Combination of alpha-ketoglutarate and 5-HMF inhibits protein carbonyl formation by reactive oxygen and nitrogen species during exposure of cigarette smoke

eingereicht von

Eberhard Gurtner

23.12.1979

zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Physiologische Chemie

unter der Anleitung von

Ao. Univ.-Prof. Mag. Dr. rer. nat. Joachim Greilberger

Graz, am 17.12.2013 Gurtner Eberhard

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 17.12.2013 *Gurtner Eberhard*

Danksagung

Momentan ist richtig
Momentan ist gut
Nichts ist wirklich wichtig
Nach der Ebbe kommt die Flut

Am Strand des Lebens
Ohne Grund, ohne Verstand
ist nichts vergebens
ich bau die Träume auf den Sand

.....

Oh, es ist schon ok
Es tut gleichmäßig weh
Es ist Sonnenzeit
Ohne Plan, ohne Geleit

Der Mensch heißt Mensch
Weil er erinnert, weil er kämpft
Und weil er hofft und liebt
Weil er mitfühlt und vergibt

Und weil er lacht,
Und weil er lebt,
Du fehlst

(Herbert Grönemeyer-Mensch)

Im Gedenken an

Ing. Winfried Gurtner

Für meinen Vater, der immer an mich geglaubt hat und kurz vor Fertigstellung dieser Arbeit verstorben ist.

TABLE OF CONTENTS

ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RONS	Reactive Oxygen and Nitrogen Species
RNS	Ribonucleic Acid
SOD	Superoxide Dismutase
CAT	Catalase
5-HMF	5- Hydroxy- Methyl- Furfurale
BSA	Bovine Serum Albumine
BHT	Butyl -Hydroxy-Toluene
DNPH	Di-Nitro-Phenyl Hydrazine
TCA	Trichloric Acid
cps	Counts per Second
e.g.	Exempli Gratia
ppm	parts per million

TABLE OF FIGURES

Zusammenfassung

Hintergrund: Unter der Einwirkung von Zigarettenrauch auf menschliches Plasma kommt es zur Bildung von oxidativem Stress und Peroxynitrit, wobei dieses eines der potentesten oxidierenden und nitrierenden Reagenzien darstellt, welches durch Zigarettenrauch gebildet wird. Reaktive Spezies sind in der Lage oxidative Veränderungen an Proteinen, Lipiden, Kohlenhydraten und DNA auszulösen und voranzutreiben, was zu vielfältigen Erkrankungen, wie degenerativen Lungen- und Herzerkrankungen, so zum Beispiel zu Bronchitis, COPD, Emphysem, Herzinfarkt und chronischer Entzündung führen kann, bis hin zu Lungenkrebs und anderen bösartigen Erkrankungen. Im Laufe dieser Diplomarbeit sollte die Frage geklärt werden, ob durch den Einsatz von alpha-ketoglutarat und 5-HMF die oxidative Schädigung von Proteinen durch Zigarettenrauch reduziert wird und ob diese Substanzen in der Lage sind die schädliche Wirkung von Peroxynitrit zu neutralisieren. Von besonderem Interesse war die Frage, ob diesen Substanzen ein höheres antioxidatives Potential als Vitamin C zuzuschreiben ist.

Methoden: Um das Ausmaß der oxidativen Schädigung von Proteinen festzustellen, wurden photometrische Messungen von Carbonylproteinen durchgeführt. Im ersten Schritt wurde die Reduktion von Carbonylproteinen durch Kombination von alpha-ketoglutarat und 5-HMF und die Reduktion von Carbonylproteinen durch Vitamin C im Vergleich zu einer BSA-Kontrollösung durchgeführt. Es wurden Konzentrationen von 0.568 mmol verwendet. Im zweiten Schritt wurde die inhibierende Wirkung auf die Carbonylproteinbildung der einzelnen Substanzen allein (alpha-ketoglutarat, Vitamin C, 5-HMF) und im Vergleich zu einander gemessen, wobei unterschiedliche Konzentrationen verwendet wurden (0.284 mmol, 0.426 mmol, 0.568 mmol). Um die Frage zu beantworten, ob alpha-ketoglutarat, 5-HMF und Vitamin C fähig sind das schädliche Peroxynitrit abzufangen, wurden Chemilumineszenzmessungen durchgeführt. Die Effekte verschiedener Konzentrationen von alpha-ketoglutarat, 5-HMF und Vitamin C (0.16mM, 0.8mM, 4mM, 20mM, 200mM) auf die durch $ONNO^-$ verursachte Oxidation von Luminol bei der Chemilumineszenz wurde untersucht. Die Resultate wurden statistisch analysiert und grafisch dargestellt.

Resultate: Aufgrund der durchgeführten Untersuchungen in Bezug auf die Reduktion der Carbonylproteinbildung, konnte nicht nur gezeigt werden, dass die Kombination aus alpha-ketoglutarat und 5-HMF den stärksten inhibierenden Effekt auf die

Carbonylproteinbildung hatte, sondern auch, dass es der Wirkung von Vitamin C klar überlegen war. Im Rahmen des Vergleichs der antioxidativen Potenz der einzelnen Substanzen untereinander, stellte sich heraus, dass auch hier alpha-ketoglutarat dem Vitamin C überlegen war, wobei 5-HMF die schwächste inhibierende Wirkung auf die Carbonylproteinbildung hatte. Durch Analyse der inhibierenden Effekte der untersuchten Verbindungen auf die Luminolreaktion, konnte gezeigt werden, dass Vitamin C im Vergleich zu alpha-ketoglutarat und 5-HMF das stärkste antioxidative Potential hatte. Das zweitstärkste antioxidative Potential konnte alpha-ketoglutarat zugeschrieben werden, wobei auch hier 5-HMF von allen drei Substanzen das schwächste antioxidative Potential entwickelte.

Conclusio: Oxidativer Stress spielt bei der Entstehung vieler Erkrankungen eine wichtige Rolle. In der Prävention und Behandlungen zahlreicher Erkrankungen besteht der Bedarf an multimodalen Konzepten, wie etwa durch die Reduktion von oxidativem Stress. Die im Rahmen dieser Diplomarbeit gefundenen Daten belegen eine Abschwächung der Radikalwirkung durch alpha-ketoglutarat und 5-HMF und untermauern die Hypothese, dass eine Reduktion, des durch Rauchen verursachten oxidativen Stresses, erreicht werden kann. Die Reduktion von oxidativem Stress durch die Nutzung von alpha-ketoglutarat und 5-HMF könnte einen weiteren multimodalen Schritt in der Prävention und Behandlung von Erkrankungen darstellen, bei denen oxidativer Stress beteiligt oder verursachend ist.

Abstract

Background: Exposure of human plasma to gas phase and whole cigarette smoke leads to oxidative stress and formation of peroxynitrite, as one of the most potent oxidizing and nitrating reagent produced from cigarette smoke. Reactive species are able to initiate or promote oxidative modifications of proteins, lipids, carbohydrates and DNA, which generally leads to various degenerative pulmonary and cardiovascular diseases like bronchitis, chronic obstructive pulmonary disease (COPD), emphysema, myocardial infarction and chronic inflammation as well as lung cancer and other malignancies. It was the primary goal of this thesis to answer the question if alpha-ketoglutarate and 5-HMF protect against oxidative damage of proteins exposed to the gas phase of cigarette smoke and if these substances are able to act as scavengers for peroxynitrite compared to ascorbic acid.

Methods: In order to determine the level of oxidative protein damage, photometric measurement of carbonyl proteins has been carried out. In a first step, measurement of the reduction of carbonyl proteins through a BSA-solution with both substances, alpha-ketoglutarate and 5-HMF in combination, compared to ascorbic acid, using concentrations of 0.568 mmol of each substances, was executed. In a second step, measurement of inhibitory effects on carbonyl protein formation of each substance alone (alpha-ketoglutarate, ascorbic acid, 5-HMF) and in comparison to each other, in different concentrations (0.284 mmol, 0.426 mmol, 0.568 mmol), was done. In order to answer the question if alpha-ketoglutarate, 5-HMF and ascorbic acid are able to act as scavengers for peroxynitrite, peroxynitrite scavenging measurements by chemiluminescence technique were accomplished. Effects of different concentrations of alpha-ketoglutarate, 5-HMF and ascorbic acid (0.16mM, 0.8mM, 4mM, 20mM, 200mM) on the oxidation of luminol through $ONNO^-$ during chemiluminescence were analyzed. The results were treated and analyzed statistically and presented graphically.

Results: Due to the examinations carried out on inhibition of carbonyl protein formation, it could not only be shown that the combination of alpha-ketoglutarate and 5-HMF had the strongest inhibiting effect on carbonyl protein formation, but also that it was clearly superior in its effect to ascorbic acid. Investigating the antioxidative potency of each substance alone against one another, it could be demonstrated that alpha-ketoglutarate was superior to ascorbic acid, whereas 5-HMF showed the weakest effects on reduction of

protein carbonyl formation. By analysis of the inhibiting effects on the luminol reaction through the compounds under investigation it could be figured out, that ascorbic acid showed the strongest antioxidative potential compared to alpha-ketoglutarate and 5-HMF. The second strongest antioxidative potential could be attributed to alpha-ketoglutarate and again 5-HMF showed the weakest antioxidative potential of all three substances.

Conclusion: Oxidative stress plays an important role in the development of many diseases. In the prevention and treatment of numerous diseases there is a need for multimodality concepts, e.g. to reduce the occurrence of oxidative stress. The generated data of this diploma thesis documented the radical scavenging effect through alpha-ketoglutarate and 5-HMF and supports the hypothesis that there could be achieved a significant reduction of oxidative stress by means of smoking. Reduction of oxidative stress through the use of alpha-ketoglutarate and 5-HMF may therefore be one further step towards introducing a multimodality approach in the prevention and treatment of several diseases caused or contributed to oxidative stress.

1 Introduction

The aim of this diploma thesis is to show on one hand if alpha-ketoglutarate and 5-HMF protect as water soluble substances oxidative damage of proteins exposed to the gas phase of cigarette smoke and on the other hand, if these substances are able to act as scavengers for $ONOO^-$ as one of the most potent oxidizing and nitrating reagent produced from cigarette smoke compared to ascorbic acid.

Exposure of human plasma to gas phase and whole cigarette smoke initiate or promote directly oxidative modifications of proteins, lipids, carbohydrates and DNA by thousands of chemicals including free radicals, reactive oxygen and nitrogen species (RONS) and saturated and unsaturated aldehydes [1; 2; 3; 4].

This leads generally to various degenerative pulmonary and cardiovascular diseases like bronchitis, chronic obstructive pulmonary disease (COPD), emphysema, myocardial infarction and chronic inflammation as well as lung cancer and other malignancies [5].

Chronic inflammation activates a variety of inflammatory cells, which induce and activates several enzymes like nitric oxide synthase, myeloperoxidase, NADPH oxidase and eosinophil peroxidase. These enzymes produce high concentrations of free radicals and oxidants including superoxide anion (O_2^{*-}), nitric oxides (NO_x), hydrogen peroxides and hypochlorous acid, which itself reacts to a more potent oxidizing, nitrating and deaminating reagent called peroxynitrite ($ONOO^-$) [6].

These species is able to damage DNA, RNS, lipids and proteins leading to increased mutations and altered functions of enzymes and proteins, like activation of oncogene products and inhibition of tumor-supressor proteins [3; 7; 8; 9].

Many studies have shown that increased concentrations of modified proteins are measured in inflamed tissues, such as plasma of lung cancer patients and cigarette smokers [10]. Oxidative damage of proteins of human plasma like albumin and degradation of microsomal proteins in pig lung, heart and liver caused mostly by the aqueous tar phase of cigarette smoke, as evidenced by the measurement of carbonyl proteins, was completely inhibited by the water soluble ascorbic acid and only partially by glutathione, but not by other antioxidants including superoxide dismutase (SOD), catalase (CAT), vitamin E and beta-carotene [3; 11].

This led to the suggestion that large dose of vitamin C protects smokers from oxidative damage and associated degenerative diseases. In terms of chemoprevention of oxidative damage the role and concentrations of antioxidants are yet not defined exactly. It is reported about the benefits of an oral supplementation of α-ketoglutarate and 5-hydroxy-methyl-furfurale (5-HMF) in patients for lung surgery due to non-small cell lung cancer resulting in an increased exercise and energy capacity and a reduced oxidative stress by measuring the carbonyl proteins [12].

1.1 Oxidative stress and free radicals

In the human body there are a lot of processes that take place at any time, having an oxidative or anti-oxidative effect to biomolecules. In terms of salutogenesis these main two processes ought to be in balance at all times. In an ideal manner a complex web of antioxidant defences guarantees this, even though it only minimizes, but does not completely prevent biomolecules from oxidative damage.
Oxidative stress describes a state in which the balance between production and subsequent elimination of free radicals (pro-oxidants) through our body´s anti-oxidative defence system is not given any more.
The result is an increased formation of free radicals, because the equilibrium shifts in favour of oxidants and to the detriment of anti-oxidants.

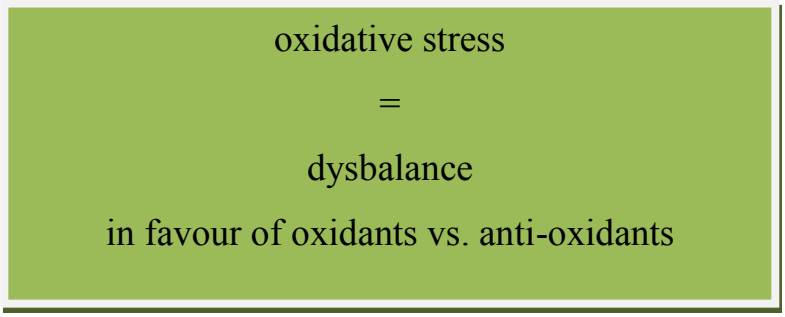


Figure 1: Definition of oxidative stress

Oxidants are rated among those compounds, that own one or more unpaired electrons in their orbital. This particular special property lends them a very high responsiveness.
Inter alia radiation, toxins (metals, pesticides, particles, drugs and many more), radicals (free radicals, RONS) and ozone are counted among the representatives of oxidants.

Anti-oxidants have effects beyond enzymatic (vitamins and trace elements containing enzymes) or non-enzymatic regulated systems (vitamin C, E, glutathione).
The group of biological anti-oxidants includes reducing agents that very easily react with oxidizing substances and therefore they protect more important molecules against oxidation. Vitamin C and E, ubiquinone, carotenoid (beta carotene, lycopene), bilirubin and glutathione are rated among those [13].
Dietetic anti-oxidants are compounds in comestibles that significantly reduce the effects of reactive substances [14].

