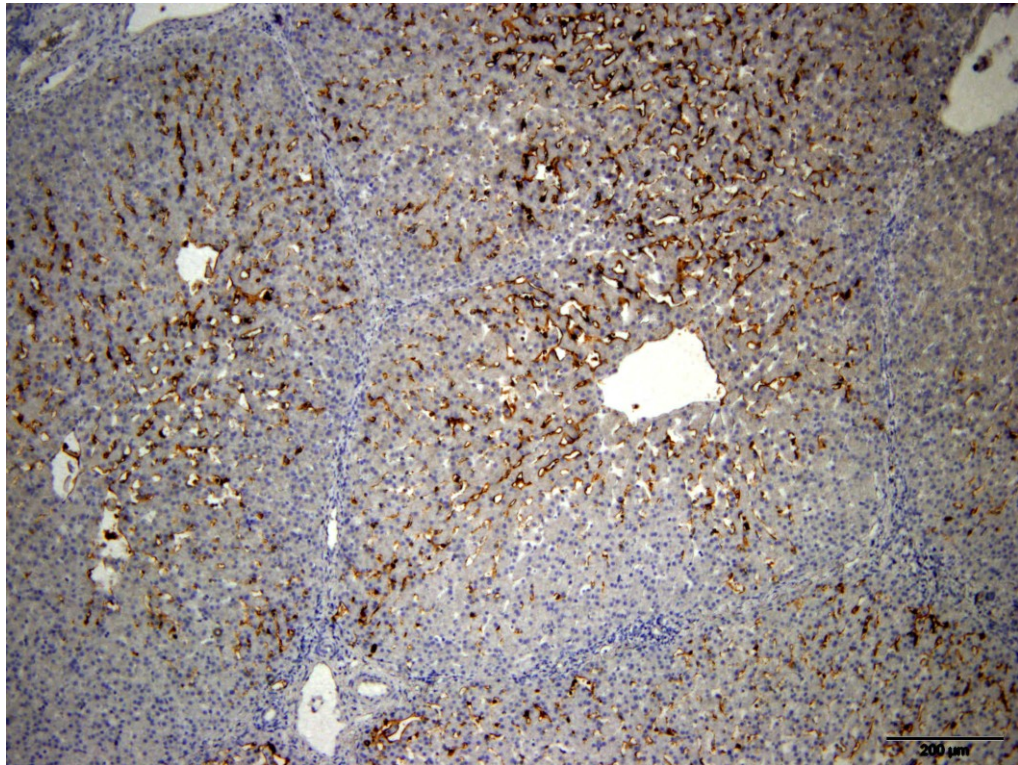


Figure 4.5d: This image shows a histological section of a radiofrequency ablated porcine liver (Fig 3) immunohistochemically stained for nitrotyrosine, magnification is 10X.

4.4.4 Pig 4

Pig 4 was scarified two months after RFA. Figure 4.6a shows HE staining and the immunohistochemically staining is demonstrated in figure 4.6b (HSP70) and 4.6c (nitrotyrosine). Both of the immunostainings show positive results.

