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Impact of coffee consumption on DNA stability and redox status in humans

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Verfasserin:	Christine Hölzl
Matrikel-Nummer:	9506561
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Betreuer:	Ao. Univ.-Prof. Dr. Siegfried Knasmüller

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SUMMARY/ZUSAMMENFASSUNG

SUMMARY

Coffee is one of the most widely consumed beverages worldwide. Epidemiological data suggest that there is an inverse association between coffee consumption and the incidence of reactive oxygen species (ROS) related diseases (such as diabetes, neurodegenerative diseases, cancer). Also animal studies and *in vitro* experiments provide evidence for antioxidant activity of coffee and coffee components. However, data from human intervention trials are scarce and partly controversial.

Aim of the present thesis was to investigate the impact of coffee consumption on DNA stability and on the redox status in humans. Therefore, intervention trials were carried out and different parameters of oxidative stress were determined. DNA stability monitored in single cell gel electrophoresis (SCGE or comet) assays was one of the main parameters. To design an optimal study plan the results of earlier SCGE assays were critically evaluated and the outcomes published in form of a review. So far 84 studies have been conducted with different food items and in about half of them protective effects were found.

In the first human intervention trial the impact of a mix of metal and paper-filtered coffee (n=8, 600 ml/P/day, 5 days) on DNA stability was studied. The results indicate that coffee consumption leads to a significant decrease of oxidised purines (by 68 %) and pyrimidines (by 48 %) (detection with DNA lesion specific enzymes). Furthermore, DNA damage caused by reactive oxygen radicals (H_2O_2 treatment) and by the heterocyclic aromatic amine 3-amino-1-methyl-5H-pyrido [4,3-b] indole acetate (Trp-P-2), a carcinogen found in fried meats, was significantly reduced (by 17 % and 35 %, respectively). Furthermore, an increase of the activity of the antioxidative enzyme superoxide dismutase (by 38 %) was detected; whereas the activity of glutathione peroxidase was not significantly altered.

The second trial was conducted with a new type of instant coffee (n=29, 800 ml/P/d, 5 days), which is rich in chlorogenic acids (CAs). CAs are well absorbed and it is assumed that they contribute substantially to the antioxidant properties of coffee. In this clinical intervention trial results of 15 different oxidative parameters (including endpoints of oxidative DNA damage, lipid peroxidation and antioxidant enzymes) as well as gene expression profiles were analysed. The results obtained with the comet assay indicate a decrease of oxidised DNA bases (oxidised purines by 6,7 %, oxidised pyrimidines by 9,8 %) and reduced sensitivity towards ROS induced DNA damage (6.1%). However, only the decrease of oxidised purines in female volunteers reached significance. Furthermore, F2-isoprostane levels in the urines of the volunteers (which is one of the most reliable marker for lipid peroxidation) and also protein nitrosation (3-nitrotyrosin levels) were significantly reduced

(by 14.9 % and 17.2 %, respectively) after coffee intake. Interestingly, the decrease of these two parameters was more pronounced in female volunteers. Other markers of oxidative stress were not affected by coffee consumption.

The third article (paper III) describes human intervention studies with unfiltered and paper filtered coffee in which the impact of coffee consumption on glutathione-S-transferase (GST) activities were monitored in saliva and plasma. GSTs are detoxifying enzymes and possess also antioxidant properties. The first part describes a study with unfiltered coffee (n=10, 1L/P/d, 5 days). The results show that coffee increased the levels of the isoenzyme GSTP (3-fold) but not of GSTA. Furthermore, no increase of the overall GST activity was found. Also other investigated biochemical parameters (creatinine, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase) were not altered. A slight but not significant increase of cholesterol levels was observed after the intervention. In the second trial, GSTP levels after consumption of unfiltered and paper filtered coffees, which differ in their levels of coffee specific diterpenoids (cafestol and kahweol), were compared. Again, a significant (3-fold) induction of GSTP was observed, regardless the type of coffee consumed. Since GST plays a key role in the detoxification of polycyclic aromatic hydrocarbons (PAHs), we conducted a further trial in which the impact of coffee intake on the sensitivity of peripheral lymphocytes towards (±)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE) induced DNA damage was monitored. BPDE is the reactive metabolite of benzo(a)pyrene, which is a model compound for PAHs. We detected a significant reduction of BPDE-induced comet formation (by 45 %) after the intervention due to coffee consumption.

The results of our experiments suggest that coffee protects humans against adverse health effects caused by DNA damage. This finding provides a possible explanation for the results obtained in epidemiological studies indicating that coffee intake is inversely related with the incidence of diseases associated with cellular damage by reactive oxygen species and DNA instability.

ZUSAMMENFASSUNG

Kaffee ist eines der am häufigsten konsumierten Getränke weltweit. Epidemiologische Studien ergaben einen inversen Zusammenhang zwischen Kaffeekonsum und der Inzidenz von Krankheiten, an deren Auslösung reaktive Sauerstoffspezien beteiligt sind (z.B. Diabetes, neurodegenerative Erkrankungen, Krebs). Auch Tierstudien und *in vitro* Experimente zeigen, dass Kaffee und dessen Inhaltsstoffe antioxidativ wirken, allerdings liegen nur wenige, teilweise widersprüchliche Ergebnisse von Humanstudien vor.

Das Ziel der vorliegenden Arbeit war die Klärung der Frage, ob Kaffeekonsum beim Menschen einen Einfluss auf den oxidativen Status und auf die DNA Stabilität hat. Es wurden humane Interventionsstudien durchgeführt und verschiedene Parameter des oxidativen Stresses bestimmt, die Biomarker für antioxidative Effekte sind. Einer der Hauptparameter war die Messung der DNA Stabilität mittels dem Einzelzellgelelektrophorese Test (Komet Assay). Um ein optimales Studiendesign zu entwickeln, evaluierten wir die bis dato vorhandenen Interventionsstudien die mit dem Komet Assay durchgeführt wurden und verfassten einen Übersichtsartikel, der die Resultate dieser Studien zusammenfasst. Bisher wurden 84 Studien mit verschiedenen Nahrungsmitteln durchgeführt und in etwa der Hälfte wurden Schutzeffekte gefunden.

In der ersten humanen Interventionsstudie wurde die Auswirkung des Konsums eines Gemisches von metallgefiltertem und ungefiltertem Kaffee (n=8, 600 ml/Person/Tag, 5 Tage) auf die DNA Stabilität untersucht. Die Resultate zeigen, dass Kaffeekonsum zu einer signifikanten Reduktion von oxidierten Purinen (68 %) und Pyrimidinen (48 %) (detektiert mittels läsionsspezifischen Enzymen) und auch zu einer erniedrigten Sensitivität gegenüber H_2O_2 induzierter DNA Schädigung (17 %) führt. Weiters konnten wir zeigen, dass durch Kaffeekonsum die Aktivität eines antioxidativ wirksamen Enzyms (Superoxid dismutase) steigt. Im Gegensatz dazu war die Aktivität der Glutathioneperoxidase nicht verändert.

Bei der zweiten Arbeit handelte es sich ebenfalls um eine Interventionsstudie (n=29, 800 ml/P/T, 5 Tage), die allerdings mit „cross-over“ Design durchgeführt wurde. Die verwendete Kaffeesorte war ein neu entwickelter Instantkaffee, der einen besonders hohen Gehalt an Chlorogensäuren aufwies. Chlorogensäuren werden sehr gut über den Magen-Darmtrakt aufgenommen und es wird angenommen, dass sie in besonderer Weise für die antioxidativen Eigenschaften von Kaffee verantwortlich sind. In dieser klinischen Studie wurden DNA Stabilitätsparameter wie in der ersten Studie gemessen, sowie zusätzlich Parameter des oxidativen Status (Lipidperoxidation und diverse antioxidativ wirksame Enzyme), weiters wurden auch Genexpressionsprofile bestimmt. In der Gruppe der

Kaffeetrinker wurde eine Reduktion der oxidierten Basen (oxidierte Purine - 6,7 % und oxidierte Pyrimidine - 9,8 %) detektiert und auch die Sensitivität gegenüber ROS-induzierten Schäden war erniedrigt (6.1 %), diese Effekte erreichten jedoch nur im Falle der oxidierten Purine bei den weiblichen Probanden statistische Signifikanz. Auch die F-2 Isoprostane im Urin und die 3-Nitrotyrosin Konzentrationen im Plasma waren deutlich nach dem Kaffeekonsum reduziert, die anderen biochemischen Parameter veränderten sich jedoch nicht. Die protektiven Eigenschaften des Kaffees waren in dieser Studie bei den weiblichen Probanden deutlich höher.