There are a number of different radicals that can be differentiated by their central atoms (oxygen, nitrogen, sulfur, hydrogen, metals). The most important for the organism are placed by oxygen radicals and nitrogen radicals, so called RONS (reactive oxygen and nitrogen species.
In principle radicals are build by wide-ranging influences on atoms and molecules. These influences can be heat, UV-radiation (photolysis), X-rays and other ionizing radiation, furthermore radicals arise electrochemically from oxidation or respectively from reduction.

Biological structures can be harmed by free radicals due to their high reactivity and as a consequence the characteristics of these biological structures can be changed disadvantgeous. Macromolecules are getting modified (collagen, elastin, nucleic acids, tissue matrix) which leads to lipid peroxidation of membranes and surface structures (lipoproteins, erythrocytes) [15].
For example, the polyunsaturated fatty acid side-chains are indispensable to the fluidity of membranes and they are the most vulnerable to free radical attack and the resulting lipid peroxidation [16].

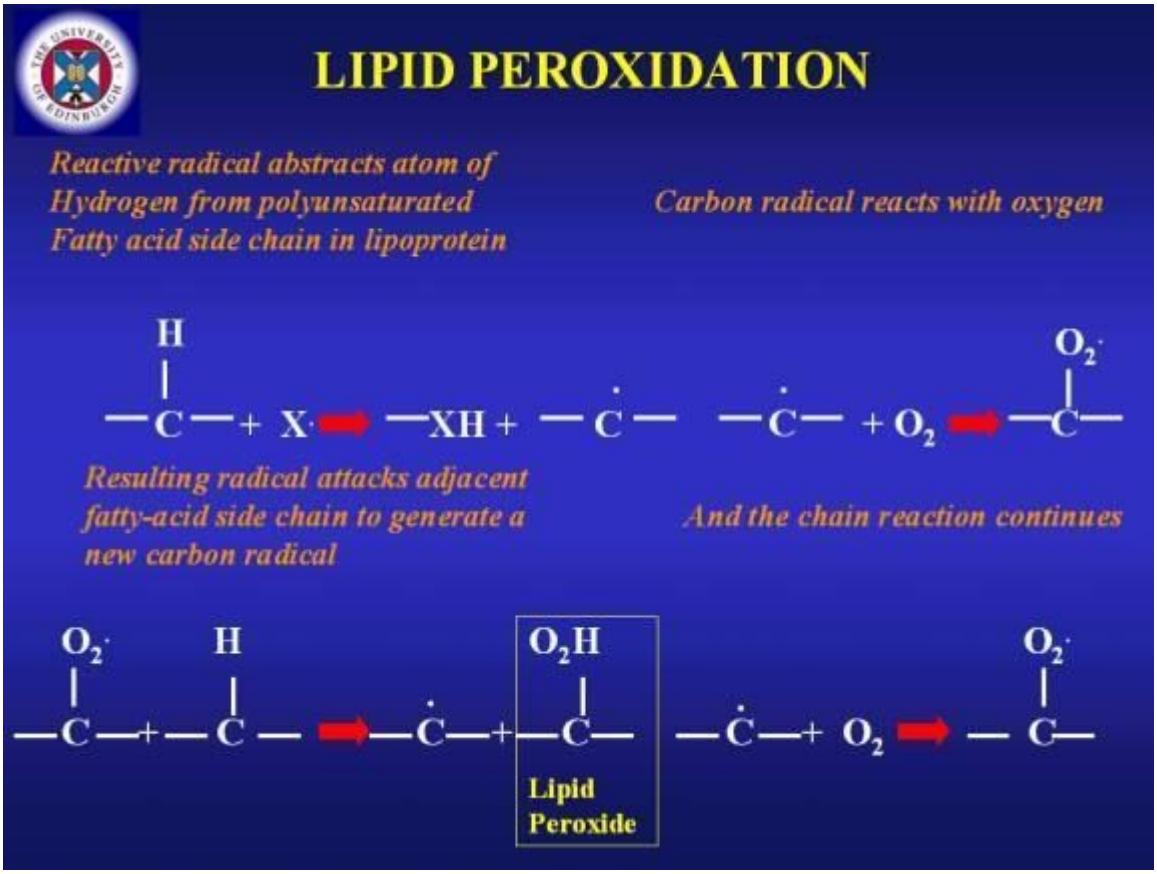


Figure 2: Development of Lipid peroxide by free radicals [17].

Most radicals are highly reactive and thus they have a rather brief short-life (< 1 second). Within a very short time free radicals react with other substances in their very surrounding area. In comparison with free radicals reactive oxygen species and nitrogen species (RONS) are long-lasting compounds that occur in the human organism caused by overloading the body with permanent burden and/or overstress (e.g. illness, surgery, injury, ischemia, reperfusion and sport).

These developments are referred to oxidative stress, whereby reactive oxygen species (ROS) are involved as well as reactive nitrogen species (RNS) and, due to their increased occurrence in the organism, they can no longer be eliminated.

- chemical reagents
  OH^{*-}, O_2^{*-}, H_2O_2, HOCl, HOBr, $^1O_2$, $ONOO^-$ [(NO^* + O_2^{*-} or NO_2^* + OH^*)]
- activated phagocytosis (oxidative burst activity)
- free metals (Fe^{2+}, Cu^{1+})
- γ-radiation in presence of O_2
- UV light, ozone
- lipid peroxidation (HNE, MDA, acrolein)

Figure 3: Overview of free radicals and RONS [18].

Two options are known for our body`s own defences (the immune system) to protect the cells from excessive radical formation:

- **endogenous:** superoxid dismutase, glutathione peroxidase, uric acid
- **exogenic:** bioflavonoids, carotenoid, tocopherol, ascorbic acid

The redox status causes an optimal cell functioning, which in turn is based on the balance between oxidants and anti-oxidants. The redox status and the redox balance are representative for the oxidation and reduction potential of a cell.
An overbalance towards oxidation can be caused by an increased production of RONS, possibly combined with a reduced anti-oxidative functioning.

In the short term this overbalance could be desirable due to regulate the vaso-tonus by way of activation of guanylate cyclase.
For the longer term RONS cause oxidative damage to nucleic acids, proteins and lipids; this leads to formation of systemic inflammation due to gene expression and as consequence apoptosis of formerly healthy cells is induced.

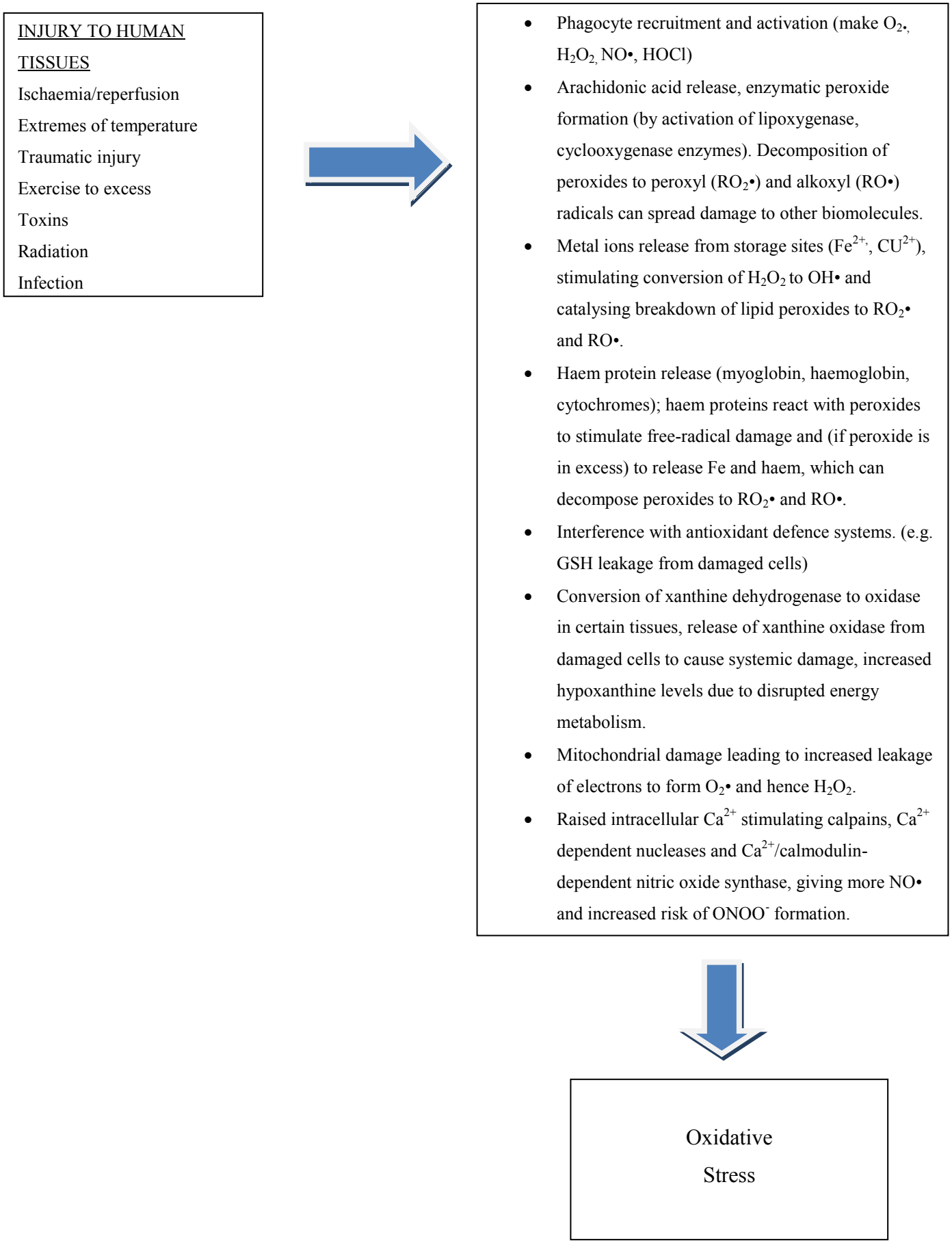


Figure 4: Some of the reasons why tissue injury leads to oxidative stress [16].

Thus RONS are not considered to be deleterious right from the start. Nevertheless, if our cells are exposed to RONS for a longer time, the immune system can´t protect the cells from damage. Oxidative damage and inflammation occur due to oxidation of different biomolecules.
Oxidative stress plays a decisive role in a hundred cases of acute or chronic illness, as an increasing number of studies have shown.
Overproduction of RONS is understood as a trigger for oxidative stress and that does involve all processes accompanied by excessive or low oxygen consumption.
These include:

- Absorption of harmful substances from the environment
- Excessive intake of nutrients
- Physical activity

In contrast to excessive radical production the human immune system uses RONS as a defensive against many pathogens and RONS are also playing an important key role in normal physiological signaling cascades [19].

Antioxidant defenses:

In the human organism there are repair systems against oxidative damage, including endogenous and diet-derived molecules.
It is the antioxidant defense systems task to scavenge reactive oxygen species and to make sure that formation of reactive oxygen species is reduced to the greatest possible extent.
That this is not always the case is underlined by the fact that low levels of products of free radical attack on biomolecules are even found in healthy tissues, proving that antioxidant defenses do not completely prevent from oxidative damage.
Hence, repair of damaged DNA is an important issue [16].

1.2 Tobacco smoke and oxidative stress by means of COPD

Tobacco smoke constitutes an important exogenous resource of free radicals and oxidants. Due to the high responsiveness of reactive oxygen species, these substances are able to oxidize phospholipids by a chain of reactions through lipid peroxidation [20].

As mentioned earlier, oxidative damage of proteins caused mostly by the aqueous tar phase of cigarette smoke is evidenced by the measurement of carbonyl proteins [3; 11]. Measurement of carbonyl proteins is a established method to prove oxidative damage to proteins [21; 22; 23].

Peroxidation of polyunsaturated fatty acids proves a negative impact on membrane functioning, furthermore it inactivates membrane receptors and enzymes and by the way, vascular permeability is increased. These processes are associated with pathogenesis of numerous pulmonary lesions [20].

Cigarette smoke or tobacco smoke consists of a complex mixture of more than 4700 chemical substances and both include high concentrations of free radicals and oxidants (> 1015 particles/pull). The prevailing oxidant is carbon monoxide (CO) with concentrations between 500 and 1000 ppm [24].

The semiquinone radical is a stable radical in the tar phase of cigarette- or tobacco smoke, which may react with oxygen thus forming superoxide, hydroxyl radical or hydrogen peroxide [25].

Pathogenesis of COPD is well studied thus it is recognized that oxidative stress influences the effects of anti-proteases and furthermore the expression of inflammatory mediators [26].

At the current state of research through COPD, specialists recognize that smoking leads to a functional deficiency of alpha-1-antitrypsin by the way of oxidation caused mostly by oxidants contained in cigarette smoke [27].

There exist interesting proof that oxidative stress is an important factor in establishing COPD [20; 28; 29].

Similar to protease-antiprotease-dysbalance, a disturbed balance between oxidants and antioxidants, is considered to be another reason for development of COPD.

Disorders of these two systems are closely meshed with one another. Oxidative stress inactivates anti-proteases as well as pro-inflammatory degradation products may generate new oxidants [30].

Cigarette smoke is the major risk factor for COPD. Inflammatory cells and structural cells like neutrophil granulocytes, eosinophil granulocytes, macrophages and epithelial cells, activated in the airways of COPD patients, produce and secrete ROS [20], especially superoxide anions and hydrogen peroxide.

Superoxide anions are generated by reduction of NADPH (nicotinamide adenine dinucleotide phosphate) and further on they are converted to H_2O_2 through superoxide dismutase. Afterwards H_2O_2 is dismutated to water and molecular oxygen by means of catalase.

In the presence of free iron, H_2O_2 can react thus forming high reactive hydroxyl radical (Fenton reaction). Superoxide anion and NO may cause formation of peroxynitrite ($ONOO^-$), which itself can generate hydroxyl radical (OH•) [6].

$ONOO^-$ reacts with tyrosine residues of proteins under formation of nitrotyrosine derivatives. NO is formed by L-arginine with the participation of NO-synthases. There are two constitutive active representatives (neuronal NOS = nNOS and endothelial NOS = eNOS) and one inducible form (iNOS). The inducible form (iNOS) is regulated by proinflammatory cytokines like TNF-α, INF-γ and IL-β and is able to produce large quantities of NO [31].

Increased NO in lung parenchyma and in the small airways might be associated with elevated iNOS in epithelial cells and macrophages [32; 33].

Among other things oxidative stress induces oxidation of arachidonic acid and consecutive formation of prostanoid mediators, so called isoprostanes. Isoprostanes have an impact on functional effects [34], like bronchoconstriction and plasma exsudation [35; 36; 37].