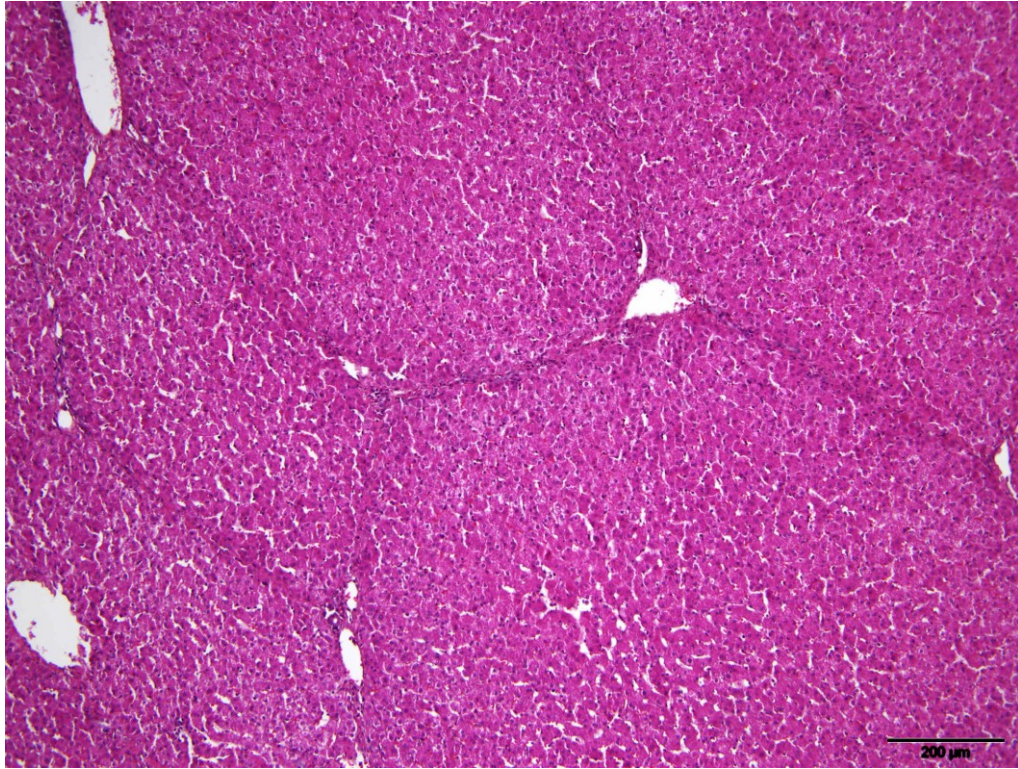


Figure 4.6a: Histological image of a radiofrequency ablated porcine liver (Pig 4) demonstrating the lesion's surrounding undamaged tissue in HE-staining, magnification is 10X.

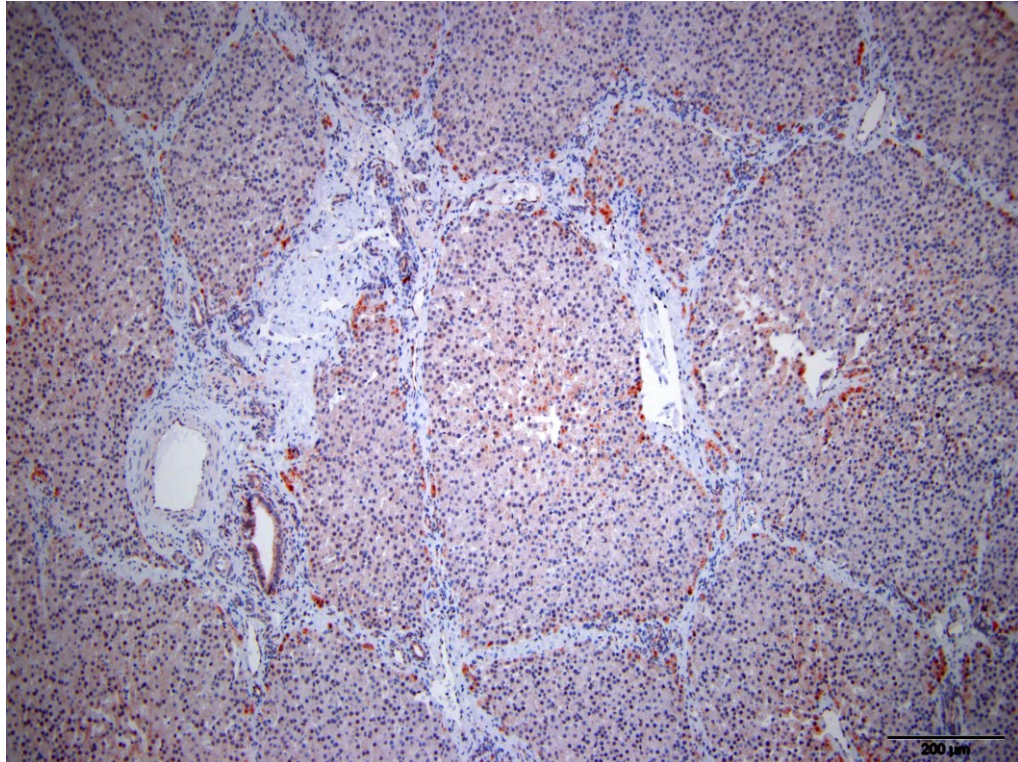


Figure 4.6b: The picture shows a histological section of a radiofrequency ablated porcine liver (Pig 4) immunohistochemically stained for HSP70, magnification is 10X.