Die dritte Arbeit betrifft humane Interventionsstudien die mit gefiltertem und ungefiltertem Kaffee durchgeführt wurden und in denen die Auswirkungen auf die Glutathione-S-Transferase (GST) Aktivität im Speichel und Plasma untersucht wurde. GST ist an der Detoxifizierung von Umweltgiften (z.B. polyzyklischen aromatischen Kohlenwasserstoffen) beteiligt und ist auch antioxidativ wirksam. Im ersten Teil der Arbeit wurde eine Studie mit ungefiltertem Kaffee (n=10, 1L/P/Tag, 5 Tage) durchgeführt und die Ergebnisse zeigten eine signifikante Erhöhung (3-fach) der Konzentration des Isoenzyms GSTP. Im Gegensatz dazu waren die GSTA Gehalte, die gesamte GST Aktivität und auch diverse klinische Parameter (Creatinin, Alanin-Aminotransferase, Aspartat-Aminotransferase, Alkalische-Phosphatase) nicht verändert; der Cholsesterinspiegel war nach Kaffeekonsum leicht jedoch nicht signifikant erhöht. In der zweiten Studie tranken die Teilnehmer 3 Tage lang ungefilterten oder papiergefilterten Kaffee (n=7, 1L/P/T), die sich im Gehalt von kaffeespezifischen Diterpenoiden (Cafestol und Kahweol) unterschieden. Unabhängig von der Art des konsumierten Kaffees waren die gemessenen GSTP Aktivitäten nach Kaffeekonsum deutlich erhöht (3-fach). Wir führten nachfolgend eine weitere Interventionsstudie durch (n=7, 1L/P/Tag, 5 Tage), in der wir die Auswirkung von ungefiltertem Kaffeekonsum auf die Empfindlichkeit peripherer Lymphozyten gegenüber (±)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxid (BPDE) untersuchten. BPDE ist der reaktive Metabolite von Benz(a)pyren und ist eine Modelsubstanz der polyzyklischen aromatischen Kohlenwasserstoffe. Die Ergebnisse zeigen, dass nach Kaffeekonsum die Zellen weniger empfindlich gegenüber dieser Substanz reagierten.

Die Ergebnisse der Interventionsstudien deuten darauf hin, dass Kaffeekonsum einen positiven Einfluss auf die DNA Stabilität hat. Die Resultate untermauern die Befunde von epidemiologischen Studien die zeigen, dass Kaffeekonsum vor Krankheiten schützt, die mit reaktiven Sauerstoffspezien und DNA Instabilität in Zusammenhang stehen.

LIST OF ABBREVIATIONS

AP-1	activator protein 1
ARE	antioxidative response element
BPDE	(±)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxide
CA	chlorogenic acid
C+K	cafestol and kahweol
ELISA	enzyme linked immunosorbent assay
FPG	formamidopyrimidine DNA glycosylase
FRAP	ferric ion reducing antioxidant parameter
GPx	glutathione peroxidase
GSH	glutathione
GST	glutathione-S-transferase
HNE	4- hydroxy- 2 nonenal
IL-1	interleukin-1
LDL	low density lipoproteins
LP	lipid peroxidation
MAPKs	mitogen activated protein kinases
MDA	malondialdehyde
MN	micronucleus
NFκB	nuclear factor kappa-B
NOS	nitric oxide synthetases
Nrf2	nuclear factor erythroid 2-related factor 2
ORAC	oxygen radical absorbance capacity test
oxLDL	oxidised LDL
RNS	reactive nitrogen species
ROS	reactive oxygen species
SCGE	single cell gel electrophoresis
SOD	superoxide dismutase
TAC	total antioxidant capacity
TEAC	trolox equivalent antioxidant capacity
TNF-α	tumour necrosis factor-alpha
TRAP	total radical trapping antioxidant parameter
3-NT	3-nitrotyrosine
4-HNE	4-hydroxy-2-nonenal
8-OHdG	8-hydroxydeoxyguanosine

INTRODUCTION

It is well documented that oxidative stress has a substantial impact on human health. It has been estimated by Blomhoff that around ten scientific articles are published every day which concern the effects of reactive oxygen species (ROS) [1], and strong attempts have been made to identify dietary compounds which protect humans against the adverse consequences of oxidative damage. A broad variety of food compounds has been identified, which inactivate radicals directly and/or induce antioxidant defence systems. Apart from vitamins, also many secondary plant constituents have been identified as antioxidants, which are contained in foods. These compounds are often by-products of the primary metabolism and play a role in the protection of plants against radical induced damage caused by light or by tissue damage and also protect them against pests. The discovery of ROS protective compounds in human foods has lead to dietary recommendations and also to the production of food supplements and production of foods, which contain such compounds [2].

The uptake of antioxidants via coffee consumption is relatively high, since coffee is a frequently consumed beverage. Some investigations indicate that the total intake of phenolic antioxidants via coffee intake is substantially higher than via fruits and vegetable consumption and other beverages [3]. Furthermore, evidence from epidemiological studies indicates that coffee consumption is inversely related to the incidence of specific forms of cancer (i.e. in the liver and colon), liver cirrhosis and fibrosis and diseases such as diabetes, Alzheimer and Parkinson [4]. It is possible that these protective effects are due to inactivation of ROS (which play a role in the etiology of these diseases) by coffee components. Results of a number of *in vitro* experiments show that specific coffee components such as hydroxycinnamic acids, Maillard reaction products, caffeine and coffee itself possess antioxidant properties [5,6]. However, at present the evidence for effects of coffee in humans is scarce and partly controversial (for details see literature overview, chapter 3).

The concentration of coffee constituents, which possess antioxidant properties depends on the selection of the cultivars used (Coffee arabica and/or robusta), on the roasting process and also on the preparation method. This offers the possibility to optimise the antioxidant properties of coffee. During the roasting process, the levels of chlorogenic acids decline and Maillard products are formed. A number of investigations indicate that the chlorogenic acids play a key role in the antioxidant properties of coffee [7-9].

The present thesis consists of a literature overview and origial articles presented in the publication section. The literature overview provides information on the general mode of action of radicals, their formation and the molecular mechanisms of antioxidants (chapter 1). Furthermore, the methodological principles of antioxidant measurements used in human

intervention trials and their advantages and limitations are disscused (chapter 2). Furthermore, the results of human intervention trials, which have been carried out with coffee so far are described and disscused in chapter 3. This information provides the basis for the design of the human intervention studies, which are described in the publication section.

The main parameter used in all trails was DNA migration, which was determined in single cell gel electrophoresis (SCGE) assays. SCGE assays are based on the determination of DNA migration in an electric field. DNA damage leads to comet shaped images, therefore this technique is also termed "Comet assay". Different parameters of DNA migration were measured, namly endogenous formation of single and double strand breaks, endogenous formation of oxidised purins and pyrimidines which can be monitored by use of lesion specific enzymes and alterations of the sensitivity of the cells towards damage caused by ROS (H_2O_2 treated) or genotoxic carcinogens contained in the human diet (such as polycyclic aromatic hydrocarbons or heterocyclic aromatic amines).

The first article (paper I) describes a small intervention trial (n=8), in which the impact of consumption of a mix of paper- and unfiltered coffee was investigated with the SCGE assay and also antioxidant enzymes have been determined (paper I, page 79).

The second intervention is a large comprehensive multiple endpoint study with cross-overdesign (n=29) in which the influence of instant coffee consumption on different parameters of oxidative damage was investigated (paper II, page 91). Beside of DNA stability (determined with the SCGE assay) also isoprostane levels were determined in the urine with GC-MS, which is regarded as the most reliable method to determine the isoprostanes and several other parameters such as malondialdehyde (MDA), oxidised low-density lipoproteins (oxLDL), total antioxidant capacity (TAC), glutathione-S-transferase (GST), cellular glutathione (GSH) levels, reactive oxygen species (ROS), levels of antioxidant enzymes (superoxide dismutase, glutathione peroxidase) and geneexpression profiles were measured.

The third article concerns the impact of coffee consumption on the activity of glutathione-S-transferase (GST) activities (paper III, page 121). This enzyme is involved in the detoxification of environmental pollutants and has also antioxidant properties [10,11]. Therefore, the impact of coffee on the sensitivity of peripheral lymphocytes towards (±)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE) induced DNA damage was monitored. BPDE is the reactive metabolite of benzo(a)pyrene, which is a model compound for PAHs.

The review article (page 135) gives an overview on the current state of knowledge of the use of single cell gel electrophoresis (SCGE) assays in human intervention trials.

LITERATURE (OVERVIEW)

CHAPTER 1
REACTIVE OXYGEN SPECIES (ROS): FORMATION, BIOLOGICAL FACTS AND DETOXIFICATION

1.1 Introduction

Oxidative stress has been defined by Blomhoff [1] as a "condition that is characterised by the accumulation of non-enzymatic oxidative damage to molecules that threaten the normal functions of a cell or the organism". Oxidative stress is involved in the aetiology of a number of human diseases (for review see Valko et al. [12]). Therefore, strong attempts have been made over the last decades to identify components that protect humans against the consequences of oxidative damage. This chapter gives a brief overview of the formation of different types of reactive oxygen species (ROS) and describes their interactions with different types of macromolecules. Furthermore, the consequences of oxidative damage, cellular defence mechanisms, and the mechanisms underlying the protective effects of dietary compounds are described.

1.2 Formation of reactive oxygen species (ROS)

"Reactive oxygen species" is a collective term that includes not only radicals, but also non-radical derivates of oxygen (O_2), for example hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and ozone (O_3) (for details see Table 1) [13]. Radicals, which are characterised by their high reactivity (except O_2), contain unpaired electrons in atomic or molecular orbitals. Therefore, they donate or receive other electrons to obtain stability. O_2 itself is a bi-radical and rather unreactive, but its univalent reduction leads to the formation of more reactive species. ROS in the cells undergo a cascade of reactions, which leads to conversion into other molecular forms and furthermore to oxidation of macromolecules. These processes and some important representatives of ROS are depicted schematically in Figure 1.