Granulocytic peroxidases like myeloperoxidase in neutrophil granulocytes play an important key role in oxidative stress. In the presence of chlorine ions H_2O_2, emerged from O_2^-•, is metabolized to hypochloric acid through myeloperoxidase, which itself acts strongly oxidative. Myeloperoxidase is able to nitride tyrosine residues in the same way as peroxynitrite [38; 39; 40].

Peroxidation of polyunsaturated fatty acids affects membrane functions, inactivates membrane-bound receptors and enzymes as well as vascular permeability increases. It is well known, that this processes are associated with pathogenesis of numerous lung diseases [20].

1.3 **Peroxynitrite**

NO* and superoxide anion (O_2^-•) are build in smooth muscle cells, macrophages, vascular endothelial cells, copper cells and many other cells. These two free radicals react very fast to peroxynitrite, which in turn is a strong oxidation and nitration reagent. Peroxynitrite is quite stable, while the protonated form (ONOOH) shows a very short half life of under one second at physiological pH.
The formation of NO is regulated by four nitrogen oxide synthases (iNOS, eNOS, nNOS, mtNOS). Due to this processes there is an average intramitochondrial NO-concentration of 20-500 nM [41; 42].

Among other things NO*-synthesis in the endothelium is triggered by acetylcholine, adenine and bradykinin [43].
Other places of origin for NO* are neutrophil granulocytes, macrophages, neurons and hepatocytes [44].
NO* is responsible for local regulation of blood flow and blood pressure, furthermore it participates in platelet aggregation and neutrophil aggregation [45].
While biochemical tasks and functions of NO* are clarified, this is not the case with tasks and functions of superoxide anion. To maintain a constant NO*-concentration in the human body NADH/NADPH oxidases, xanthine oxidase, cyclooxygenase, NO* synthase (NOS) and arachidonic acid metabolizing enzymes are of special importance [46].

Superoxide anion is generated as by-product of normal cell metabolism, as well as through release from endoplasmatic reticulum and due to electron transport processes.
Other reasons for an increased production of superoxide anion and NO* are pathological processes, like:

- Sepsis
- Arteriosclerosis
- Hyperoximia
- Xenobiotics metabolism
- Inflammation, Ischaemia [41; 43]

In aqueous milieu the reaction of NO* and superoxide anion is running very fast, but in gas phase the reaction is diffusion-limited.
If NO* concentration can be increased by ten times, as well as superoxide anion concentration, peroxynitrite formation rate is increased a hundredfold [43].
Elevated $ONOO^-$ production in mitochondria causes malfunction and a loss of integrity. The intact mitochondrial membrane is permeable for NO, but it is not for superoxide anion. As a consequence the mitochondrial membrane can be harmed due to formation of peroxynitrite. Thus $ONOO^-$ has a short half-life, this radical is able to react with a number of biomolecules like proteins, lipids, hydrocarbons, nucleotides and antioxidative compounds. $ONOO^-$ owns oxidation potential as well as nitration potential and therefore it attacks proven molecules on different places [42].

The most common effects of peroxynitrite are lipid peroxidation, nitration of proteins and different DNA damages, like tyrosine nitration, thiol oxidations and DNA single strand breakages. These modifications towards normal physiological conditions lead to major consequences:

- Inhibition of mitochondrial respiration and mitochondrial permeability
- Several dysfunctions and even promotion of cell death

Previous studies have highlighted the fact that an alteration in the pH value from physiological range to alkaline range causes an increase in the half-life of peroxynitrite and as a consequence attack on target cells is prolonged.
Moreover, it is widely believed that cells, that produce larger quantities of NO after stimulation are developing a safeguard mechanism against self-produced $ONOO^-$ [47].

In neutral solutions $ONOO^-$ breaks down to additional products that can cause oxidative damage. These additional products are nitronium ion (NO_2^+), nitrogen dioxide (NO_2) and hydroxyl radical, one of the strongest free radicals [48].
Not only biomolecules are attacked by $ONOO^-$ but also cells and tissues are getting harmed, leading to tissue damage, cytotoxicity and apoptosis [49].

Enzymatic and non-enzymatic reactions are defanging small amounts of $ONOO^-$ and therefore direct toxic effects can be neutralized. Due to inhibition of nitration reactions uric acid is used as an $ONOO^-$ radical catcher in vivo and in vitro [50].

Summarizing the above, it can be said, that the adverse effects of $ONNO^-$ are caused by different mechanisms [41]:

- Proton catalyzed decomposition of $ONOO^-$ leading to formation of potent secondary oxidants
- Direct reactions of $ONOO^-$ with biomolecules
- Reactions of $ONOO^-$ with bicarbonate leading to formation of secondary toxic reactive compounds

On the basis of the fact that the unpaired electrons of NO* and O_2 link themselves to a new N-O- binding, it has to be said, that $ONOO^-$ is not a free radical strictly spoken [51].

1.4 Consequences of lipid peroxidation

Aldehydes and lipid peroxides come into being due to oxidation of lipids. At very low and nontoxic concentrations aldehydes and lipid peroxides are known to act as signaling transducers of metabolic reactions triggered by reactive oxygen species. It is common knowledge that they modulate cell functions. Just to mention a few, gene expression and cell proliferation are among the modulated cell functions [52].
Due to these processes it comes to formation of lipid-derived products, which in high concentrations, easily react with proteins, DNA and phospholipids. This is not the only reason why they are considered to be the more damaging species, in addition it is also known, that intra- and intermolecular toxic covalent adducts are generated, which is a further reason for persistency of oxidative stress.
In vivo lipid hydroperoxides come into being due to the impact of reactive oxygen species on polyunsaturated fatty acids, they are products of lipoxygenase and COX activities [53].

4-hydroxynonenal (HNE) is the most recurrent lipid-derived product arising from ROS. Depending on its concentration it expedites cell proliferation or ensures oxidative alterations of DNA [54] and apoptosis [55; 56; 57]. 4-HNE takes part in cell cycle control [58].
Due to mutation in the p53 gene expression it plays a role in human cancer [59] and furthermore it is involved in formation of adducts with DNA and upregulates the expression of COX-2 [60].

COX-2 expression is an important factor for production of ROS. LOX and phospholipid hydroperoxide glutathione-peroxidase (PH-GPx) are two enzymes with opposed activities that control the peroxidation of cell membranes.
To maintain a normal redox status in cells the regulatory relation of these enzymes is an important issue. If this regulation is disturbed, cytotoxic molecules are generated which in turn play a role in carcinogenesis [61].
Just to underpin the importance of this fact, it is known, that the inhibition of LOX in carcinosarcoma cells induces apoptosis and furthermore it hinders proliferation of these carcinoma cells [62].

2 Characterization of the relevant compounds

As mentioned in the introduction this diploma thesis will clarify if alpha-ketoglutarate and 5-HMF protect as water soluble substances oxidative damage of proteins exposed to the gas phase of cigarette smoke and on the other hand, if these substances are able to act as scavengers for $ONOO^-$ compared to ascorbic acid.
There follows a short summary of the proposed compounds.

2.1 Alpha-ketoglutarate

Alpha-ketoglutarate, just for reasons of simplicity only called AKG, belonging to the group of alpha-keto acids, owns 5 carbon atoms, 6 hydrogen atoms as well as 5 oxygen atoms. The quotidian production in the human organism is up to two kilogram AKG. AKG further plays an important role in the cells energy production, besides antioxidative effects are relegated to AKG. Due to these characteristics, AKG is able to remove free radicals that could harm proteins, nucleic acids and lipids.
AKG doesn´t only play a role in energy production and as a substance with antioxidative effects, it carries out additional tasks too [18].

2.1.1 AKG and the citric acid cycle

AKG is a physiological intermediate product of the citric acid cycle. In the first step of the reaction of this cycle acetyl radical is transferred on oxalacetate, producing citrate. The citric acid cycle takes its name from this first step of reaction [63].

The citric acid cycle is a metabolic pathway localized in mitochondria, having catabolic and anabolic functions. On the one hand the citric acid cycle initiates the final oxidation of energy substrates and on the other hand the generated intermediate products are converted to glucose, amino acids, lipid acids and isoprenoids [13].

In the citric acid cycle there can be found eight reaction steps, if those are taken into account, in which stable intermediates are formed, not including other reaction steps, in which unstable intermediates are built. Due to NAD^+-dependent oxidation of isocitrate in the 3rd reaction step oxalic succinate comes into being.
In the following spontaneous decarboxylation of oxalic succinate is now emerging AKG. Taking up an amino group AKG can be converted into the amino acid glutamate in a single step. This reaction is an important intersection between the citric acid cycle and the metabolism of amino acids [63].

Hereafter AKG plays an important key role in urea production from amino acids. In this connection glutamate is transported into mitochondria in exchange for OH and AKG is removed from mitochondria in exchange for malate. Following to this reaction re-change of malate against phosphate occurs [64].

In the 4th reaction step succinyl coenzyme-A is built from AKG, an oxidative decarboxylation with following cofactors:

- Thiamine pyrophosphate (TPP)
- Lipon amide
- Coenzyme-A
- FAD and NAD^+

CO_2 and NADH are built in this reaction step. CO_2, which is generated here, represents the large majority of the CO_2 in exhaled air [63].
Furthermore AKG is involved in gluconeogenesis from lactate [64].

2.1.2 AKG in collagen and glutamic acid synthesis

Among other things collagen consists of the two amino acids proline and lysine and is particularly part of tendons, connective tissue, dermis and the gingiva. In order that collagen is formed, a oxygen atom needs to be integrated into lysine and proline. AKG is required for termination of this reaction, whereby AKG is a substance needed for collagen synthesis. Furthermore AKG may be used for formation of glutamic acid, if there was supplied too little with the diet [18].

2.2 **Hydroxymethylfurfurale**

5-HMF is an agent with very complex biochemical effects. On the one hand it prevents the formation of RONS, like H_2O_2 and $ONOO^-$, by consuming singlet-oxygen and its interaction with hydroxyl radicals and superoxide, and on the other hand it increases the expressions of antioxidative enzymes like glutathione and superoxide dismutase, so that higher antioxidative enzymatic capacities are available [65; 66]

5-HMF was shown to reduce the decline of the mitochondrial membrane potential in human cells by pretreatment before hypoxia administration [67].

5-HMF is a chemical compound that contains a aldehyde group and a furan ring. 5-HMF is formed in the so called Maillard-reaction through thermal decomposition of sugar and carbohydrates, therefore it can also be found in heat treated aliments like milk, honey and fruit juices [68; 69].

It is further known that heated sterilization of parenteral solutions induces hexose decomposition to 5-HMF at low pH [70].

5-HMF also affects the pre-systemic metabolism and pharmacokinetics of glycyrrhizin in vivo [71].

In the following a short overview on the characteristics of 5-HMF: [18]

- 5-HMF is a very potent radical catcher and has much greater effect on reactive nitrogen species than vitamin C
- 5-HMF is able to scavenge ammonia and free radicals
- Due to its antioxidative capacity 5-HMF protects molecular oxygen from radical attack and as a consequence oxygen utilization is much better
- 5-HMF prevents from iron (II) and hydrogen peroxide induced cell damage
- 5-HMF is said to have a mood-lifting effect

2.2.1 Side effects of 5-HMF

In recent literature, there is no evidence that 5-HMF poses a serious health risk, even though the highest concentrations in specific foods approach the biologically effective concentration range in cell systems [72].
Dosages of parenterally administered 5-HMF exceeding 75mg/kg body weight have led to some toxic effects including increased activity of hepatic enzymes, altered serum-protein fractions, and hepatic fatty degeneration. Approximately 50% of parenterally administered 5-HMF is oxidized and eliminated by the kidneys [70].

2.3 **Vitamin C**

Vitamin C is a water soluble vitamin, which serves as an important co-factor for a lot of enzymes in the human body as well as it is a strong reducing agent. The biologically active form is L-ascorbic acid that can be derived from most mammals out of glucose. The human being is unable to do so and therefore man must partake vitamin C through food [73; 74].
Vitamin C is an important antioxidant that scavenges hydroxyl-, alcohol-, and peroxide radicals, as well as nitrite and $ONOO^-$. It has the capacity to regenerate other antioxidants like tocopherole, uric acid and beta-carotene.
Vitamin C is present in foodstuffs, above all in fresh fruit (orange, sweet lime, grapefruit, cherry) and fresh vegetables (tomato, broccoli, collard).
Storage and preparation is very important for the vitamin content due to luminous sensitivity and temperature sensitivity of vitamin C [74; 75].

2.3.1 Absorption of vitamin C

Absorption of vitamin C is partly be done in the mouth, but in particular it is done in a Na^+-dependent carrier transport in the jejunum and ileum, in high concentrations increasingly passive.
Taken in normal doses 80 % of the ingested vitamin C is reabsorbed. The transport of vitamin C in the blood is mainly free, only 24 % are transported linked on plasma proteins.
Vitamin C is decomposed into oxalic acid, threose, erythrulose and dioxo gluconic acid.
The metabolites are excreted via urine, in higher doses (3000 mg/d) metabolites are increasingly excreted through the intestines [73; 74].

There is recommended a minimum daily intake of 90 mg/d for men and 75 mg/d for women.

In case of an intake of approximately 100 mg/d, 80-90 % of vitamin C is getting reabsorbed. Gastrointestinal disturbances and diarrhea are the case by ingestion of approximately 2-6 g/d. These side effects can easily be resolved through reduced supply with vitamin C.

If a insufficient supply with vitamin C is the case the clinical picture of scurvy occurs. Leading symptoms are spongy swollen bleeding gums, dry skin, open sores on the skin, fatigue, impaired wound healing and depression.

Stress, cigarette smoke, alcohol abuse, fever and viral infections cause a serious breakdown of plasma level concentration of vitamin C.

These days, the full picture of the disease has come almost to a standstill due to adequate supply of vitamin C through food or dietary supplements.

Studies have shown that a daily intake of 100-120 mg/d of vitamin C have led to a risk reduction of heart disease, stroke and cancer in healthy individuals [75].

3 Material and Methods

H_2O_2, $NaNO_2$, HCl (38%), alpha-ketoglutarate, bovine serum albumine (BSA), guanidine-HCl, butyl-hydroxy-toluene (BHT) were from Sigma Aldrich. Luminol (5-amino-2, 3-dihydro-1,4-phthal-azinedione) and di-nitro-phenyl-hydrazine (DNPH) were obtained from Fluka and 5-hydroxy-methyl-fufurale (5-HMF) was from Degussa, Germany.