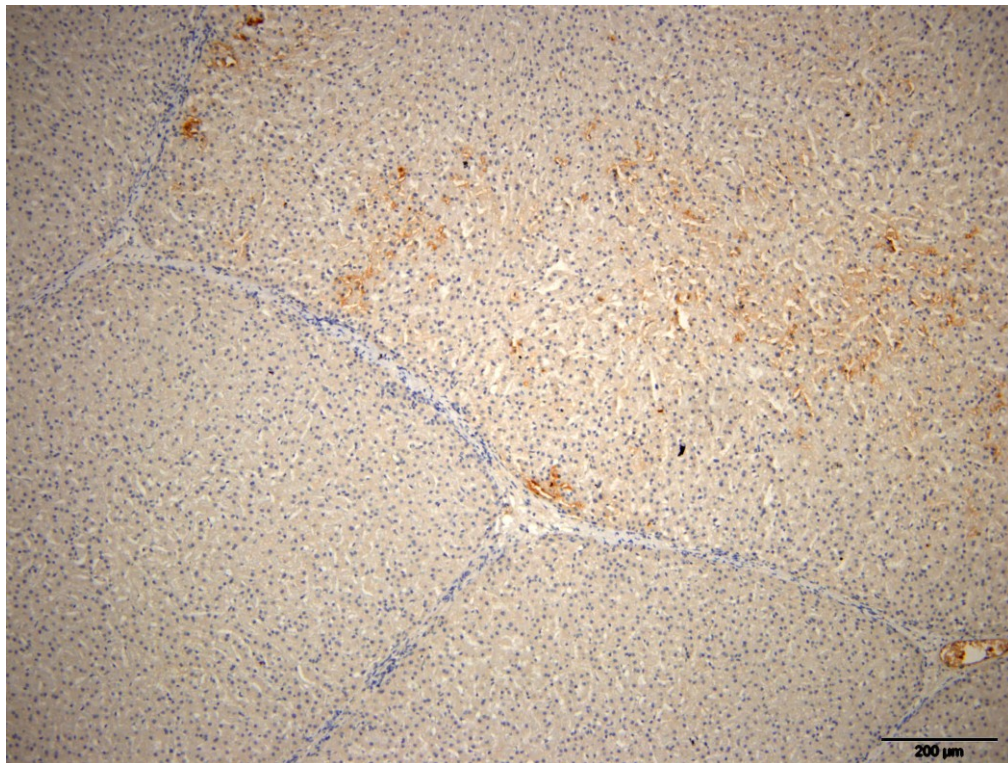


Figure 4.6c: Histological picture of a radiofrequency ablated porcine liver (Pig 4) immunohistochemically stained for nitrotyrosine, magnification is 10X.

4.4.5 Pig 5

Pig 5 survived three months after RFA. As in Pig 3 the chosen, images obviously show the transition zone between ablated region and untreated tissue.