Table 1: Chemical structures of different ROS (modified from [13])

Radicals		Non radicals	
Name	**Chemical structure**	**Name**	**Chemical structure**
Superoxide	$O_2^{\bullet-}$	Hydrogen peroxide	H_2O_2
Hydroperoxyl	HO_2	Peroxynitrite[a]	$ONOO^-$
Hydroxyl	OH^-	Peroxynitrous acid[a]	ONOOH
Peroxyl	RO_2	Nitrosoperoxycarbonate	$ONOOCO_2^-$
Alkoxyl	RO^-	Hypochlorous acid[b]	HOCl
Carbonate	CO_3^-	Hypobromous acid, [c]	HOBr
Carbon dioxide	CO_2^-	Ozone	O_3
Singlet oxygen	$O_2\ ^1\Sigma g^+$	Singlet oxygen	$O_2\ ^1\Delta g$

can also be designated as [a] "reactive nitrogen species", [b] "reactive chlorine species", [c] "reactive bromine species"

The one electron reduction product of oxygen is the superoxide radical ($O_2^{\bullet-}$). It is a by-product of the respiration chain in the mitochondria of the cells produced by NADPH

oxidases and formed as a consequence of reduction of oxygen required for ATP production. $O_2^{\bullet-}$ forms at low pH hydroxyperoxyl radicals (HO_2) which cross membranes due to their uncharged nature. Due to the pH of most body tissues, the ratio of $[O_2^{\bullet-}]/[HO_2^{\bullet}]$ is rather large (e.g., 1000/1 at pH 7.8), but the HO_2 radicals are more reactive [13,14]. Under stress conditions, $O_2^{\bullet-}$ acts as an oxidant of [Fe–S] cluster-containing enzymes (e.g., metalloproteins) and facilitates hydroxyl radical (OH^-) production from H_2O_2 by making Fe^{2+} available for the Fenton reaction [15].

If two electrons are transferred (e.g., enzymatically via superoxide dismutase), the product is H_2O_2, which can be detoxified enzymatically (catalase, glutathion peroxidase). H_2O_2 is a source of more deleterious species such as HOCl and $OH^{\bullet}$. The enzyme myeloperoxidase catalyses the production of HOCl by interaction between H_2O_2 peroxides and chlorides [16]. H_2O_2 itself is a poor oxidant and reacts to a less extend with [Fe-S] clusters and metallo enzymes. The highly reactive $OH^{\bullet}$ radicals with a very short *in vivo* half-life of approx. 10−9 s [17] react very close to its side of formation. It can be formed *in vivo* via the Haber-Weiss Reaction ($\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$), which combines a Fenton reaction and the reduction of Fe^{3+} by $O_2^{\bullet-}$, yielding Fe^{2+} and oxygen ($Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$) [18].

The different ROS have distinct biological properties, which include their high chemical reactivity, their short half-live and their lipid solubility. Moreover it is notable that "reactive" is a relative term; $O_2^{\bullet-}$ and H_2O_2 are highly selective in their reactions with biological molecules, leaving most of them unscathed, whereas $OH^{\bullet}$ attacks all types of biomolecules (see Figure 1, grey box) [13].

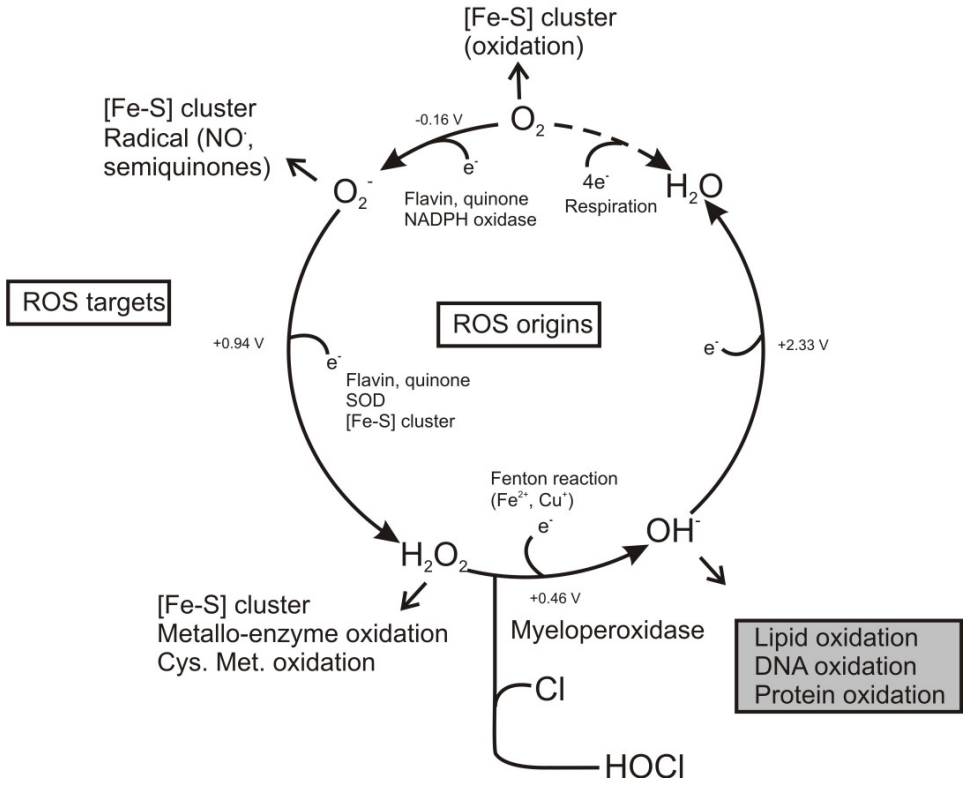

Figure 1: ROS sources and biochemical properties (adapted from D'Autreaux et al. [19]).

Also reactive nitrogen species (RNS) belong to ROS; one of the most important is nitric oxide ($NO^{\bullet}$). It is produced by oxidation of the terminal guanidine-nitrogen atoms of arginine [20,21]. This reaction is catalysed by nitric oxide synthetases (NOS, i.e. neuronal NOS, endothelial NOS and inducible NOS). $NO^{\bullet}$ can react with different radicals, the most important reaction under physiological conditions is the formation of peroxynitirite ($ONOO_2$) in which O_2 is involved [21,22]. $ONOO_2$ can cause damage similar to that induced by $OH^{\bullet}$ [20].

1.3 Sources of ROS

Beside of exposure to exogenous sources, ROS are produced by normal cellular functions, in particular during respiration in the mitochondria. It has been estimated that the average person encounters approximately 10,000–20,000 free radical attacks per cell per day. In well trained athletes this number can increase by 50 % [23].

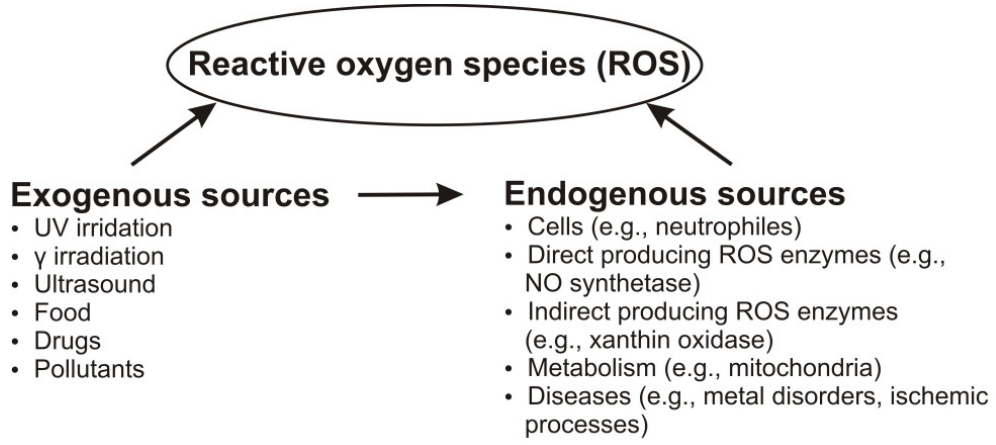

Figure 2: Schematic overview on exogenous and endogenous sources of ROS exposure (adapted from Kohen and Nyska [24]).

Several **exogenous factors** can contribute to oxidative stress. Air pollutants such as cigarette smoke [25,26] and car exhausts [27], drugs (e.g., bleomycin, doxorubicine) [28] as well as pesticides and herbicides [29] and industrial chemicals [30] are sources of ROS that can attack the organism [24]. Ionising radiation (caused by e.g., medical applications such as radiation therapy, X-rays, natural sources such as cosmic and solar radiation) may cause toxic effects in organisms primarily via ionisation of intracellular water [31]. Even exposure to non-ionizing irradiation such as UV-C (< 290 nm), UV-B (290–320 nm), and UV-A (320–400 nm) can indirectly produce a variety of ROS including $^1O_2$, H_2O_2, and $O_2^{\cdot-}$ radicals; also hemolytic cleavage of H_2O_2 by UV radiation yields OH^- Radicals [24]. Also O_3 can be formed in the presence of UV light. In the upper atmosphere it is required to scavenge UV-C radiation, but on the other hand it is a powerful oxidising agent [32]. Also infections by pathogenic microorganisms may produce ROS either by direct release from the pathogen or as a consequence of endogenous responses of phagocytes and neutrophils.