Measurements of protein damage

Protein damage by cigarette smoke was assessed by using following setup according to Levine at al. [76]. 50mg/mL bovine serum albumin (BSA) was dissolved in phosphate buffered saline. 4mL of BSA solution was transferred in a suction bottle, which was connected to a water pump. To estimate the concentration dependent protective effect of alpha-ketoglutarate and 5-HMF of oxidative damaged proteins by cigarette smoke different concentrations of α-ketoglutarate and 5-HMF were used (0, 0.035, 0.071 and 0.106mM) and compared to the antioxidative capacity of ascorbic acid.
Before smoking two 100 µL aliquots of BSA-solution were diluted with 1150 µL 10mM PBS pH 7.4 containing 40µM BHT to obtain a 4mg/mL BSA solution. 1mg of protein was precipitated by pipetting 250µL of the 4mg/mL protein solution with 250µL 20 % trichloric acid (TCA) solution. After smoking within 2 minutes further aliquots were diluted and precipitated with TCA. The glass was closed and incubated at 37°C. After 15, 30 and 60 minutes aliquots were taken out, diluted and precipitated. All precipitated samples were centrifuged at 5000g for 3 minutes and the supernatant was removed. Pellets were dissolved in 10mM DNPH containing 6 M guanidine pH 2.5 and incubated for 45 minutes at room temperature. Protein damage was measured spectrophotometrically with the carbonyl protein assay according to Levine et al.[76].

Peroxynitrite scavenging measurements by chemiluminescence technique

$ONOO^-$ was prepared according to the method previously described [77] by incipient mixing of equal volumes of 0.7 M H_2O_2-solution in 0.6 M HCl and 0.6 M $NaNO_2$ on ice and immediate termination of the reaction with 1.5 M NaOH. Surplus H_2O_2 was removed by adding MnO_2 and following filtration of the suspension. The $ONOO^-$ concentration was

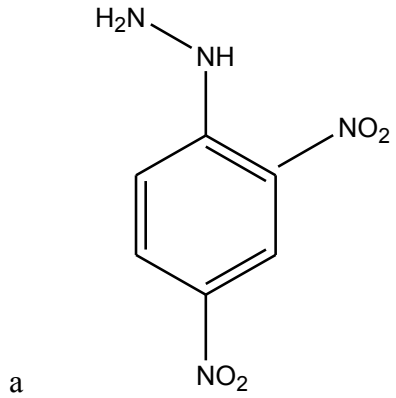
determined spectrophotometrically at 302nm with a coefficient of extinction of 1670 $M^{-1}cm^{-1}$.

Consumption of peroxynitrite was measured with luminescence technique according to Radi et al. [41].

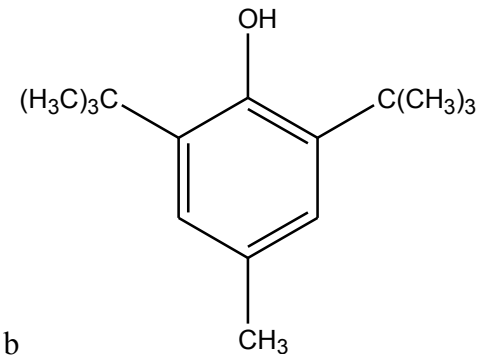
1 µL of 20mM $ONOO^-$ was transferred in a well of a white microtitration plate (Nunc). 5µL of 0, 0.16, 0.8, 4, 20, 200 and 1M separate solutions of alpha-ketoglutarate, 5-HMF and ascorbic acid in PBS was pipetted directly to $ONOO^-$. Chemiluminescence signals were detected on a BMG Luminescence plate reader (Lumistar, BMG, Germany) for each second between 0 and 40 seconds. Luminescence signal is expressed with counts per second (cps).

3.1 **Measurement of oxidative protein damage**

During the reaction of the intermediates of the lipid peroxidation (aldehydes) with proteins it comes to formation of carbonyl groups on the side chains of amino acids. If these proteins are reacting with the reagent Dinitrohydrazine which specifically reacts with carbonyl groups, it comes to formation of protein adducts which are featuring a special coloration that can be quantified photometric.



a



b

Figure 5: Dinitrophenylhydrazine (DNPH); Figure 6: Butylhydroxytoluene (BHT)

Execution of the measurement

The measurement of the oxidative protein damage was accomplished on three days running. It follows a explanation how this was done.

Following reagents, materials and equipment were required:

Required reagents:

PBS-buffer (pH = 7.4)

Reagent 1 (biquinoline acid)

Reagent 2 (4% $CuSO_4$-solution)

Test solution of unknown concentration

Required materials and equipment

Test tubes

Pipette

Photometer

Cuvette

Water bath (37°C)

3.2 **Production of the working solution**

To produce the working solution it was necessary to pipette 10 ml reagent and 200 µl reagent 2 into a test tube (green colored solution)

Production of the standard with different concentrations

The standard had a concentration of 1 mg/mL. Diluting this standard with a PBS-buffer different solutions, with different concentrations were established:

1 mL Std + 1 mL PBS = Std 1 (0.5 mg/mL)
1 mL Std 1 + 1 mL PBS = Std 2 (0.25 mg/mL)
1 mL Std 2 + 1 mL PBS = Std 3 (0.125 mg/mL)

Attenuation of the sample solution of unknown concentration

The assay was diluted 1:300 with PBS-buffer

1.) 100 µl assay + 900 µl PBS (1:10 = Dilution 1)
2.) 10 µl Dilution 1 + 290 µl PBS (1:30 = Dilution 2)

Photometric determination of the protein amount

1. Repeat determinations were done
2. The photometer was enabled and the wavelength was adjusted to 562 nm

- 75 µl of the diluted assay of the different standards as well as PBS-buffer were pipetted into a labeled test tube (double preparation, 10 test tubes in all)
- Afterwards 750 µl working solution was added to every test tube, then the samples had to be stired well.
- All test tubes were incubated in a water bath for 30 minutes (60°C)
- After the incubation the test tubes had to be cooled down (water pipe)
- A bulb was filled with PBS-buffer, which was necessary to adjust the point of origin on the photometer.
- Afterwards the assays were measured, starting with the zero value.
- The extinctions had to be noted and interpreted with the aid of a computer afterwards.
- To determine carbonyl the amount of protein had to account 50 mg/ml, to that effect the assay had to be diluted with PBS- buffer.

3.3 **Oxidation solution**

Required reagents:

- PBS-buffer (Phosphate buffered saline, pH 7.4)
- Albumin solution (BSA)
- 20 % TCA- solution (+ 4°C, trichloroacetic acid)
- 10 mM DNPH in 2M HCL (lightproof)
- Ethylacetate / ethanol (1:1)
- BHT-solution: 20 mmol /L (Butylhydroxitoluen, Abb.1b)
- Guanidine-buffer (6M, pH 2.5)
- Antioxidant

Required materials and equipment

- Cigarette
- Pipe
- Feeding bottle with circuit points
- Eppendorf tube (1.5 mL)
- Pasteurpipettes
- Pipettes
- Vortexer (magnetic stirrer with heating)
- Plastic spattle
- Waste bin for organic solvent
- Centrifuge (Eppendorf-tubes)
- Refrigerator
- Photometer
- Half-micro cuvette
- Incubator (37°C)

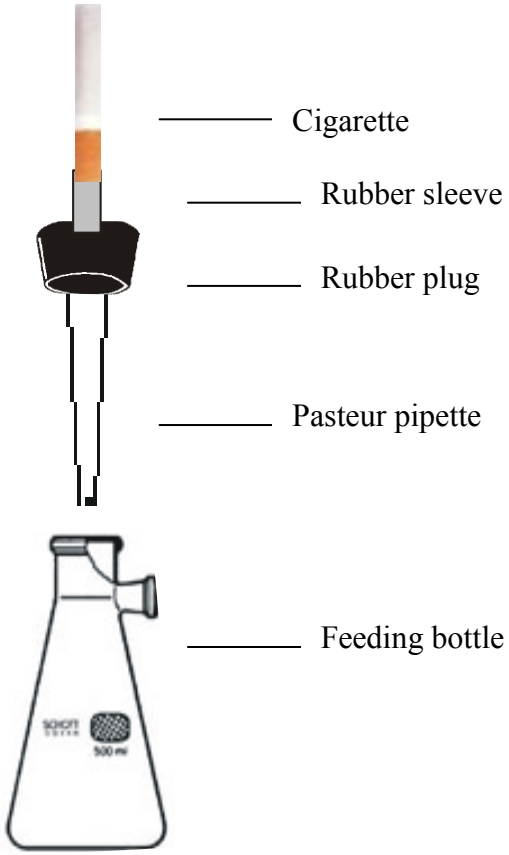


Figure 7: Test assembly for simulated smoking

3.4 **Instruction sheet**

1.) 3 ml of the BSA-solution ought to be given to an unknown amount of antioxidant into the feeding bottle.
2.) Afterwards a cigarette should be "smoked" (5-6 min). Assembling of the equipment as shown in the illustration. Timekeeping starts with beginning smoking.
3.) After smoking the feeding bottle needs to be occluded and incubated at 37°C.
4.) On the eve of beginning smoking, after smoking (5-7 min) and after 30 minutes two times 100 µl aliquots need to be taken from the feeding bottle. Lettering as follows:

Option number + description (a = before smoking, b = directly after smoking, c = 30 minutes after smoking)

A.) Ever one 100 µl aliquot needs to be pipetted into an Eppendorf - tube and frozen at -20°C.
B.) For the second aliquot following ought to be primed:

- 1150 µL PBS-buffer given into a labeled Eppendorf- tube
  + 2.5 µl BHT-solution (being on the verge of addition of the BSA-solution)
  + 100 µl oxidized BSA-solution
- Vortexer

<u>The work needs to be continued with the assays of the second aliquot:</u>

1. 250µl from the oxidation lug (option number a, option number b, option number c) are pipetted in a Eppendorf tube (two times, with the same description)
2. 250 µl from the 20% TCA-solution need to be added to each tube, then everything needs to be mixed and afterwards it has to be put into the refrigerator for 10 minutes at 4°C.
3. Taken out from the refrigerator all needs to be centrifugalized for 3 minutes at 5000 rpm.
4. The supernatant needs to be taken off with a pipette and abolished.

3.5 DNPH-reaction and photometric measurement

1. 500 µL DNPH-solution needs to be pipetted to the precipitate and mixed up with a plastic spattle.
2. The lugs need to be incubated 45 minutes at room temperature and mixed up all 15 minutes.
3. 500 µL 20 % TCA-solution needs to be added and mixed up.
4. All needs to be centrifugalized for 3 minutes at 1100 rpm.
5. The supernatant needs to be taken off with a pipette and abolished.
6. The pellet needs to be spiked with 1 mL ethyl acetate/ethanol and mixed up.
7. All needs to be centrifugalized for 3 minutes at 1100 rpm.
8. Steps 9 to 11 are to repeat twice
9. 500 µL of the 6 M guanidine-buffer need to be added to the pelett.
10. The last step was to solve everything for 15 minutes at room temperature and then all had to be mixed in constant intervals.

Photometric measurement:

Wavelength: 360 nm
Blank: 500 µL 6 M guanidine-buffer
Calculation: Carbonyl protein nmol/mg = Extinction / 0.044

Statistics

The indicated data are means ± SD. Data was compared using a paired t-test. Significance of correlation is based on linear regression.

4 Results

In order to determine the level of oxidative damage in human cells, measurement of carbonyl proteins is a very sensitive method. Combination of AKG and 5-HMF was estimated to inhibit protein carbonyl formation during exposure of cigarette smoke by reactive oxygen and nitrogen species. Another question was if inhibition of carbonyl formation trough AKG and 5-HMF was more efficient than inhibition through ascorbic acid.

4.1 Measurement of oxidative protein damage

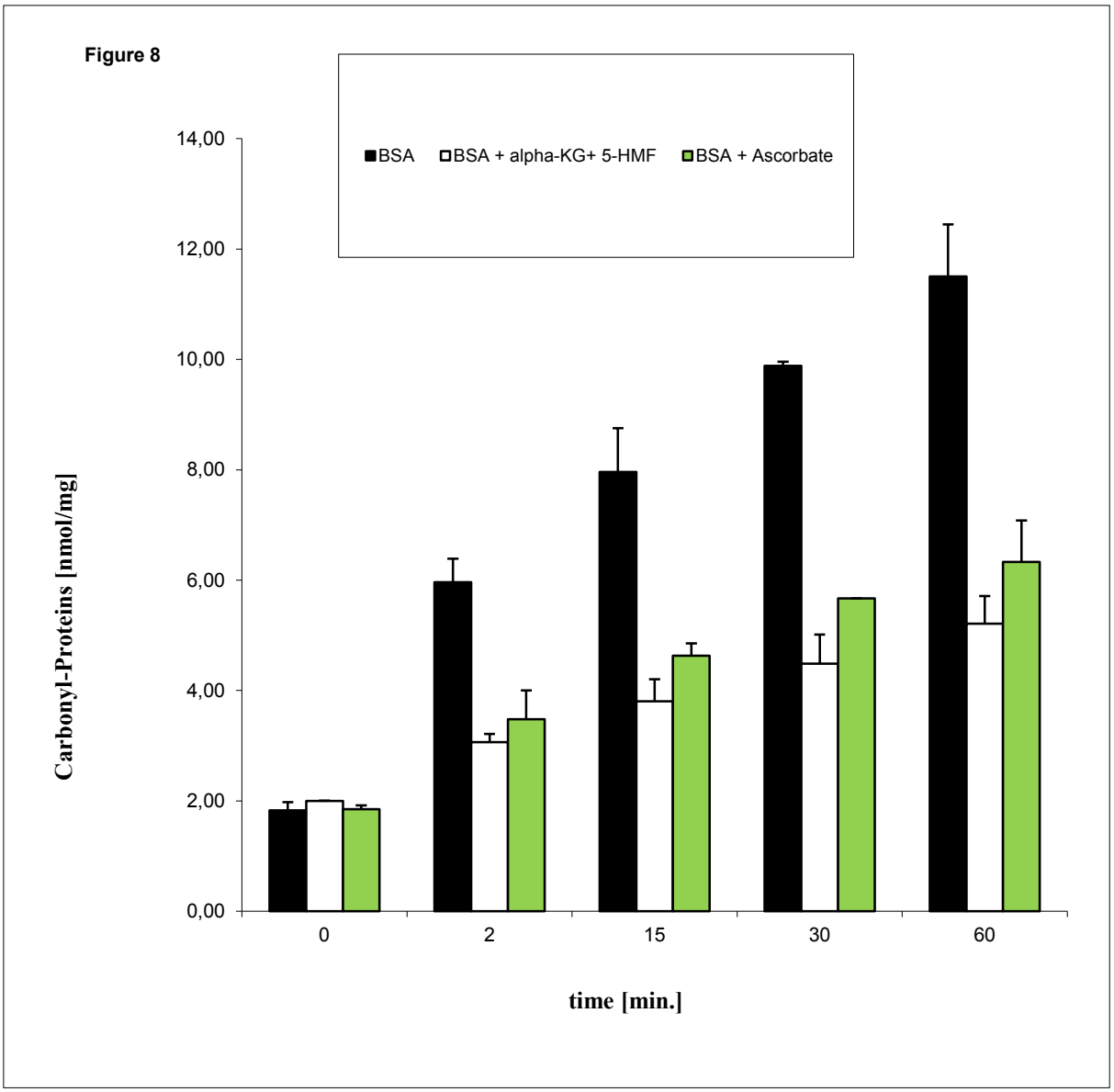


Figure 8: Inhibition of carbonyl proteins of a BSA-solution with alpha-ketoglutarate and 5-HMF during exposure of cigarette smoke compared to a 0.568 mmol ascorbic acid solution and BSA-control solution

Figure 8 showed effectively the inhibition of carbonyl proteins of a BSA-solution with both substances, alpha-ketoglutarate and 5-HMF, during exposure of cigarette smoke compared to ascorbic acid solution or compared to BSA control solution.