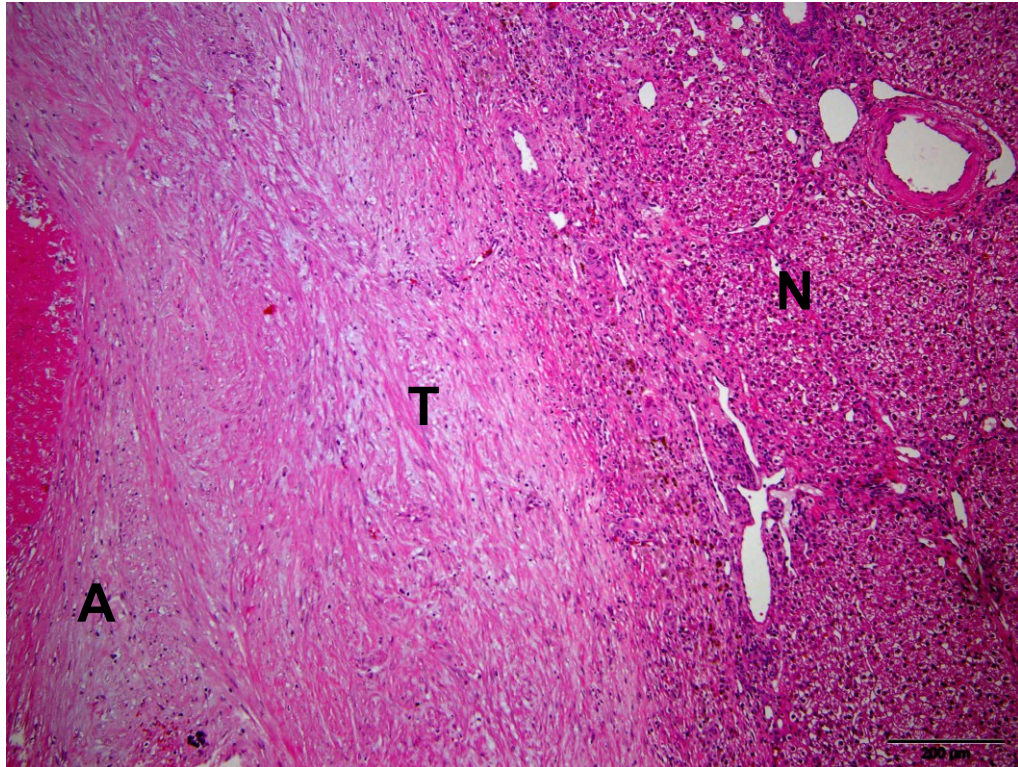


Figure 4.7a: Histological image of a radiofrequency ablated porcine liver (Pig 5) demonstrating the transition zone (T) between normal tissue (N) and the ablated region (A) in HE-staining, magnification is 10X.

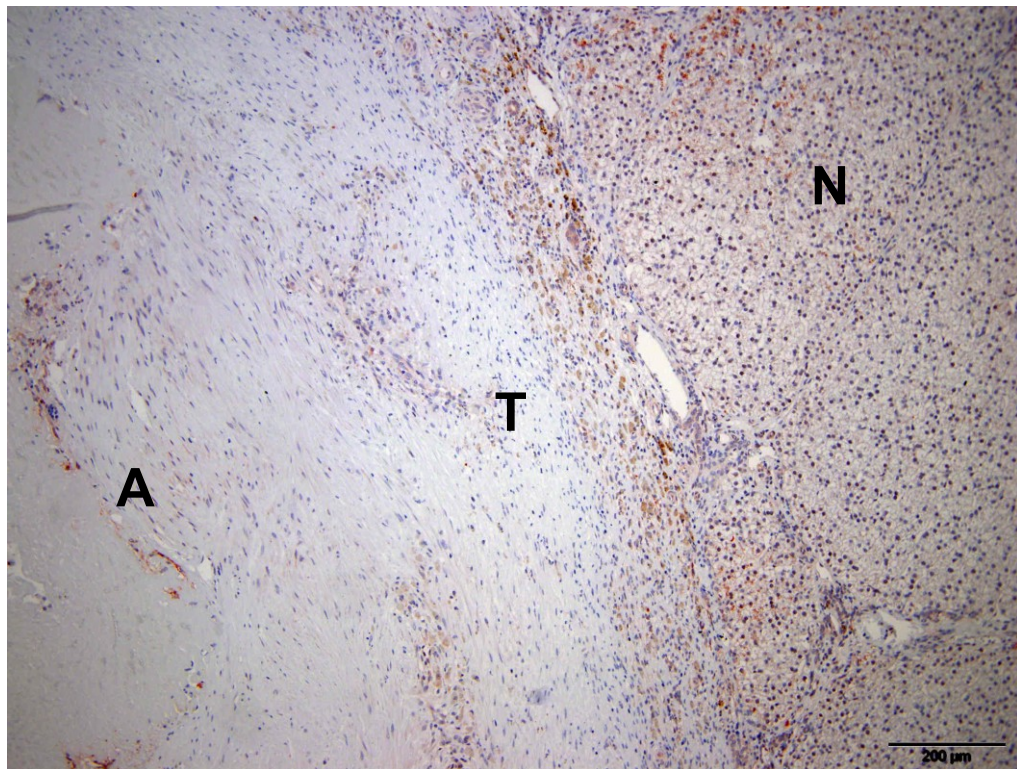


Figure 4.7b: Histological section of a radiofrequency ablated porcine liver (Pig 5) immunohistochemically stained for HSP70. The image demonstrates the transition zone (T) between normal tissue (N) and the ablated region (A), magnification is 10X.