Probably the most relevant external source is nutrition. Most of the foods we consume are oxidised and contain oxidants such as peroxides, aldehydes, fatty acids and transition metals [33,34]. Although the exogenous exposure of the organisms to ROS can be high, the ROS formation due to endogenous sources is more extensive, because it is a continuous process of every cell in the organism during life time [24].

The most important endogenous source is the respiration processes in the mitochondria and it was shown, that the massive continuous production of radicals is even increased in ageing cells [35,36]. Also enzymes are a source of ROS, most of them produce ROS as a by-product (e.g., xanthine oxidase), but there are also enzymes which are designed to produce ROS (e.g., NOS). There are also some diseases which are related to an excess of ROS (for

reviews see [12,37]), but ROS are not only the cause but also the consequence of certain diseases [13,38]. More than 200 clinical disorders have been described in which ROS are involved either in the beginning of the disease and/or produced during its course.

1.4 Oxidative damage of DNA, Lipids and Proteins

Continuous exposure of the organism to ROS results in oxidative damage of cell components and leads to alterations of many cellular functions. Three main categories of macromolecules namly proteins, lipids and DNA are targets of oxidative damage. Some of these changes can be used as markers of oxidative stress.

Oxidative **DNA damage** by ROS can induce mutations, which lead to genomic instability and furthermore to formation of aberrant cells (cancer cells). DNA is a rather stable molecule, thus ROS can interact with it and various different types of damage (e.g., modification of DNA bases, loss of purines, single and double strand breaks, damage to the deoxyribose sugar, DNA protein crosslinkage and damage to the DNA repair system) can be formed [23,24]. The major portion of DNA damage is attributable to the highly reactive $OH^{\cdot}$ radical. $OH^{\cdot}$ reacts with all parts of the DNA, damaging the DNA bases (purines and pyrimidines) and also the deoxyribose backbone. The formed reaction products are various (e.g., 5-hydroxycyteine, uracil glycol) depended on different factors (e.g., absence or presence of oxygen and or transition ions). The most extensively investigated product is 8-hydroxy-desoxyguanosine (8-OHdG) [13,39]. 8-OHdG is very easily formed and it is mutagenic and therefore a potential biomarker of carcinogenesis [23]. DNA damage can occur also indirectly through lipid peroxidation (LP) products. Lipid radicals during the chain reaction can cause adduct formation, strand breaks and DNA protein crosslinks (for review see [40]). Also $NO^{\bullet}$ can damage DNA indirectly via formation of peroxinitrite [41,42].

Proteins can undergo direct and indirect damage due to interactions with ROS. The consequences are fragmentation, degradation, changes in their tertiary structure, peroxidation and damage of specific amino acid residues. Within the proteins, the peptide bonds or the sidechains may be targeted, and it has been shown that many of the reactions triggered by ROS are site-specific [43]. Radicals react in particular with nucleophilic amino acids for example with tryptophane, histidine and cysteine [44,45]. The major products are aldehydes, keto compounds and carbonyls. The formation of the latter is due to oxidation of specific amino acid residues (e.g., lysine, arginine, and proline residues), cleavage of the peptide backbone or cleavage due to oxidation of glutamyl residues. An important marker of oxidative protein damage is the carbonyl derivate 4- hydroxy- 2 nonenal (4-HNE) which is formed due to reaction of some amino acid side chains with lipid oxidation product [46].

Another important marker of oxidative damage is 3-nitrotyrosin (3-NT). It is produced via interaction of $ONOO^-$ and other nitrogen reactive radicals with the amino acid tyrosine [43]. The $NO^{\cdot}$ radical reacts in particular with Fe-S centers of proteins which transport electrons and this affects the functions of mitochondria [47]. Another important feature is its reaction with thiol groups of proteins; a typical example is the S-nitrosylation of caspases, which are part of cell signalling processes [48].

Also **Lipids** can be damaged by ROS, which leads to their oxidation, which occurs in three steps. The first step of the lipid peroxidation (LP) reaction ("initiation step") is characterised by the attack of radicals to double bonds of the fatty acids, which leads to formation of fatty acid radicals. In the second step ("propagation") the chain reaction takes place, lipid radicals are formed continuously. The last stage ("chain determination") occurs because of the interactions of $ROO^{\bullet}$ (peroxyl radical) or with other radical types and/or antioxidants (for details see [13,49]). A schematic overview of the LP process is given in Figure 3.

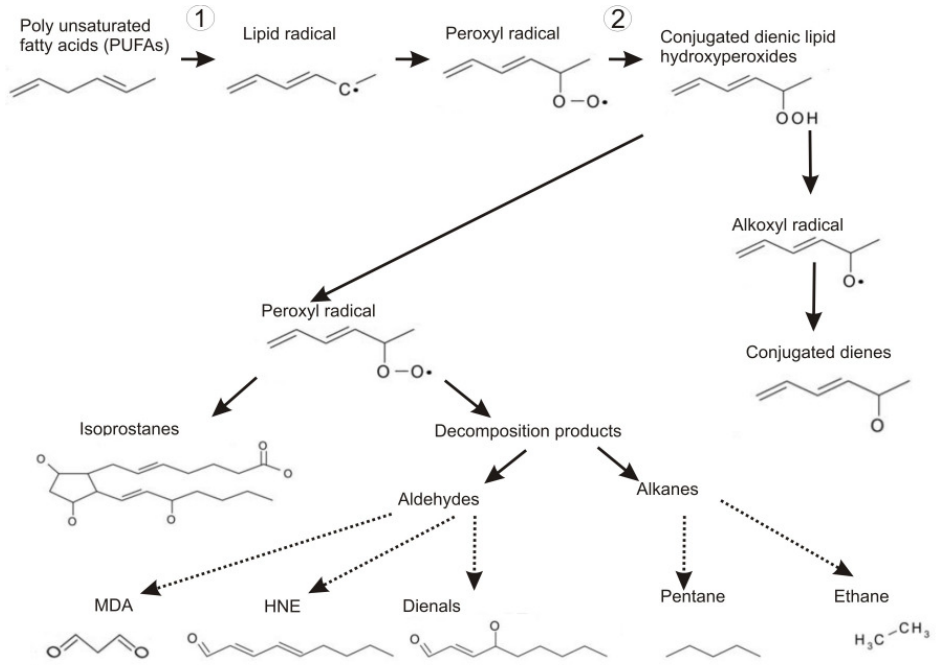

Figure 3: Schematic overview of the lipid peroxidation (LP) process. (1) Initiation phase: attack of radicals to the double bound (2) formation of hydroxyperoxides (adapted from Dotan et al. [50]).

All cellular membranes are vulnerable to oxidative damage because of their high content of unsaturated fatty acids. Important markers of LP are aldehyds and ketones, such as

malondialdehyde (MDA), which forms DNA adducts [51], the family of isoprostanes, which is excreted via the urine and 4-HNE, formed via reaction with amino acid residues.

Oxidative modification of low density lipoproteins (LDL) is one of the earliest events in atherosclerosis. LDLs are responsible for the transport of cholesterol and triglycerids from the liver to the peripherie. Oxidised LDL (oxLDL) represents a variety of modifications of both lipid and apolipoprotein B (apoB) components by LP [52]. OxLDL particles are taken up by macrophages inside the aterial walls and form foam cells, which cause artherosclerotic plaques [53].

A detailed overview of the interaction of different forms of ROS with macromolecules described above is given in Figure 4.

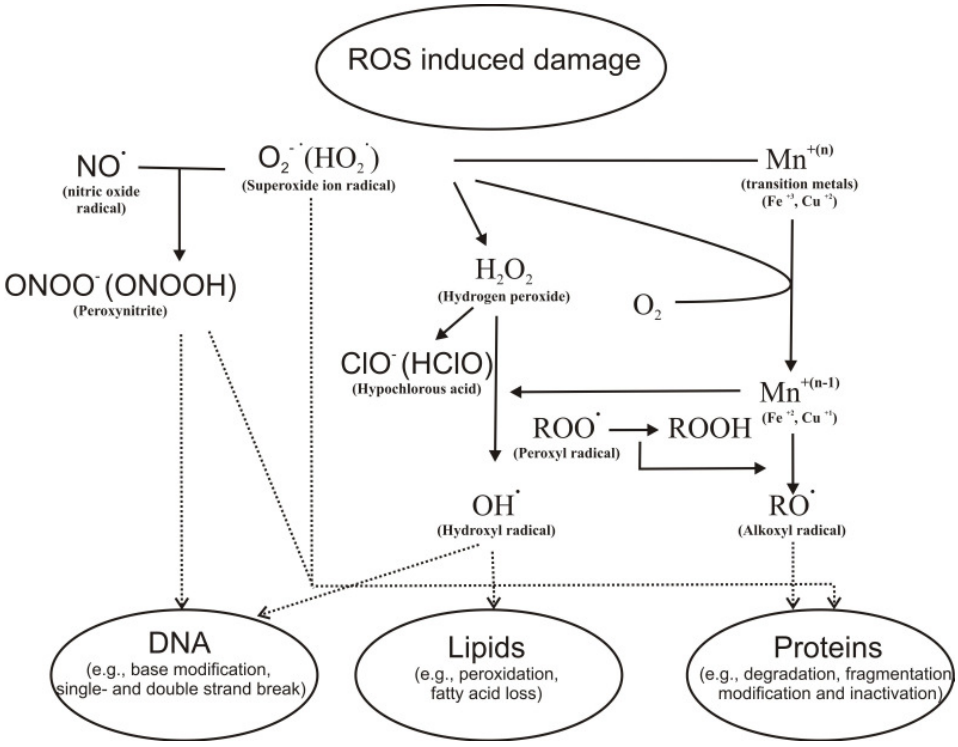

Figure 4: Oxidative damage of different macromolecules caused by ROS (adapted from Kohen and Nyska [24]). Non-continuous arrows indicate the ultimate macromolecule damaging pathways.