The reduction of damaged proteins by alpha-ketoglutarate and 5-HMF together was effective and highly significant lower after 2 minutes [5.96 +/- 0.43 nmol/mg (BSA) vs. 3.06 +/- 0.15 nmol/mg (BSA+ AKG/5-HMF), $p < 0.001$], after 15 minutes [7.96+/- 0.79 nmol/mg (BSA) vs. 3.8 +/- 0.4 nmol/mg (BSA + AKG/5-HMF), $p < 0.001$], after 30 minutes [9.89 +/- 0.07 nmol/mg (BSA) vs. 4.49 +/- 0.53 nmol/mg (BSA +AKG/5-HMF), $p < 0.001$] and after 60 minutes [11.51 +/- 0.9 nmol/mg (BSA) vs. 5.21 +/- 0.5 nmol/mg (BSA + AKG/5-HMF), $p< 0.001$] compared to BSA control solution.

Using equimolare concentrations of ascorbic acid (0.568 mmol) like alpha-ketoglutarate and 5-HMF an effective decrease of carbonyl proteins was estimated compared to control BSA solution. At all time points reduction of damaged proteins by ascorbic acid was highly significant but not as effective as by the combination of alpha-ketoglutarate and 5-HMF.

Already after 2 minutes of direct cigarette smoke exposure a significant reduction has been observed [5.96 +/- 0.43 nmol/mg (BSA) vs. 3.48 +/- 0.52 nmol/mg (BSA + Ascorbate), $p< 0.001$].

This effect was seen over all time points, whereby at this point reduction of carbonyl proteins through ascorbic acid constantly was a little bit weaker than reduction through the combination of alpha-ketoglutarate and 5-HMF [7.96 +/- 0.79 nmol/mg (BSA) vs. 4.63 +/- 0.22 nmol/mg (BSA + Ascorbate), $p<0.001$ after 15 minutes; 9.89 +/- 0.07 nmol/mg (BSA) vs. 5.67 +/- 0.0 nmol/mg (BSA + Ascorbate), $p<0.001$ after 30 minutes; 11.51 +/- 0.94 nmol/mg (BSA) vs. 6.33 +/- 0.75 nmol/mg (BSA + Ascorbate), $p<0.001$ after 60 minutes].

To have some insight which of the three substances namely alpha-ketoglutarate, ascorbic acid and 5-HMF had more inhibitory effect on protein damage compared to BSA control solution, several concentrations of each substances in BSA solutions were exposed to cigarette smoke. Inhibitory effects of alpha-ketoglutarate, ascorbic acid and 5-HMF on protein damage compared to BSA control solution are illustrated in Figure 9, 10 and 11.

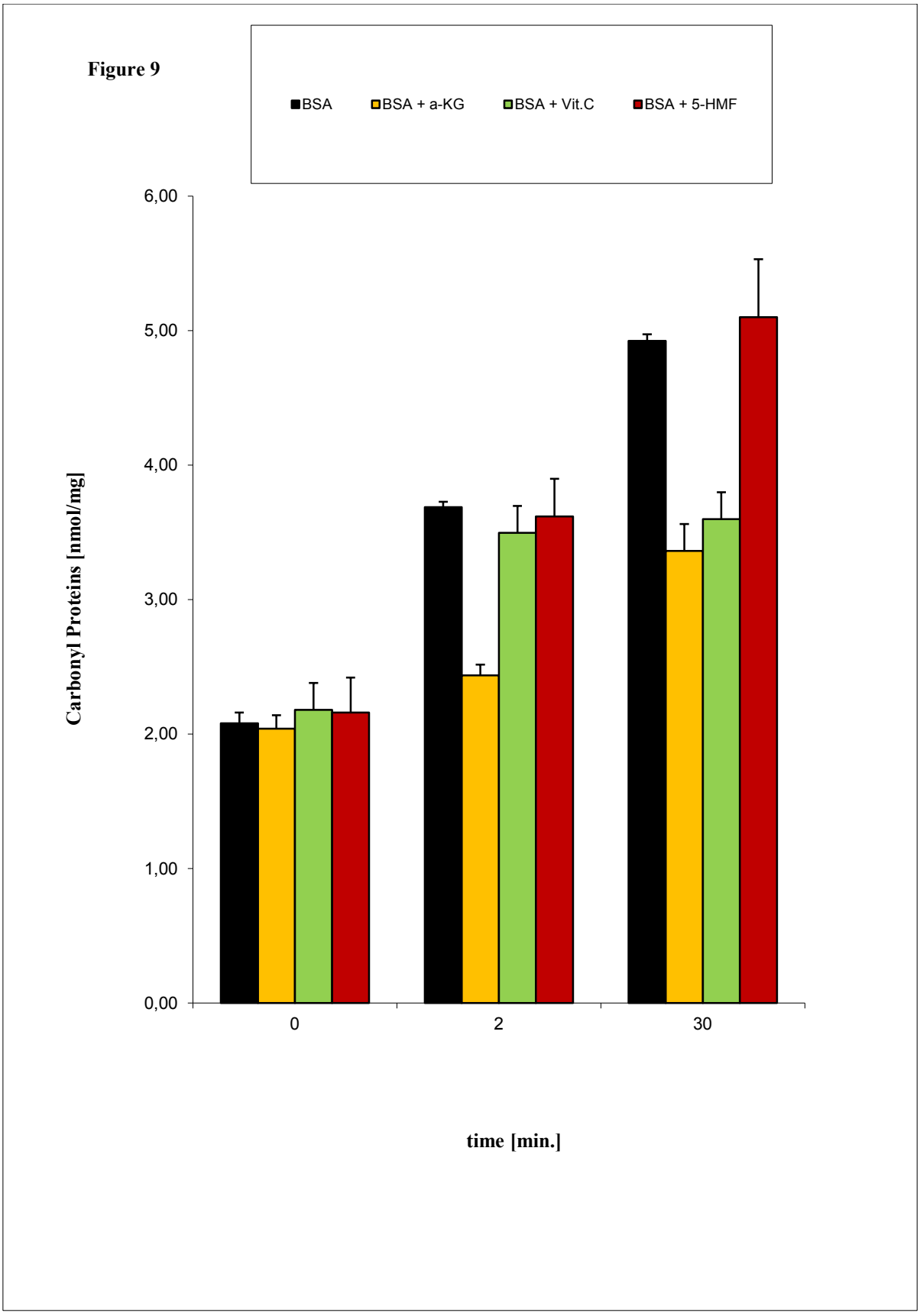


Figure 9: Inhibitory effects of 0.284 mmol of α-ketoglutarate, 0.284 mmol of ascorbic acid and 0.284 mmol of 5-HMF on protein damage compared to BSA

Figure 9 already showed a significant reduction of carbonyl proteins through alpha-ketoglutarate compared to BSA solution after 2 minutes [3.69 +/- 0.0 nmol/mg (BSA) vs. 2.44+/- 0.08 nmol/mg (BSA + AKG) , $p<0.001$] and after 30 minutes [4.92 +/- 0.1 nmol/mg (BSA) vs. 3.36+/- 0.2 nmol/mg (BSA + AKG), $p<0.001$]. This effect could have been seen over all time points in Figure 10 after 2 minutes [3.69 +/- 0.12 nmol/mg (BSA) vs. 2.49+/- 0.3 nmol/mg (BSA + AKG), $p<0.001$] and after 30 minutes [4.92 +/- 0.17 nmol/mg (BSA) vs. 2.66+/- 0.08 nmol/mg (BSA + AKG), $p<0.001$] as well in Figure 11 after 2 minutes [3.69 +/- 0.2 nmol/mg (BSA) vs. 2.44+/- 0.1 nmol/mg (BSA + AKG), $p<0.001$] and after 30 minutes [4.92 +/- 0.1 nmol/mg (BSA) vs. 2.63+/- 0.12 nmol/mg (BSA + AKG), $p<0.001$].

Reduction of carbonyl proteins through ascorbic acid compared to BSA control solution was given, while not as effective as reduction through alpha-ketoglutarate and not significant at lowest concentration after 2 minutes in Figure 9. This could have been shown in Figure 9 after 2 minutes [3.69 +/- 0.0 nmol/mg (BSA) vs. 3.5+/- 0.2 nmol/mg (BSA + Ascorbate), $p<0.001$] and after 30 minutes [4.92 +/- 0.1 nmol/mg (BSA) vs. 3.6+/- 0.2 nmol/mg (BSA + Ascorbate), $p<0.001$].

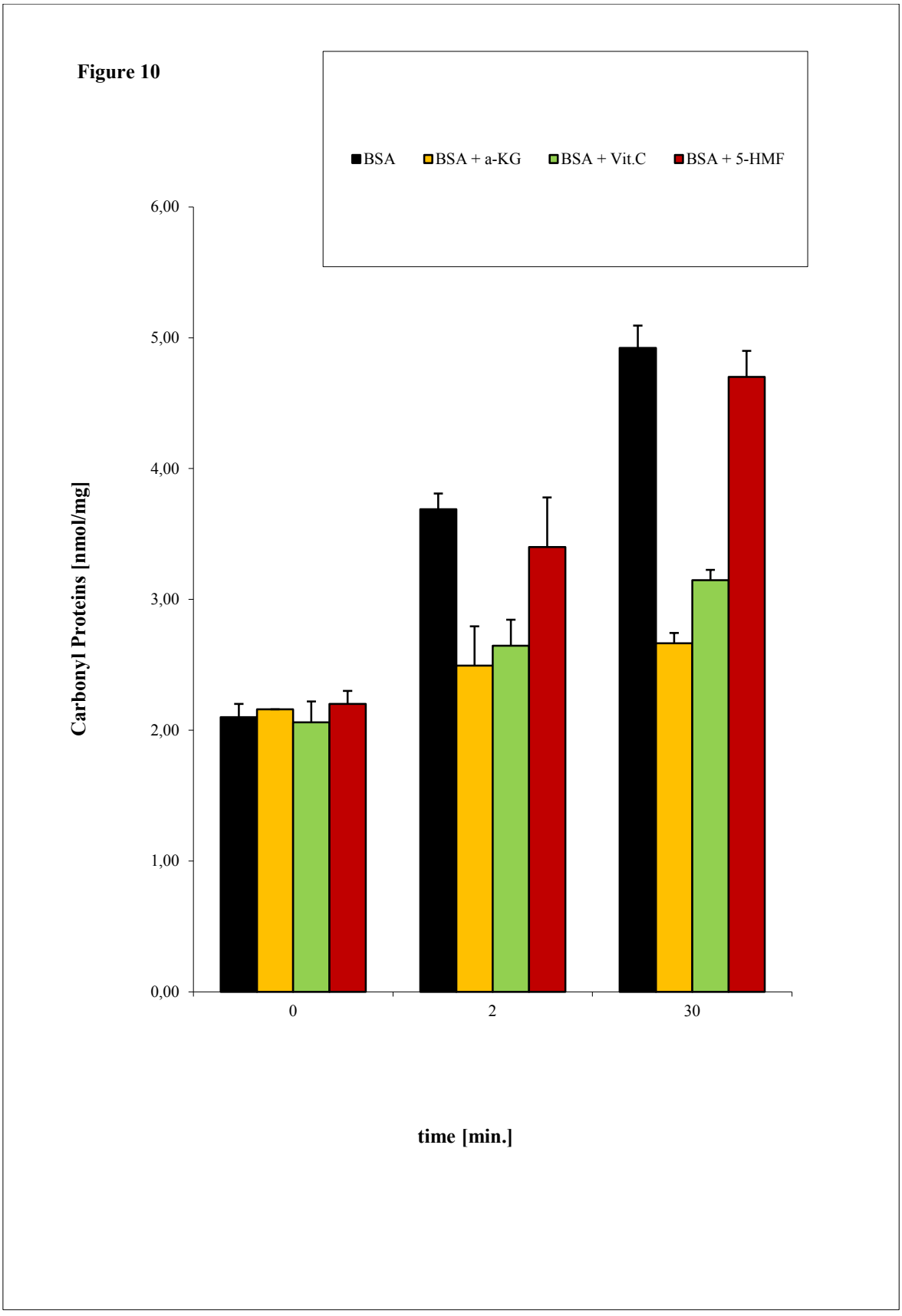


Figure 10: Inhibitory effects of 0.426 mmol of α-ketoglutarate, 0.426 mmol of ascorbic acid and 0.426 mmol of 5-HMF on protein damage compared to BSA control

At higher concentrations reduction of carbonyl proteins through ascorbic acid compared to BSA control solution again was significant, even though weaker than reduction through alpha-ketoglutarate. This became apparent in Figure 10 after 2 minutes [3.69 +/- 0.12 nmol/mg (BSA) vs. 2.64+/- 0.2 nmol/mg (BSA + Ascorbate), $p<0.001$] and after 30 minutes [4.92 +/- 0.17 nmol/mg (BSA) vs. 3.15+/- 0.08 nmol/mg (BSA + Ascorbate), $p<0.001$] and as well at highest concentration in Figure 11 after 2 minutes [3.69 +/- 0.2 nmol/mg (BSA) vs. 2.74+/- 0.1 nmol/mg (BSA + Ascorbate), $p<0.001$] and after 30 minutes [4.92 +/- 0.1 nmol/mg (BSA) vs. 2.93+/- 0.2 nmol/mg (BSA + Ascorbate), $p<0.001$].