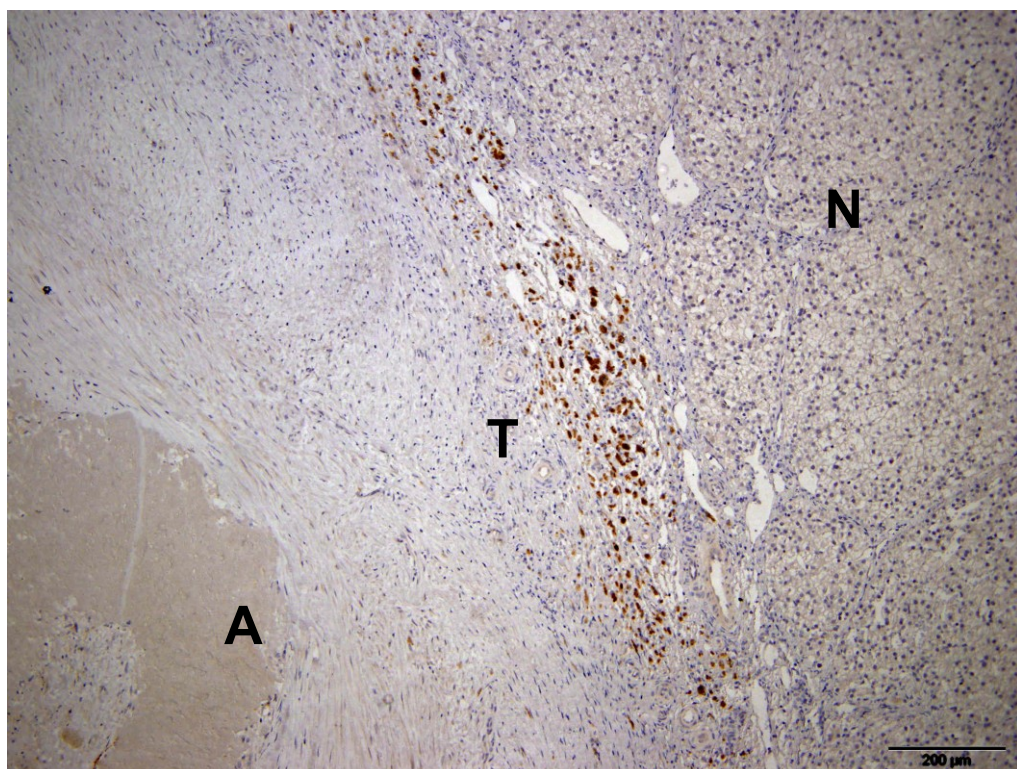


Figure 4.7c: The picture shows a histological section of a radiofrequency ablated porcine liver (Pig 5) demonstrating the transition zone (T) between normal tissue (N) and the ablated region (A) and immunohistochemical staining for nitrotyrosine, magnification is 10X.

5 DISCUSSION

The aim of the present study was to examine, whether there is an increase of oxidative stress induced cell damage during RFA treatment. This fact would enhance the risk for an aggravated healing process and the occurrence of other pathological diseases such as infections, cirrhosis of the liver, viral hepatitis and cardiovascular diseases.

For analyses serum of five pigs was collected for detection of CPs and nitrotyrosine by ELISA before, during the first and second RFA and after the ablation as well as immediately prior to scarification. Furthermore liver tissue was extracted for immunohistochemical stainings against HSP70 and nitrotyrosine as markers of oxidative or nitrosative stress induced cell damage.

On the basis of the accomplished analyses a significant enhancement of oxidative stress markers was not verifiable. For a more accurate statistical evaluation and a meaningful hypothesis it would be necessary to include more pigs in the study.

A reason why a significant enhancement of oxidative stress markers is not measurable may be that the pigs are exposed to an enormous stress during the narcotization. Furthermore already the transport of the pigs from the stable to the operating room in a small transport box may cause a physical strain. A further disadvantage is the intramuscular narcotization with ketamine and midazolam. This stress factors may lead to an increase of the stress markers already before blood is taken so that the first sample shows a high baseline level that does not increase any more during RFA.

A possibility to reduce the stress exposure of the pigs would be to narcotize them already in the stable. Another alternative would be the implantation of a Port-a-Cath (PAC)-system that would be mounted two weeks before RFA. So it would be possible to apply the narcotics unproblematic and stress free.

It also might be that the detection of CP and nitrotyrosine by ELISA is not the appropriate method for the examination of the presence of oxidative stress. There

exist a variety of other suitable markers, for instance MDA, 8-isoprostan or interleukin6.

Blood sampling was performed after narcotization, before the first, during the first and second RFA and after the ablation at the operating room and the paediatric clinic, which are located far from the laboratory in the centre for medical research. Therefore blood samples were not cooled and remained unprocessed for a certain time, what probably might be a reason for changes in detected parameters.

For all the reasons mentioned above, further experiments will be carried out under optimized conditions in order to be able to verify if there is an increase in oxidative or nitrosative stress induced cell damage during RFA.

Since now, no references concerning these subjects could be found in literature and therefore from our point of view, these experiments seem to be very interesting in order to ameliorate RFA protocols for patients suffering from HCC.

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