1.5 Antioxidant defence system

In the course of the evolution, pro- and eukaryotic organisms developed a variety of antioxidant defence mechanism, which can be grouped into two main categories namely: (a) the synthesis of molecules which are able to inactivate ROS by direct scavenging (b) indirect defence systems which are based on the activation of transcription factors via signalling

pathways leading to induction of antioxidant enzymes. In this context, it is notable that besides of the antioxidant mechanisms, which are described in this chapter in detail, the organism possesses additionally other defence mechanisms against free radical induced oxidative stress for example complex repair mechanisms [12].

1.5.1 Direct scavenging mechanisms

Direct scavenging of ROS might take place due to antioxidants contained in foods or via substances synthesised in the organisms. The most important endogenous formed antioxidants are uric acid, hämoglobin and the tripeptide glutathione (GSH). GSH is present in a high concentration in the cell and beside its function as co-substrate of glutathione peroxidase (GPx) and –transferases (GST) it is a direct scavenger of ROS.

Also plants contain highly potent antioxidants, which are often termed "secondary constituents" as they are not directly involved in their primary metabolism. These components comprise flavonoids and other phenolic components and fulfill an important biological role as they protect plants against ROS, caused by cellular damage or light. Furthermore, they may act as "natural pesticides" and possess potent toxic activities.

Dietary antioxidants and vitamins differ strongly in regard to their tissue distribution and scavenging spectrum. In the case of phenolics, it is assumed that the radicals are trapped by the molecules and become resonance-stabilised. Phenoxy radical intermediates are stable and are unlikely to participate in the initiation of new radical chain reaction (see Figure 5). Important antioxidants, which are either synthesised by higher organisms or taken up via foods are vitamin C, E and A; the latter can be also formed by cleavage from certain carotenoids which also possess potent ROS protective properties per se.

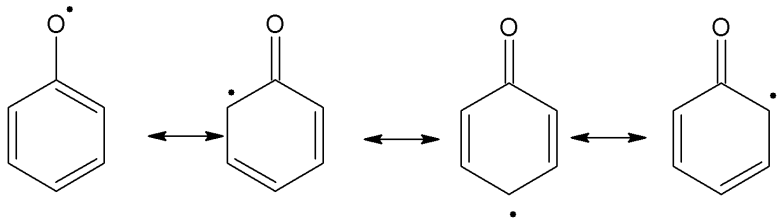

Figure 5: Stabilisation of the phenoxyradical due to delocalisation of the unpaired electron in the aromatic ring (adapted from Shahidi and Wanasundara [54])

1.5.2 Activation of transcription factors

Activation of transcription factors and complex signalling chains, which control these factors, is the concern of numerous investigations published in the last years. The general

characteristic of these defence processes is induction of cytosolic proteins (mainly enzymes such as mitogen activated protein kinases- MAPKs), which cause transcription of genes encoding for ROS defence mechanism. ROS themselves are involved in these processes, but also dietary factors can interact with the processes related to alteration of transcription. ROS have an impact on cell signalling via two different mechanisms, namely receptor- or non-receptor mediated pathway. Growth factors or cytokine receptors activate intracellular receptor mediated signalling which affect MAPKs. Also non-receptor proteins can be activated by ROS (e.g., Src, Ras –family)[55], which, as a consequence, trigger MAPK signalling (see Figure 6). In general, MAPKs relay signals generated by exogenous or endogenous stimuli to the intracellular space via phosphorylation of proteins [56]. The kinases interact also during this process with downstream mediators including transcription factors [57]. It has been shown that these processes are type and stimuli specific and it is interesting that endogenously produced H_2O_2 (e.g., by respiratory burst) induces extracellular signal-related kineases (ERK) but not p38, while exogenous peroxide treatment activates the latter enzyme [2,58,59]. The activation of the transcription factor, which controls the expression of protective genes, arrests division of damaged cells and induces apoptosis (programmed cell death) is one of the most significant effects of ROS on MAPKs. ROS activate several transcription factors, such as AP-1 (activator protein 1), NFκB (nuclear factor kappa-B), Nrf2 (Nuclear factor erythroid 2-related factor 2). Furthermore, they have also an impact on additional factors, for example on p53, also termed a “tumour suppressor” since it arrests cell cycle and induces apoptosis [60] (for detail see [2]). The complex interaction between ROS and signalling pathways is depicted in Figure 6.

Phytochemicals, such as quercitin, cumarin, genistein, and resveratrol can interact with signalling pathways [61-63]. The molecular mechanisms by which phytochemicals interact with signal transmission cascades are not fully understood. One possible mechanism is that the down regulation of transcription factors may be due to the direct scavenging of ROS [2].

A wide variety of chemopreventive phytochemicals is described in the literature. They prevent carcinogenesis either by enhancing cellular antioxidative and detoxification enzymes via activation of Nrf2 or by suppressing induction or overamplification of proinflammatory and growth promoting gene expression driven by NFκB or AP-1 [63]. In the subsequent section the most important transcription factors are described, which are affected by dietary factors or can be activated/inhibited via ROS.

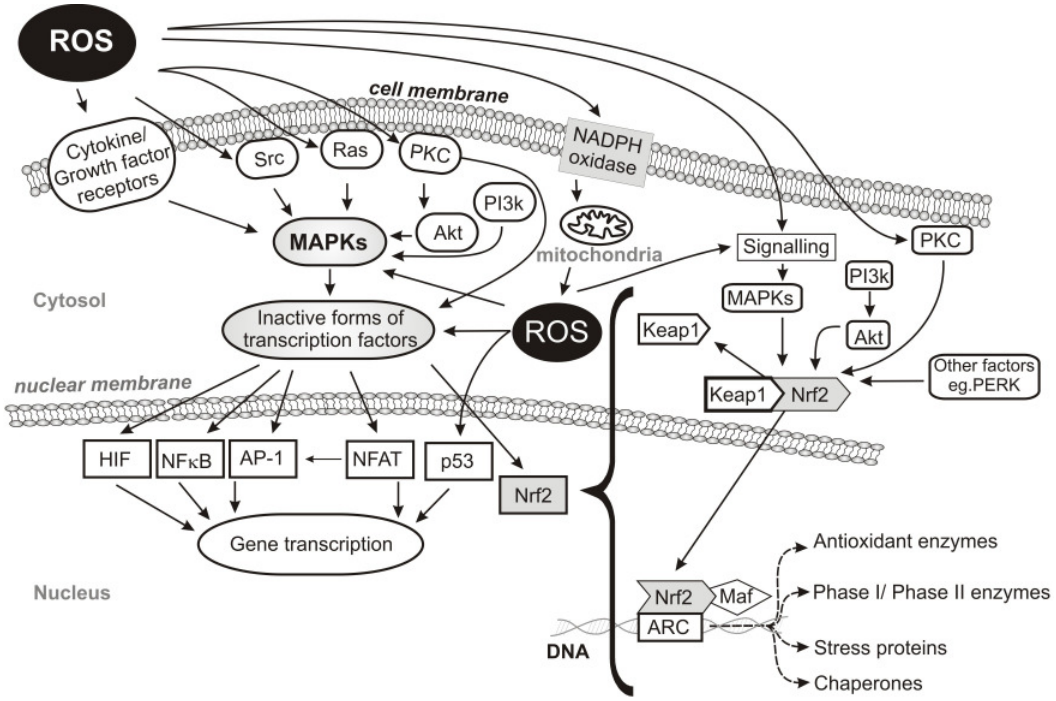

Figure 6: Reactive oxygen species (ROS) and their impact on signalling pathways (adapted from Knasmüller et al. [2])

NFκB (nuclear factor kappa-B) is a ubiquitous redox-regulated transcription factor that remains sequestered in the cytoplasm as an inactive complex with its inhibitory counterpart IκB. Exposure to oxidative and inflammatory stimuli leads to phosphorylation of IκB and subsequently to proteasomal degradation of IκB, thereby releasing free NFκB dimmers for translocation to the nucleus [63]. Hereby, ROS have been implicated as second messengers involved in the activation of NFκB via TNFα and IL-1 [64,65]. It is generally accepted that the degradation of IκB is important for the activation of NFκB, but there are also studies which show that the activation is independent of IκB [66,67]. In the nucleus, the transcription factor activates genes involved in inflammatory processes, transformation and angiogenesis [68]. A number of investigations demonstrated that the activation can be blocked by antioxidants including N-acetylcysteine, cysteine, vitamin E, thiols and green tea polyphenols [12].