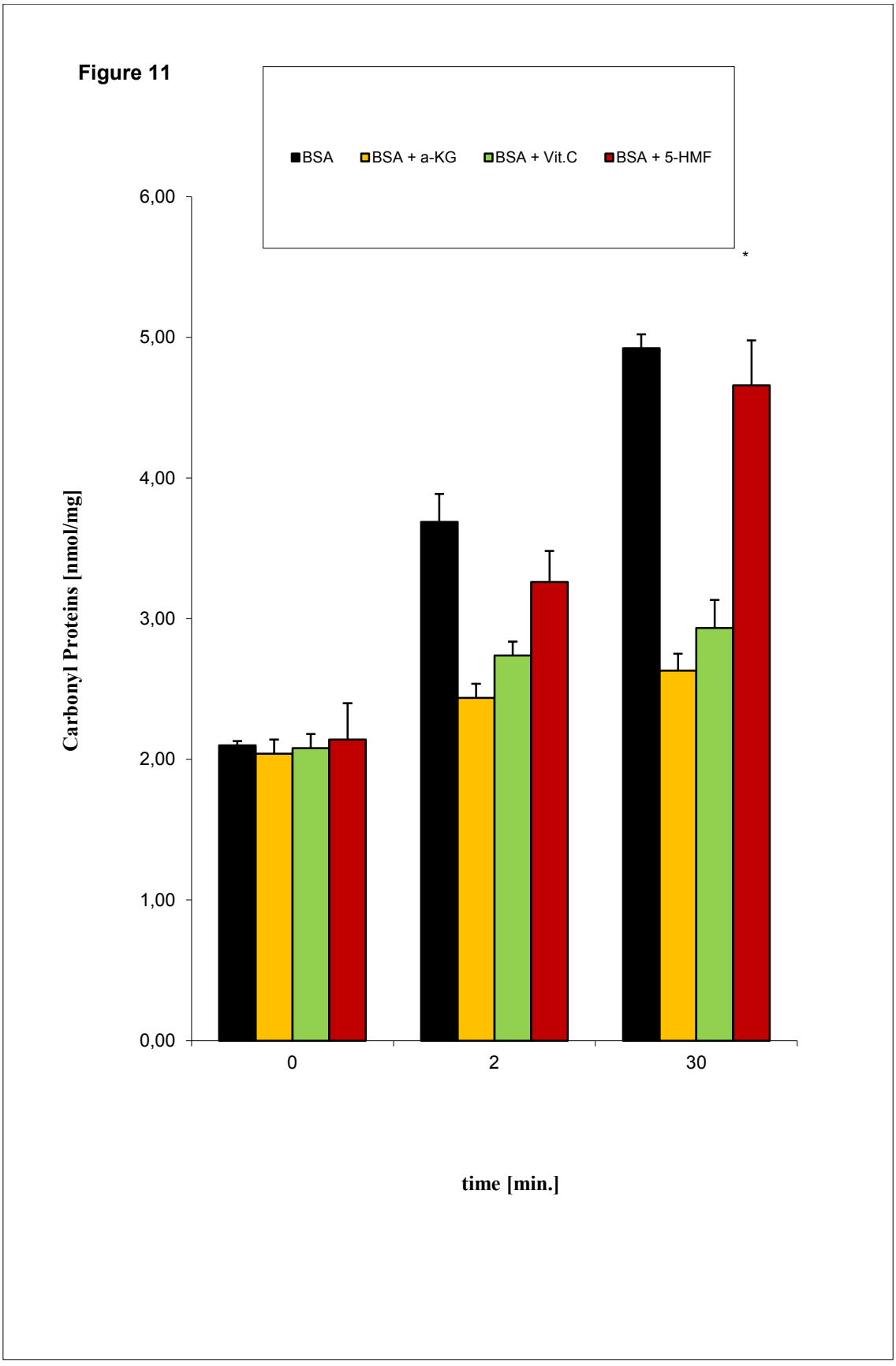


Figure 11: Inhibitory effects of 0.568 mmol of α-ketoglutarate, 0.568 mmol of ascorbic acid and 0.568 mmol of 5-HMF on protein damage compared to BSA control

Both alpha-ketoglutarate and ascorbic acid decreased the carbonyl content of the BSA solution better than 5-HMF as shown in Figure 9 after 2 minutes [2.44 +/- 0.08 nmol/mg (BSA + AKG) vs.3.5 +/- 0.2 nmol/mg (BSA + Ascorbate) vs. 3.62 +/- 0.28 nmol/mg (BSA + 5-HMF)] and after 30 minutes [3.36 +/- 0.2 nmol/mg (BSA +AKG) vs. 3.6+/- 0.2 nmol/mg (BSA + Ascorbate) vs. 5.1 +/- 0.43 nmol/mg (BSA + 5-HMF)].

The same results could have been seen in Figure 10 after 2 minutes [2.49 +/- 0.3 nmol/mg (BSA + AKG) vs. 2.64+/- 0.2 nmol/mg (BSA + Ascorbate) vs. 3.4 +/- 0.38 nmol/mg (BSA + 5-HMF)] and after 30 Minutes [2.66 +/- 0.08 nmol/mg (BSA + AKG) vs. 3.15+/- 0.08 nmol/mg (BSA + Ascorbate) vs. 4.70 +/- 0.2 nmol/mg (BSA + 5-HMF)] and Figure 11 after 2 minutes [2.44 +/- 0.1 nmol/mg (BSA + AKG) vs. 2.74+/- 0.1 nmol/mg (BSA + Ascorbate) vs. 3.26 +/- 0.22 nmol/mg (BSA + 5-HMF)] and after 30 minutes [2.63 +/- 0.12 nmol/mg (BSA + AKG) vs. 2.93+/- 0.2 nmol/mg (BSA + Ascorbate) vs. 4.66 +/- 0.32 nmol/mg (BSA + 5-HMF)]

Interestingly at lowest concentrations (0.284 mmol) there wasn´t seen a significant reduction of carbonyl proteins through 5-HMF compared to BSA control solution as shown in Figure 9 after 2 minutes [3.69 +/- 0.0 nmol/mg (BSA) vs. 3.62 +/- 0.28 nmol/mg (BSA + 5-HMF)] and after 30 minutes [4.92 +/- 0.1 nmol/mg (BSA) vs. 5.1 +/- 0.43 nmol/mg (BSA + 5-HMF)] while at the next at higher concentration (0.426) in Figure 10 there was seen a decrease after 2 minutes [3.69 +/- 0.12 nmol/mg (BSA) vs. 3.40 +/- 0.38 nmol/mg (BSA + 5-HMF), p=0.1957846] and after 30 minutes [4.92 +/- 0.17 nmol/mg (BSA) vs. 4.70 +/- 0.2 nmol/mg (BSA + 5-HMF), p=0.14470159].

At highest concentration of 5-HMF in a BSA solution protein damage was decreased significant, but only slightly which could have been seen in Figure 11 after 2 minutes [3.69 +/- 0.2 nmol/mg (BSA) vs. 3.26 +/- 0.2 nmol/mg (BSA + 5-HMF), p=0.02760356] and after 30 minutes [4.92 +/- 0.1 nmol/mg (BSA) vs. 4.66 +/- 0.32 nmol/mg (BSA + 5-HMF), p=0.1718758].

4.2 Peroxynitrite scavenging measurements by chemiluminescence technique

In order to answer the question if α-ketoglutarate, 5-HMF and ascorbic acid are able to act as scavengers for peroxynitrite ($ONOO^-$), measurement by chemiluminescence technique is a well established method.

Chemiluminescence is understood as light emission in connection with a chemical reaction, whereby the light isn`t of thermal origin. The respective energy for the electrons excitation comes from the chemical reaction itself. The energy released from the particles on returning to their original state is emitted in form of light [78].

Peroxynitrite needs to react with luminol for the creation of the luminescence signal, so that electrons stemming from $ONOO^-$ can be transferred to the luminol molecule for the purpose of oxidation.

Chemiluminescence signals were detected on a BMG luminescence plate reader for each second between 0 and 40 seconds. Luminescence signal is expressed with counts per second. The blank values arose from the single influence of $ONNO^-$ on the luminol molecule.

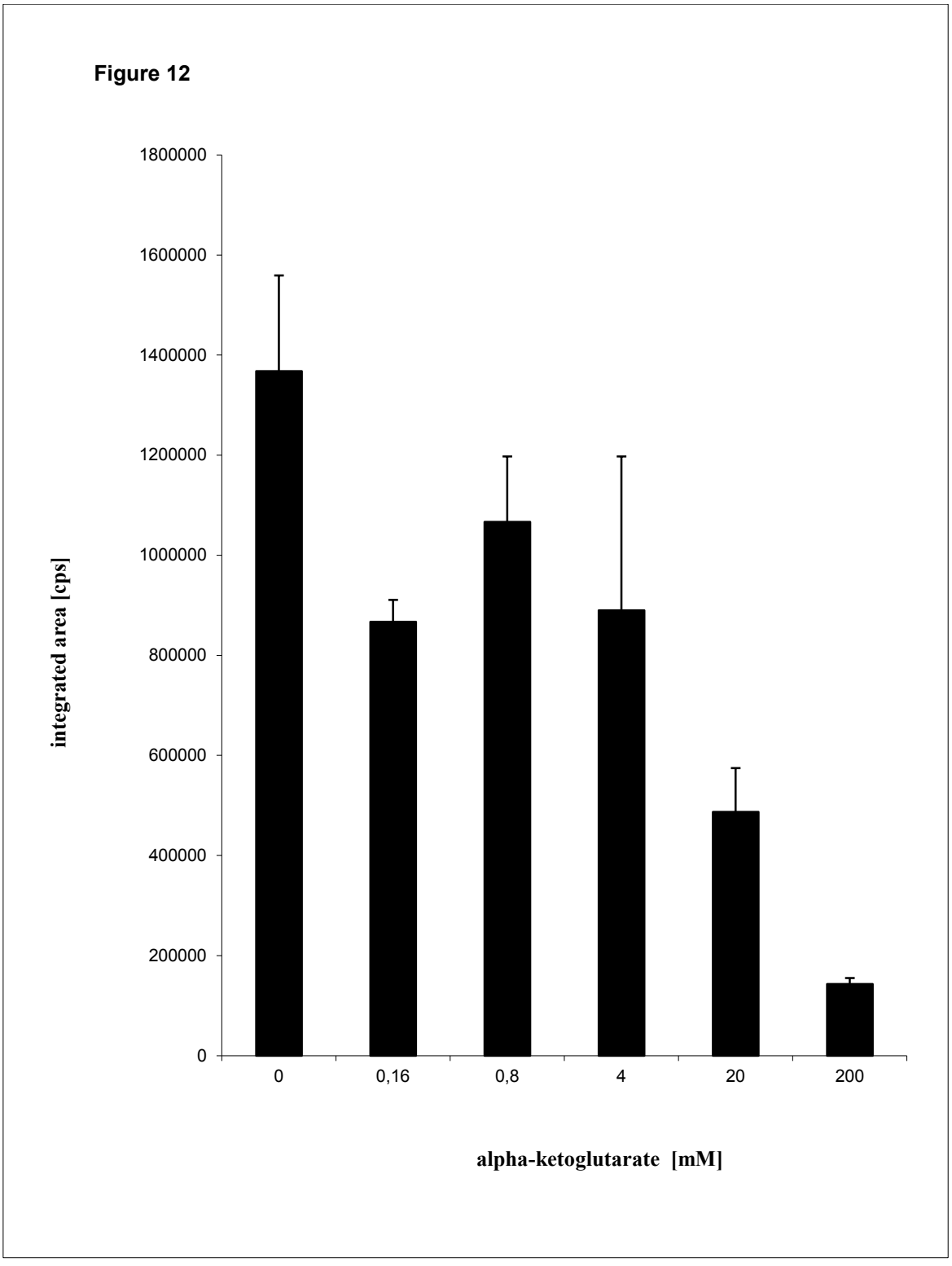


Figure 12: Inhibiting effects of α-ketoglutarate on the luminol reaction. Effects of different concentrations of α-ketoglutarate (0.16mM, 0.8mM, 4mM, 20mM, 200mM) on the oxidation of luminol through $ONOO^-$ during chemiluminescence were analyzed. The measured counts per second (cps) against time and hence the computed integrated areas are shown on the y-axis, the different concentrations of α-ketoglutarate that were used are represented on the x-axis.

Figure 12 showed the inhibiting effects of α-ketoglutarate on the luminol reaction. By the addition of α-ketoglutarate in different concentrations (0.16mM, 0.8mM, 4mM, 20mM, 200mM) its antioxidative potential could have been shown impressively, due to the significant reduction of the measured signals through all concentrations used. All measured values differed considerably from the measured blank value ($p<0.01$). At a concentration of 200mM α-ketoglutarate showed an almost complete reduction of the measured value. In comparison to the other investigated compounds (ascorbic acid, 5-HMF), α-ketoglutarate showed second largest antioxidative potential.

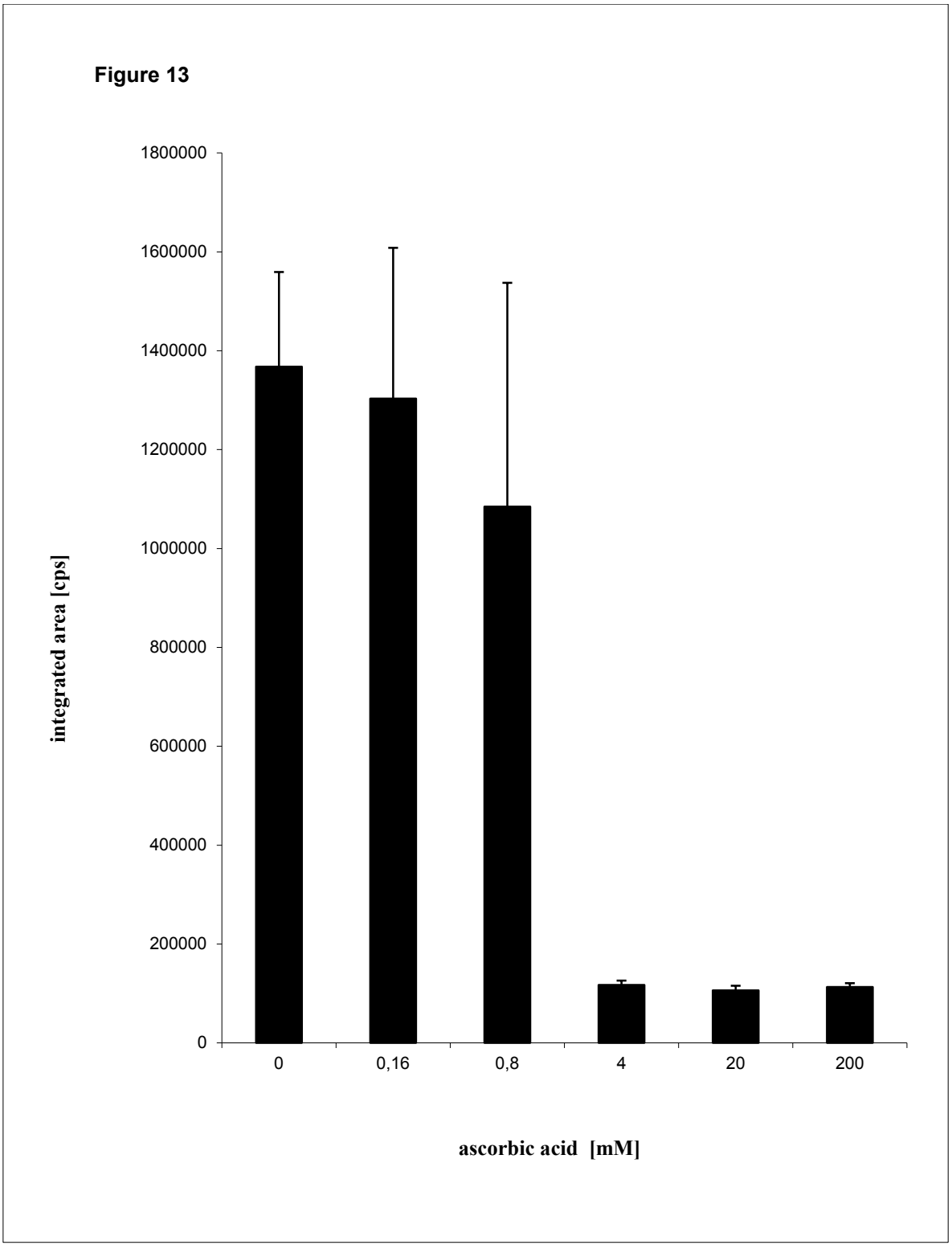


Figure 13: Inhibiting effects of ascorbic acid on the luminol reaction. Effects of different concentrations of ascorbic acid (0.16mM, 0.8mM, 4mM, 20mM, 200mM) on the oxidation of luminol through $ONOO^-$ during chemiluminescence were analyzed. The measured counts per second (cps) against time and hence the computed integrated areas are shown on the y-axis, the different concentrations of ascorbic acid that were used are represented on the x-axis.

Figure 13 showed the inhibiting effects of ascorbic acid on the luminol reaction. Effects of different concentrations of ascorbic acid (0.16mM, 0.8mM, 4mM, 20mM, 200mM) on the oxidation of luminol through $ONOO^-$ during chemiluminescence were analyzed in the same manner as through α-ketoglutarate before. At the lowest concentration level of 0.16mM ascorbic acid showed no antioxidative potential, due to the fact that there wasn´t a significant reduction of the measured values in comparison to the blank value. At a concentration of 0.8mM ascorbic acid showed an almost complete attenuation of the measured signal ($p<0.0.5$). In concentrations of 4mM, 20mM and 200mM ascorbic acid showed its antioxidative potential since there was seen a highly significant and nearly complete inhibition of the chemiluminescence signal ($p<0.01$).Compared with α-ketoglutarate and 5-HMF, ascorbic acid showed the strongest antioxidative potential.

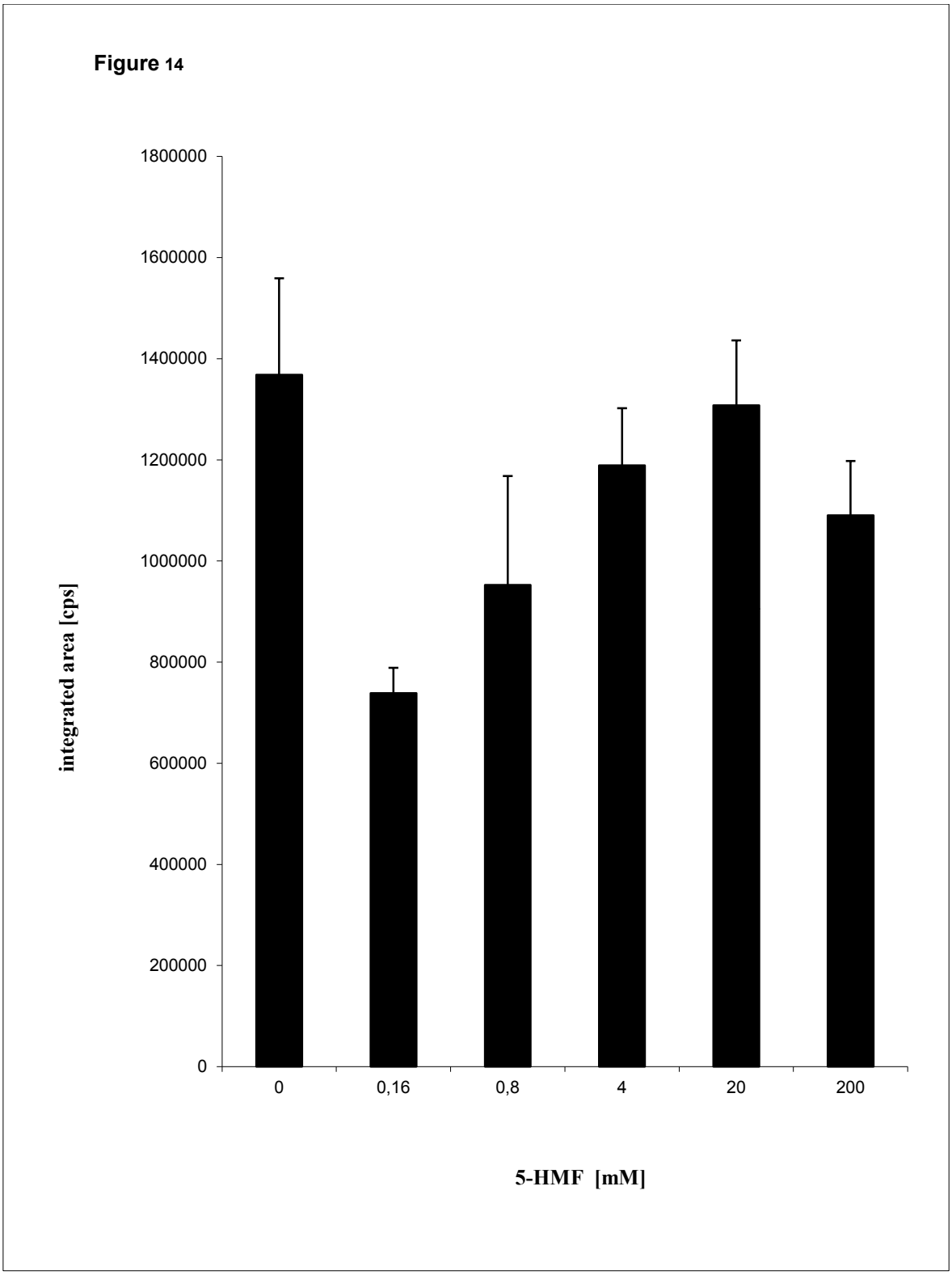


Figure 14: Inhibiting effects of 5-HMF on the luminol reaction. Effects of different concentrations of 5-HMF (0.16mM, 0.8mM, 4mM, 20mM, 200mM) on the oxidation of luminol through $ONOO^-$ during chemiluminescence were analyzed. The measured counts per second (cps) against time and hence the computed integrated areas are shown on the y-axis, the different concentrations of 5-HMF that were used are represented on the x-axis.

In Figure 14 again inhibiting effects, in this case through 5-HMF, on the luminol reaction were under investigation. It has already been shown, that ascorbic acid had the strongest antioxidative potential and α-ketoglutarate the second strongest. 5-HMF showed the weakest antioxidative potential of all three substances.

Figure 14 showed that at lowest concentration levels 5-HMF had the biggest effects. In concentrations of 0.16mM and 0.8mM of 5-HMF there was seen a significant attenuation of the measured signals in comparison to the blank value ($p<0.01$). At the next higher concentrations (4mM, 20mM, 200mM) this effect has not been observed, there was no more significant attenuation of the measured signals in comparison to the blank value ($p>0.05$).

This phenomenon follows from the redox properties of 5-HMF depending on the relation between 5-HMF concentration and radical concentration. At low concentrations of 5-HMF $ONOO^-$ can be scavenged by 5-HMF, but at higher concentrations 5-HMF strengthens the luminescence signal

5 Discussion

This diploma thesis tried to outline the question, if α-ketoglutarate and 5-HMF protect as water soluble substances oxidative damage of proteins exposed to the gas phase of cigarette smoke and on the other hand, if these substances are able to act as scavengers for $ONOO^-$ as one of the most potent oxidizing and nitrating reagent produced from cigarette smoke compared to ascorbic acid. Due to the fact that there is an increasing and various number of diseases resulting from oxidative stress there is a particular significance attached to this question as well as to the question which opportunities are existing in the treatment of these diseases.

There is proven knowledge that exposure of human plasma to gas phase and whole cigarette smoke initiates or directly promotes oxidative modifications of proteins, lipids, carbohydrates and DNA by thousands of chemicals including free radicals, reactive oxygen species (RONS) and saturated and unsaturated aldehydes [1; 2; 3; 4]

Tobacco smoke constitutes an important exogenous resource of free radicals and oxidants. Due to the high responsiveness of reactive oxygen species, these substances are able to oxidize phospholipids by a chain of reactions through lipid peroxidation resulting in the formation of so called protein carbonyls [20].

Measurement of protein carbonyls is a very sensitive method to determine the level of oxidative stress in human cells.

Within the measurements on the inhibitory effects of carbonyl protein formation through α-ketoglutarate in combination with 5-HMF on the one hand and ascorbic acid alone on the other hand compared to a BSA control solution realized in Figure 8 the antioxidative potential of these compounds could have been illustrated impressively. The evaluation of the results in these measurements led to the following findings that are discussed below.

Figure 8 showed effectively the inhibition of carbonyl proteins of a BSA-solution with both substances, α-ketoglutarate and 5-HMF, during exposure of cigarette smoke compared to ascorbic acid solution or compared to BSA control solution.

Compared to BSA control solution the reduction of damaged proteins by α-ketoglutarate and 5-HMF together was effective and highly significant at all points of measurement. A significant reduction of carbonyl proteins through ascorbic acid could have been found too over all time points, but not as effective.

The question if combination of α-ketoglutarate and 5-HMF inhibits protein carbonyl formation by reactive oxygen and nitrogen species during exposure of cigarette smoke could have been answered clearly with "yes", but it is not just that - the findings clearly reveal that the combination of α-ketoglutarate and 5-HMF has the greater antioxidative effect in comparison to ascorbic acid.

Since the superior effect of the combination of α-ketoglutarate and 5-HMF could have been shown, the three substances, namely α-ketoglutarate, ascorbic acid and 5-HMF have been compared amongst each other in Figure 9, 10, and 11, with special regard to their antioxidative potential and their inhibitory effects on protein damage as single substances. Therefore several concentrations (0.284 mmol, 0.425 mmol and 0.568 mmol) of each substance in BSA solutions were exposed to cigarette smoke.
Evaluation of the data showed a significant reduction of carbonyl proteins through α-ketoglutarate in all figures, even stronger than through ascorbic acid. Hence, with attention to carbonyl protein inhibition 5-HMF provided the worst results, compared to α-ketoglutarate and ascorbic acid.
It could have been shown, that ascorbic acid and especially α-ketoglutarate fulfil their role as radical scavengers. Protein carbonyl formation could have been reduced significant through both substances. However, it seems that 5-HMF could only fulfil its role as radical scavenger in combination with α-ketoglutarate.

Another target of this diploma thesis was to answer the question, if α-ketoglutarate, ascorbic acid and 5-HMF can act as scavengers for peroxynitrite ($ONOO^-$). Therefore measurements by chemiluminescence technique were recorded and represented in Figure 12,13 and 14.
The main findings of present data are that there could have been found a significant reduction of measured signals. α-ketoglutarate, ascorbic acid and 5-HMF showed severe inhibiting effects on the luminol reaction.
Ascorbic acid showed the strongest antioxidative potential, followed by α-ketoglutarate.
Interestingly, at low concentrations 5-HMF (0.16mM and 0.8mM) showed the biggest antioxidative potential, even bigger than α-ketoglutarate and ascorbic acid at same concentrations. As mentioned earlier, this observation is probably due to the redox properties of 5-HMF.

Recent findings in the prevention and treatment of numerous diseases have shown that there is a need for multimodality concepts, e.g. to reduce the occurrence of oxidative stress. Such efforts represent an extension of conventional ´clinical pathways´. Therefore, reaction of reactive oxygen and nitrogen species and free radicals with proteins forming carbonyl proteins and peroxynitrite was on one hand an indicator for oxidative stress determination but on the other hand an effective tool proving antioxidative capacity of α-ketoglutarate, ascorbic acid and 5-HMF.

This is in good agreement with the results of this diploma thesis showing significant decreased levels of oxidative stress parameters. The reduction of the most sensitive parameters, carbonyl proteins and peroxynitrite, for detection of oxidative stress during exposure of cigarette smoke documented the radical scavenging effect through α-ketoglutarate and 5-HMF.

However, the generated data of this diploma thesis supports the hypothesis that there could be achieved a significant reduction of oxidative stress that occurs by means of smoking.

In conclusion, reduction of oxidative stress through the use of α-ketoglutarate and 5-HMF, may therefore be one further step towards introducing a multimodality approach in the prevention and treatment of several diseases caused or contributed to oxidative stress.

6 References

[1]. **Dube, Michael F. und Green, C.R.** *Methods of the collection of smoke for analytical purpose.* 1982.

[2]. **Joseph N. Schumacher, Charles R. Green , Freddie W. Best , Marjorie P. Newell.** Smoke composition: an extensive investigation of the water soluble portion of cigarette smoke. *J. Argric. Food Chem.* 1977.

[3]. **Panda, Koustubh, et al.** Cigarette smoke induced protein oxidation and proteolysis is exclusively caused by its tar phase: prevention by Vitamin C. *Toxicology Letters.* 2001, 123, S. 21-32.

[4]. **Pryor, W A, Prier, D G und Church, D F.** Electron spin resonance study of mainstream and side-stream cigarette smoke: nature of the free radicals in gas phase smoke and cigarette tar. *Environ Health Perspect.* 1983, 47, S. 345-355.

[5]. **Ohshima, Hiroshi, Tatemichi, Masayuki und Sawa, Tomohiro.** Chemical basis of inflammation-induced carcinogenesis. *Archives of Biochemistry and Biophysics.* 2003, 417, S. 3-11.

[6]. **JS, Beckman und WH, Koppenol.** Nitric oxide, superoxide and peroxynitrite: the good, the bad and the ugly. *Am. J. Physiol.* 1996, Bd. 271, C1424-C1437.