AP-1 (activator protein 1) regulates the expression of genes, which are involved in cellular adaptation, differentiation and proliferation. It consists of either homo- or heterodimers between members of the JUN and FOS families, which interact via a leucine-zipper domain. This transcription factor is also regulated by the MAPK-signalling cascade [62]. Bioactive substances, e.g. curcumin, capsaicin, genistein and resveratrol (for detail review see [62]) have an impact on the AP-1 and NFκB transcription factors. [62].

Nrf2 (Nuclear factor erythroid 2-related factor 2) controls the expression of phase II enzymes, which are involved in the detoxification of carcinogens and protect against oxidative stress. A number of investigations showed, that dietary factors induce phase II enzyme expression via Nrf2. The induction of these genes is mediated by the antioxidative response elements (ARE) within the promoter regions of genes encoding for these enzymes. Nrf2, which belongs to the CNC (cap ´N´-collar) basic leucine zipper family, is the key mediator of ARE dependent activation. Nrf2 is sequestered in the cytoplasm by an actin binding protein Keap1 (Kelch-like ECH associating protein 1). Oxidative stress, as well as chemopreventive agents, leads to dissociation of Nrf2 from Keap1. As a consequence, Nrf2 translocates to the nucleus and binds to AREs. Numerous upstream signalling pathways (e.g., mitogen-activated protein kinases, protein kinase C) are involved in the regulation (for details see Figure 7). Chemopreventive agents can act for example via activation of signalling factors, direct disruption of Nrf2 –Keap1 complex or inhibition of proteasomal degradation [62,69].

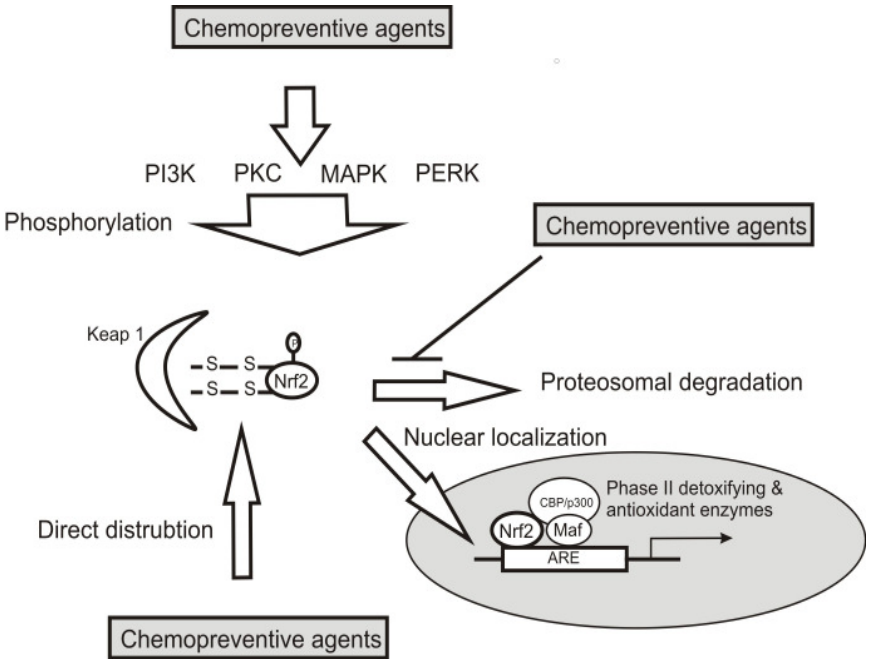

Figure 7: Induction of phase 2 detoxifying and antioxidant enzymes through activation of the Nrf2/ARE (modified after Jeong et al. [69]).

1.5.3 Antioxidant enzymes: properties and biological implications

The organism possesses a number of different enzymatic detoxification systems and it is well documented that dietary components can induce some of them. The most efficient

enzymatic antioxidants include superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) [70].

Superoxide dismutase (SOD) catalyses the dismutation of the highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2. The reaction product (H_2O_2) can be destroyed via catalase and glutathione peroxidase (GPx). In humans there are three main forms of SOD, namly mitochondrial Mn-SOD, cytosolic Cu/Zn-SOD and extracellular SOD [70]. Mn-SOD is a homotetramer and contains one manganese atom per subunit, which cycles from Mn (III) to Mn (II) during the dismutation reaction. The major source of free radicals is the respiration chain in the mitochondria. It has been shown that cytokines have an impact on induction or depression of Mn-SOD, whereas oxidants influence Mn-SOD only moderately [71]. There is also evidence that the transcription factors NFκB and AP-1 have impact on the expression of Mn-SOD [72]. Mn-SODs are more labile to denaturation by heat, organic solvents or detergents than Cu/Zn-SODs. In most animals and yeast, Mn-SOD is usually entirely located in the mitochondria [73]. The Cu/Zn-SODs have two identical subunits of about 32 kDa and each of them contains a metal cluster [70]. Investigations on the distribution of SODs demonstrated that Cu/Zn and Mn-SOD had the highest contents in the liver. Among various tissues Mn-SOD contents were half as large as the Cu/Zn-SOD [74]. Extracellular superoxide dismutase (EC-SOD) contains also Cu and Zn and was found in the interstitial spaces of tissues and extracellular fluids. It accounts also for the SOD activity in the plasma, lymph and synovial fluid [75].

Catalase promotes the conversion of H_2O_2 to water and molecular oxygen. It has a very high turnover rate (1 molecule of catalase converts 6 millions of H_2O_2 molecules into water within 1 minute) [76]. The catalase is present in all organs, high amounts are found in the liver. The enzyme is mainly located in the peroxisomes [13,76].

Glutathione peroxidase (GPx) contains selenium and catalyse the reduction of adifferent hydroperoxides (e.g., ROOH and H_2O_2) using GSH as a substrate. Oxidised glutathion (GSSG) can be reduced via glutathione reductase. In mammals at least five isoforms of GPx are known which are located in different parts of the cell (e.g., cytosol, membrane, mitochondria). Although their expression is ubiquitous, the levels of each isoform vary, depending on the tissue type [77]. Figure 8 depicts the impact of different antioxidant enzymes in the detoxification of ROS.

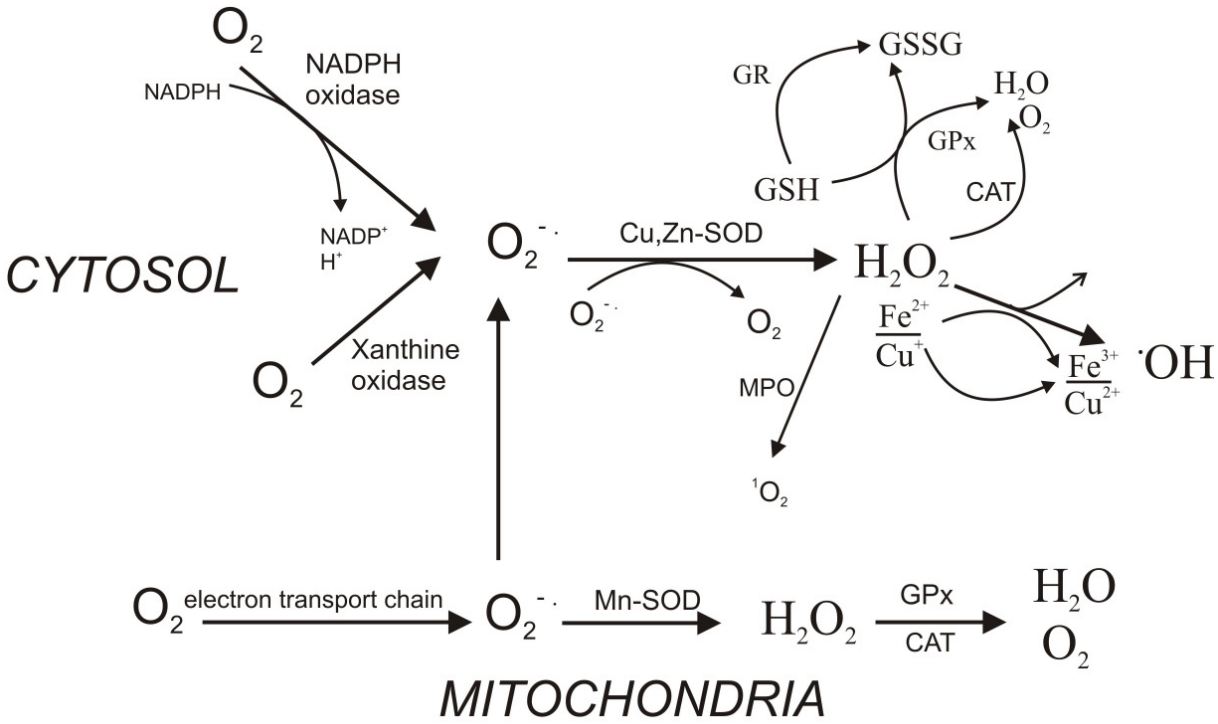

Figure 8: Important antioxidant enzymes and their impact on the detoxification of ROS (modified after Mates et al. [70]). CAT; catalase, GPx; glutathione peroxidase, GR; glutathione reductase, GSH; glutathione (reduced form), GSSG; glutathine (oxidised form), MPO; myeloperoxidase, SOD; superoxide dismutase.

Furthermore the organism also possesses enzymes (e.g., NADPH: quinone oxidoreductase and glutathione S- transferases) that catalyse metabolic detoxification of xenobiotics, drugs and carcinogens and, thus, protect the cells against oxidative stress [77]. GSH is not only involved in the detoxification catalysed by GPx, but involved in the metabolism of xenobiotics.

Many compound are metabolised by conjugation with GSH, catalysed by **glutathion-S-transferases (GST)** [13]. The GST family of enzymes comprises a long list of cytosolic, mitochondrial, and microsomal proteins that are capable of multiple reactions with a multitude of substrates and especially the liver is rich in GSTs. In mammals the alpha, mu and pi class are most abundant [13]. GSTs, like GPx, are involved in the detoxification of secondary products of ROS damaged macromolecules; this is mandatory to prevent further intracellular damage, degradation of cell components and eventual cell death and thus have antioxidant properities [11,78].

1.6 Conclusion: some remarks

ROS play an important role in living organism. They are essential in regard to the functioning of the immune system, and are continuously generated in the cause of energy metabolism. Excess in formation leads to adverse effects, including numerous diseases and it shortens the life span. In this case it is notable that excess production of SOD, and high intrinsic levels of antioxidants such as uric acid are associated with a longer life expectancy in vertebrates and invertebrates [79,80]. Therefore it is likely that an improvement of the ROS protection system via dietary strategies; i.e. via consumption of components which act as direct scavengers or which activate transcription factors controlling antioxidative enzymes may have beneficial effects in humans. Numerous antioxidants have been detected in the diet. However it has been stressed that in many cases evidence for protection is restricted to results from *in vitro* experiments, which do not enable to draw firm conclusions if beneficial effects can be expected in man [13].

CHAPTER 2
METHODS FOR THE DETERMINATION OF ANTIOXIDANT EFFECTS IN HUMAN INTERVENTION TRIALS

2.1 Introduction

A broad variety of different approaches has been developed, which are applied to monitor the redox status in humans. The research of antioxidants in human nutrition has increased considerably over the last few decades and many intervention trials have been performed to elucidate if dietary factors cause antioxidant effects *in vivo*. Several individual antioxidants, combination of several antioxidants and antioxidant rich foods and other plant constituents (e.g., gallic acid, green tea polyphenols) have been studied in human intervention trials. Also under disease conditions (e.g., cardiovascular disease, diabetes) intervention studies have been performed with antioxidative food components. The most frequent materials used in human studies are body fluids and blood cells (e.g., plasma, erythrocytes, lymphocytes, monocytes, granulocytes). The present chapter will describe methods, which are applied in human intervention trials.

Basically, the different methods can be divided into two main categories namely a) direct markers and b) indirect markers of antioxidant activity. A short overview based on the different markers is given in Figure 9.

2.2 Direct markers of antioxidant activity

2.2.1 Total antioxidant capacity (TAC)

To measure the TAC, two main techniques have been developed. One measures the **ability of a substance to transfer one electron (electron transfer)**. Due to this property radicals, carbonyls or metals are reduced. The most common tests, which belong to this category, are the trolox equivalent antioxidant capacity (TEAC), the ferric iron reducing antioxidant parameter (FRAP) and the diphenyl-1-picrylhydrazyl test (DPPH). The other one comprises methods are described which are based on the **ability to quench free radicals by hydrogen donation (hydrogen atom transfer)**. A variety of test systems, such as the total radical trapping antioxidant parameter (TRAP) and the oxygen radical absorbance capacity test (ORAC), as well as methods measuring the inhibiton of linoleic acid or LDL oxidation fall into this category.

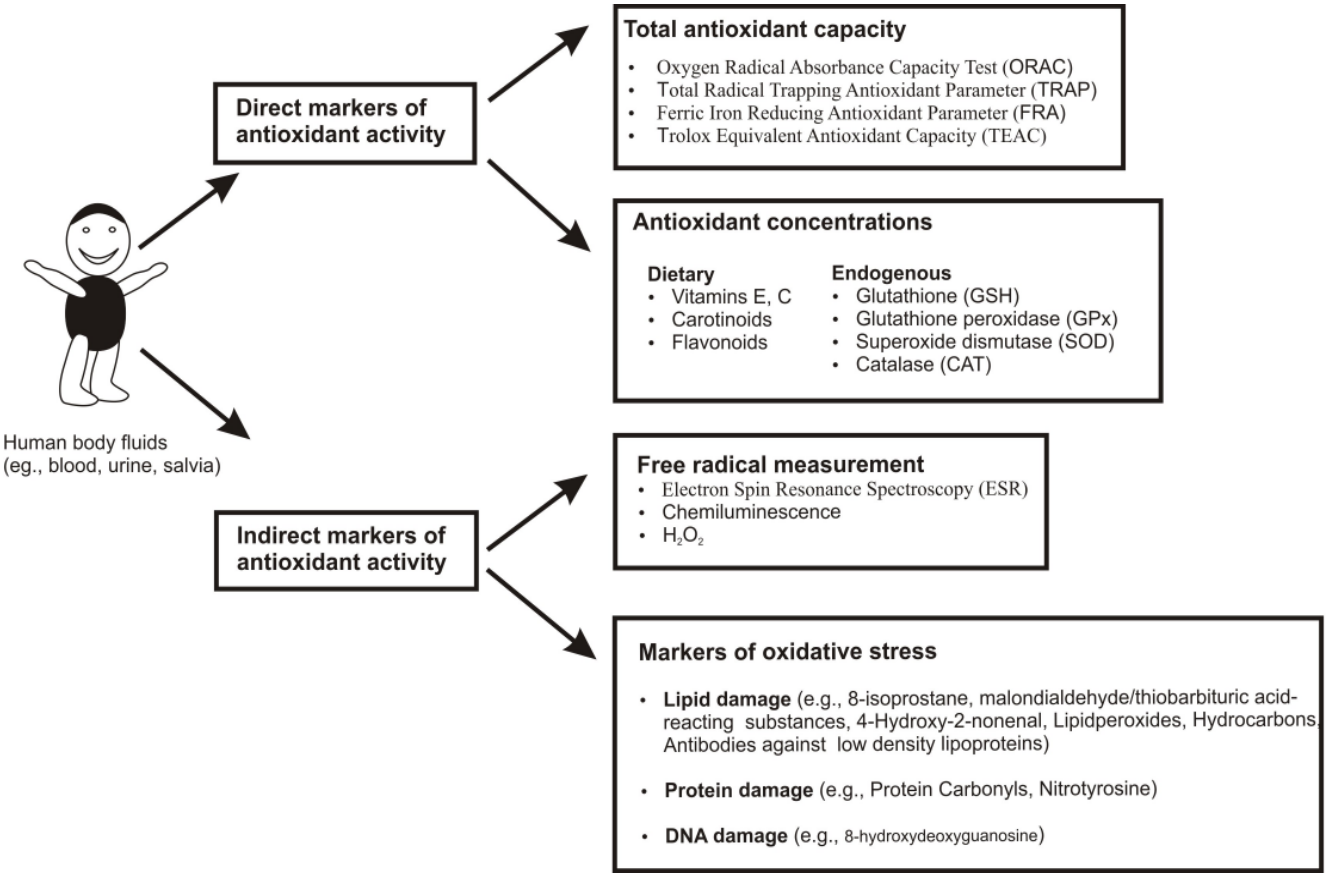

Figure 9: Biomarker of oxidative stress (adapted from Wood et al. [81])

The measurement of TAC can be used in human and animal studies (determination of TAC in plasma/serum), but to a great extent, these measurements are used to determine the antioxidant potential of foodstuffs. The pitfalls and limitations of each method are described in more detail in recent reviews [2,81-83].

The principle of the **electron transfer (ET) methods** is, that the sample itself is an oxidant that abstracts an electron from an antioxidant. The total antioxidant capacity indicates therefore the total reducing capacity.

For example, the TEAC assay measures the antioxidant capacity of plasma compared with the activity of a strong Vitamin E analogue ("Trolox") [84]. The TEAC assay is based on the ability of antioxidants to scavenge blue-green colored 2,2´-azinobis (3-ehtylbenzthiazoline-6-sulfonic acid) radicals ($ABTS^{\cdot}$). This chromophore has the advantage of being soluble in organic solvents and aqueous conditions and its reactivity is not affected by ionic strength, therefore it can be applied to determine lipophilic and hydrophilic antioxidants [83]. The concentration of the amount of $ABTS^{\cdot}$ which accumulates within a fixed time period is used to quantify the antioxidant capacity, which is calculated as Trolox equivalents [85].