[7]. **Ducrocq, C., et al.** Peroxynitrite: an endogenous oxidizing and nitrating agent. *Cellular and Molecular Life Sciences.* 1999, Bd. 55, 8-9, S. 1068-1077.

[8]. **H, Ischiropoulos.** Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch. Biochem Biophys.* 1998, 356, S. 1-11.

[9]. **Szabó, Csaba und Ohshima, Hiroshi.** DNA Damage Induced by Peroxynitrite: Subsequent Biological Effects. 1997, Bd. 1, 5, S. 373-385.

[10]. **IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.** *Tobacco smoking.* International Agency for Research on cancer. Lyon,France : s.n., 1986. 38.

[11]. **Panda, Koustubh, et al.** Vitamin C prevents cigarette smoke-induced oxidative damage in vivo. *Free Radical Biology and Medicine.* 2000, Bd. 29, 2, S. 115-124.

[12]. **Maier, A, et al.** Metabolic effects of oral supplementation of Alpha-Ketoglutaric acid and 5-Hydroxy-methyl-furfural in preoperative nutritional support of lung surgery. 2006.

[13]. **Jan, Koolman und Klaus-Heinrich, Röhm.** *Taschenatlas der Biochemie.* 3.Auflage. s.l. : Georg Thieme Verlag, 2003.

[14]. **Coulter, Ian D, et al.** Antioxidants Vitamin C and Vitamin E for the Prevention and Treatment of Cancer. *Journal of General Internal Medicine.* 2006.

[15]. **Berg, Aloys und König, Daniel.** Oxidativer Stress und Sport. *DEUTSCHE ZEITSCHRIFT FÜR SPORTMEDIZIN.* (5) 2000.

[16]. **Halliwell, Barry.** Free Radicals and other reactive species in disease. [Hrsg.] Nature Publishing Group. *ENCYCLOPEDIA OF LIFE SCIENCES.* 2001.

[17]. [Online] http://faculty.ksu.edu.sa/70917/Pictures%20Library/Forms/DispForm.aspx?ID=17.

[18]. [Online] 2013. http://www.cyl-pharma.com/index.php?route=common/page&id=311.

[19]. **Dröge, Wulf.** Oxidative stress and ageing: is ageing a cysteine deficiency syndrome? *Philosophical Transactions of the Royal Society.* 2005, 360(1464), S. 2355-2372.

[20]. **MacNee, W.** Oxidative stress and lung inflammation in airways disease. *European Journal of Pharmacology.* 2001, 429, S. 195-207.

[21]. **Stadtman, Earl R. und Oliver, Cynthia N.** Metal-catalyzed Oxidation of Proteins. *The Journal of Biological Chemistry.* 2005-2008, 1991, 266.

[22]. **Murphy, M E und Kehrer, J P.** Oxidation state of tissue thiol groups and content of protein carbonyl groups in chickens with inherited muscular dystrophy. *Biochemical Journal.* 260, 1989, S. 359-364.

[23]. **Stadtman, Earl R.** Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Radical Biology and Medicine.* 1990, Bd. 9, 4, S. 315-325.

[24]. **WA, Pryor und K, Stone.** Oxidants in cigarette smoke. Radicals, hydrogen peroxide, peroxynitrate, and peroxynitrite. *Annals of the New York Academy of Sciences.* 1993, 686, S. 12-27.

[25]. **CHOW, CHING K.** Cigarette Smoking and Oxidative Damage in the Lung. *Annals of the New York Academy of Sciences.* 1993, 686, S. 289-298.

[26]. **H, Takizawa, et al.** Increased expression of transforming growth factor-beta1 in small airway epithelium from tobacco smokers and patients with chronic obstructive pulmonary disease (COPD). *American Journal of Respiratory and Critical Care Medicine.* 2001, 163, S. 1476-1483.

[27]. **REPINE, JOHN E., BAST, AALT und LANKHORST, IDA.** Oxidative stress in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine.* 1997, Bd. 156, 2, S. 341-357.

[28]. **JE, Gadek, GA, Fells und RG, Crystal.** Cigarette smoking induces functional antiprotease deficiency in the lower respiratory tract of humans. *Science.* 1979, 206, S. 1315-1316.

[29]. **PA, Henricks und FP, Nijkamp.** Reactive oxygen species as mediators in asthma. *Pulmonary Pharmacology & Therapeutics.* 2001, Bd. 14, 6, S. 409-420.

[30]. **Hogg, J und Senior, R.** Chronic obstructive pulmonary disease c : Pathology and biochemistry of emphysema. *THORAX.* 2002, Bd. 57, 9, S. 830-834.

[31]. **Ricciardolo, Fabio L.M., et al.** Reactive nitrogen species in the respiratory tract. *European Journal of Pharmacology.* 2006, Bd. 533, 1-3, S. 240-252.

[32]. **FL, Ricciardolo, et al.** Nitrosative stress in the bronchial mucosa of severe chronic obstructive pulmonary disease. *The Journal of Allergy and Clinical Immunology.* 2005, Bd. 116, 5, S. 1028-1035.

[33]. **M, Ichinose, et al.** Increase in reactive nitrogen species production in chronic obstructive pulmonary disease airways. *American Journal of Respiratory and Critical Care Medicine.* 2000, Bd. 162, S. 701-706.

[34]. **JD, Morrow.** The isoprostanes: their quantification as an index of oxidant stress status in vivo. *Drug Metabolism Reviews.* 2000, Bd. 32, 3, S. 377-385.

[35]. **I, Kawikova, et al.** 8-Epi-PGF2 alpha, a novel noncyclooxygenase-derived prostaglandin, constricts airways in vivo. *American Journal of Respiratory and Critical Care Medicine.* 1996, Bd. 153, 2, S. 590-596.

[36]. **Janssen, L. J.** Isoprostanes: an overview and putative roles in pulmonary pathophysiology. *American Journal of Physiology - Lung Cellular and Molecular Physiology* . 2001, Bd. 280, S. 1067-1082.

[37]. **A, Okazawa, et al.** 8-Epi-PGF2alpha induces airflow obstruction and airway plasma exudation in vivo. *American Journal of Respiratory and Critical Care Medicine.* 1997, Bd. 155, 2, S. 436-441.

[38]. **VLIET, ALBERT van der, et al.** Reactive Nitrogen Species and Tyrosine Nitration in the Respiratory Tract: Epiphenomena or a Pathobiologic Mechanism of Disease? *American Journal of Respiratory and Critical Care Medicine.* 1999, Bd. 160, 1, S. 1-9.

[39]. **Eiserich, Jason P., et al.** Formation of nitric oxide-derived inflammatory oxidants by myeloperoxidase in neutrophils. *Nature.* 1998, Bd. 391, S. 393-397.

[40]. **JP, Gaut, et al.** Myeloperoxidase produces nitrating oxidants in vivo. *The Journal of clinical investigetion.* 2002, Bd. 109, 10, S. 1311-1319.

[41]. **Radi, R, et al.** Peroxynitrite-induced luminol chemiluminescence. *Biochemical Journal.* 1993, Bd. 290, S. 51-57.

[42]. **Schöpfer, F, et al.** Oxidation of ubiquinol by peroxynitrite: implications for protection of mitochondria against nitrosative damage. *Biochemical Journal.* 2000, Bd. 349, S. 35-42.

[43]. **Radi, R, et al.** Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. *The Journal of Biological Chemistry.* 1991, Bd. 266, S. 4244-4250.

[44]. **Ischiropoulos, Harry, et al.** Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Archives of Biochemistry and Biophysics.* 1992, Bd. 298, 2, S. 431-437.

[45]. **Pietraforte, D und Minetti, M.** One-electron oxidation pathway of peroxynitrite decomposition in human blood plasma: evidence for the formation of protein tryptophan-centred radicals. *Biochemical Journal.* 1997, Bd. 321, S. 743-750.

[46]. **Amirmansour, Charles, Vallance, Patrick und Bogle, Richard G.** Tyrosine nitration in blood vessels occurs with increasing nitric oxide concentration. *British Journal of Pharmacology.* 1999, Bd. 127, 3, S. 788-794.

[47]. **Cantoni, Orazio, et al.** Cell signaling and cytotoxicity by peroxynitrite. *Environmental Health Perspectives.* 2002, Bd. 110, 5, S. 823-825.

[48]. **Vliet, Albert Van der, et al.** Interactions of peroxynitrite with human plasma and its constituents: oxidative damage and antioxidant depletion. *Biochemical Journal.* 1994, Bd. 303, S. 295-301.

[49]. **Ippoushi, Katsunari, Takeuchi, Atsuko und Azuma, Keiko.** Prevention of peroxynitrite-induced oxidation and nitration reactions by ellagic acid. *Food Chemistry.* 2009, Bd. 112, 1, S. 185-188.

[50]. **Radi, Rafael.** Nitric oxide, oxidants, and protein tyrosine nitration. *PNAS-Proceedings of the National Academy of Sciences of the United States of America.* 2004, Bd. 101, 12, S. 4003-4008.

[51]. **JS, Beckman, et al.** Oxidative chemistry of peroxynitrite. *Methods in Enzymology.* 1994, Bd. 233, S. 229-240.

[52]. **Uchida, Koji.** 4-Hydroxy-2-nonenal: a product and mediator of oxidative stress. *Progress in Lipid Research.* 2003, Bd. 42, 4, S. 318-343.

[53]. **IA, Blair.** Lipid hydroperoxide-mediated DNA damage. *Experimental gerontology.* 2001, Bd. 369, S. 1473-1481.

[54]. **Nair, Jagadeesan, et al.** Etheno DNA-base adducts from endogenous reactive species. *Mutation Research.* 1999, Bd. 424, S. 59-69.
[55]. **J, Ruef, et al.** Induction of rat aortic smooth muscle cell growth by the lipid peroxidation product 4-hydroxy-2-nonenal. *Circulation.* 1998, Bd. 97, 11, S. 1071-1078.
[56]. **Cheng, Ji-Zhong, et al.** Effects of mGST A4 Transfection on 4-Hydroxynonenal-Mediated Apoptosis and Differentiation of K562 Human Erythroleukemia Cells. *Archives of Biochemistry and Biophysics.* 1999, Bd. 372, 1, S. 29-36.
[57]. **MU, Dianzani, G, Barrera und M, Parola.** 4-Hydroxy-2,3-nonenal as a signal for cell function and differentiation. *Acta biochimica Polonica.* 1999, Bd. 46, 1, S. 61-75.
[58]. **VM, Fazio, et al.** Control of neoplastic cell proliferation and differentiation by restoration of 4-hydroxynonenal physiological concentrations. *Molecular Aspects of Medicine.* 1993, Bd. 14, 3, S. 217-228.
[59]. **W, Hu, et al.** The major lipid peroxidation product, trans-4-hydroxy-2-nonenal, preferentially forms DNA adducts at codon 249 of human p53 gene, a unique mutational hotspot in hepatocellular carcinoma. *Carcinogenesis.* 2002, Bd. 23, 11, S. 1781-1789.
[60]. **T, Kumagai, et al.** 4-hydroxy-2-nonenal, the end product of lipid peroxidation, is a specific inducer of cyclooxygenase-2 gene expression. *Biochemical and Biophysical Research Communications.* 2000, Bd. 273, 2, S. 437-441.
[61]. **H, Kühn und A, Borchert.** Regulation of enzymatic lipid peroxidation: the interplay of peroxidizing and peroxide reducing enzymes. *Free Radical Biology & Medicine.* 2002, Bd. 33, 2, S. 154-172.
[62]. **DG, Tang und KV, Honn.** Apoptosis of W256 carcinosarcoma cells of the monocytoid origin induced by NDGA involves lipid peroxidation and depletion of GSH: role of 12-lipoxygenase in regulating tumor cell survival. *Journal of Cellular Physiology.* 1997, Bd. 172, 2, S. 155-170.
[63]. **Rassow, Joachim, et al.** *Biochemie-Duale Reihe.* Stuttgart : Georg Thieme Verlag KG, 2006. S. 113-115.
[64]. **Kurt, Jungermann und Hans, Möhler.** *Biochemie.* Berlin,Heidelberg : Springer Verlag, 1980.
[65]. **C, Janzowski, et al.** 5-Hydroxymethylfurfural: assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutathione. *Food and Chemical Toxicology.* 2000, Bd. 38, 9, S. 801-809.

[66]. **YX, Li, et al.** In vitro antioxidant activity of 5-HMF isolated from marine red alga Laurencia undulata in free-radical-mediated oxidative systems. *Journal of Microbiology and Biotechnology.* 2009, Bd. 19, 11, S. 1319-1327.

[67]. **MM, Li, et al.** The protective role of 5-HMF against hypoxic injury. *Cell Stress & Chaperones.* 2011, Bd. 16, 3, S. 267-273.

[68]. [Online] http://de.wikipedia.org/wiki/Hydroxymethylfurfural.

[69]. [Online] http://de.wikipedia.org/wiki/Maillard-Reaktion.

[70]. **Ulbricht, Richard J., Northup, Sharon J. und Thomas, John A.** A review of 5-hydroxymethylfurfural (HMF) in parenteral solutions. *Fundamental and Applied Toxicology.* 1984, Bd. 4, 5, S. 843-853.

[71]. **YC, Hou, et al.** Effects of glucose, fructose and 5-hydroxymethyl-2-furaldehyde on the presystemic metabolism and absorption of glycyrrhizin in rabbits. *The Journal of Pharmacy and Pharmacology.* 2005, Bd. 57, 2, S. 247-251.

[72]. **Janzowski, C, et al.** 5-Hydroxymethylfurfural: assessment of mutagenicity, DNA-damaging potential and reactivity towards cellular glutathione. *Food and Chemical Toxicology.* 2000, Bd. 38, 9, S. 801-809.

[73]. **Horn, Florian, et al.** *Biochemie des Menschen.* 2.korrigierte Auflage. Stuttgart : Georg Thieme Verlag, 2002/2003. S. 454.

[74]. **Ernst, Jürgen, Krantz, Sven und Witt, Martin.** *Chemie Physik Biologie.* Heidelberg : Springer Medizin Verlag, 2007. S. 234.

[75]. **Naidu, K Akhilender.** Vitamin C in human health and disease is still a mystery ? An overview. *Nutrition Journal.* 2003.

[76]. **RL, Levine, et al.** Carbonyl assays for determination of oxidatively modified proteins. *Methods in Enzymology.* 1994, Bd. 233, S. 346-357.

[77]. **Hughes, M. N. und Nicklin, H. G.** The chemistry of pernitrites. Part I. Kinetics of decomposition of pernitrous acid. *Journal of the Chemical Society.* 1968, S. 450-452.

[78]. [Online] Oktober 2013. http://www.unituebingen.de/straehle/kristallstrukturanalyse/pdf/farbstoff3.pdf.