In the FRAP assay the reduction of 2,4,6-tripyridyl-s-triazine (TPTZ) to a colored product is determined. In order to maintain the solubility of Fe, this approach is conducted at acidic conditions (pH of 3.6) [86]. This assay (similar to other "reducing assays") does not enable to monitor compounds that quench radicals, for example thiolic compounds such as glutathione or proteinsm, therefore the antioxidant capacity of the samples may be underestimated. A modified version is the copper reducing assay (CUPRAC) in which iron is replaced by Cu (Cu^{2+} is reduced to Cu^{1+}). The advantage of this protocol is that thiol type antioxidants can be determined (such as GSH), Cu has a lower redox potential (more selective) and high stability.

Another assay developed by Brand Williams et al. [87] uses the 2,2 diphenyl-1-picrylhydrazyl radical, which is commercially available. Compared to $ATBS^{-}$, the DPPH measurement is carried out under nearly physiological conditions (i.e. pH 7.0) [88]. The different compounds used for the TAC assays described above are depicted in Figure 10.

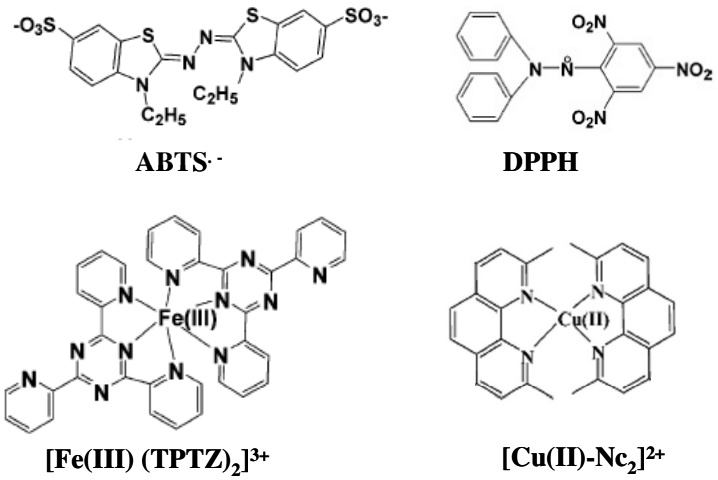

Figure 10: Substrates, which are used to determine the total antioxidant capacity based on electron transfer reactions. $ABTS^{\cdot -}$ - 2,2´- azinobis(3-ethylbenzthiazoline-6-sulfonic acid); DPPH - 2,2 diphenyl-1-picrylhydrazyl radical; [Fe(III) $(TPTZ)_2$] (TPTZ; 2,4,6-tripyridyl-s-triazine), $[Cu(II)\text{-}Nc_2]^{2+}$ (Nc- Neocuprine, 2,9-dimehtyl-1,10-phenantroline).

The majority of the hydrogen atom transfer methods uses a reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through decomposition of azo compounds. The first TAC method was the TRAP assay developed by Wayner et al. [89] using 2,2´- azobis (2,4-amidinopropane) dihydrochloride, a hydrophilic azo-compound which generates peroxyl ($ROO^{\cdot}$) radicals. Meanwhile, different other approaches have been developed which differ from each other in terms of substrates, reaction

conditions and quantitation methods [90]. In general, the samples react with $ROO^{\cdot}$ radicals at low concentration and a significant spectroscopic change occurs between the native (original) sample and the oxidised sample. Only when antioxidants have been depleted the $ROO^{\cdot}$ radicals will attack lipids and cause lipid peroxidation. A modification of the TRAP assay is the chemiluminescence (CL) assay, which is based on reactions with oxidants that emit CL (for details see [91]).

Also the ORAC assay based on inhibition of $ROO^{\cdot}$ induced oxidation by antioxidants. The radical reacts with a fluorescent probe thereby forming a non-fluorescent product which can be quantified. In the first version developed by Cao et al. [92], ß-phycoerythrin was used as a fluorescent agent, but due to shortcomings and inconsistencies of the results, fluorescein or dichlorofluorescein are currently used. These fluorescent agents are less reactive and more stable [83]. One advantage of the ORAC assay is that it can be easily automated [93] and excellent results have been obtained with well plates coupled with a microplate reader [94]. A disadvantage of this method and for TRAP is interference of proteins which contribute by ≥ 80% to the total antioxidant capcity [83,90]. In order to obtain reproducible data, trolox can be used as internal standard or the samples must be deproteinized prior to the measurements. Other hydrogen atom transfere methods are the Crocin bleaching assay, inhibited oxygen uptake (IOU) measurements or approaches, which detect the inhibition of linoleic acid oxidation.

Several critical reviews (e.g. [82,83]) emphasize, that no single test reflects the overall antioxidative capacity and the situation even becomes more complex, if we want to measure the redox status in *in vivo* studies. It is notable, that the different TAC assays do not provide information on bioavailability, *in vivo* stability, retention of antioxidants in tissues and reactivity *in situ* [82]. Additionally to the assay validity, special attention has to be paid to confounding factors such as matrix effects. Also clinical, metabolical, or physical conditions, which are not necessarily related to oxidative stress may influence the antioxidant capacity. For example, in human plasma, the TRAP values are around 10^3 µmol $ROO^{\cdot}$ and it is known, that the major contributors are urate (35-65%) and plasma proteins (10-50%), followed by ascorbate (up to 24%) and vitamin E (5-10%). Due to pathologies, the urate levels can raise because of alterations in the purine metabolism, therefore in some studies an increase of plasma TAC was observed in diseased people. In some studies, males had higher TAC values than females, which may be due to higher urate levels [13]. It can be seen in Table 2 that the TAC values differ strongly when different methods are used. These differences appear because of the sensitivity of the chemical reactions on which the measurements are based and

the specific experimental designs (e.g., the duration of the measurements). Some assays run to completion (e.g., ORAC) while others run only for a fixed time period (e.g., the $ATBS^{\cdot}$ assay) and in some a lag period is determined.

Also the anticoagulants used to prepare plasma may affect the outcome. The use of heparinized tubes is better than EDTA tubes, because heparin stabilizes plasma against loss of TAC [95]. It has also be seen that the TAC values are general higher in males than in females [95,96].

Table 2: Total antioxidant capacity in human body fluids by various assays (modified after Halliwell and Gutterdige [13])

Method	Principle	Representative results (µM)	Ref.
$ABTS^{\cdot}$ assay	ET	~ 2000	[97]
FRAP	ET	1030 ± 220	[86]
TRAP	HAT	~ 1000	[13]
Enhanced chemiluminescence	HAT	829 ± 77	[98]
ORAC	HAT	3100 ± 490	[99]
Crocin assay	HAT	~ 2000	[13]

Abbreviations: ABTS; 2,2´- azinobis(3-ethylbenzthiazoline-6-sulfonic acid), FRAP; ferric reducing anti-oxidant power of plasma, TRAP; the total radical trapping antioxidant parameter, ORAC; oxygen radical absorbance capacity test, ET; electron transfer, HAT; hydrogen atom transfer.

2.2.2 Determination of the concentrations of selected antioxidants

Measurements of **dietary antioxidants** such as vitamin C [100], vitamin E [101,102], carotenoids (e.g., α- and β-carotene, lycopene, lutein, zeaxanthin and β-cryptoxanthin) [102,103] and flavonoids (e.g., quercetin, epicatechin, proanthocyanidins) are usually performed with high performance liquid chromatography (HPLC) linked with various detection systems (e.g., UV spectra, fluorescence, electrochemical detection).

HPLC is a sensitive and highly accurate technique, however due to the expensive equipment, the requirement of a high degree of technical expertise, and the time consuming sample preparation procedures this technique is not available in all laboratory settings. Furthermore some technical difficulties may arise due to the fact that certain antioxidants are instable. Precautions include for example, protection of samples from fluorescent and direct light, cool temperatures during extraction and processing of the samples, and the storage of the samples at –80°C.

Humans possess an **endogenous defense system** against ROS, which includes antioxidative enzymes catalysing the enzymatic detoxification of ROS as well as

endogenously formed compounds (e.g., glutathione, uric acid, plasma proteins), which act as radical scavangers. The activation of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and gluthatione peroxidase (GPx) are usually determined spectrophotometrically and commercial kits are available for this assays [104-106]. Specific isoenzymes can be monitored with enzyme linked immunosorbent assay (ELISA) kits.

The reduced or oxidised form of glutathione can be determined with HPLC or with spectrophotometric assays [107,108]. The measurement of plasma proteins (e.g., albumin) is often carried out with HPLC but for protein measurements also enzymatically methods are available, which makes analyses for more laboratories accessible [109,110].

2.3 Indirect markers of antioxidant activity

2.3.1 Measurements of free radicals

An indirect approach to determine the antioxidant properties (in body fluids) is the measurement of free radicals. However, this determination is difficult due to the highly reactive nature of ROS. The **electron spin resonance spectroscopy (ESI)** method measures the absorption of energy, which results from the interaction of ROS with an applied external magnetic field. For this method, spin trapping agents are required to form spin adducts, which are more stable than the original ROS. Because of the toxicity of the trapping agents, these methods have limitation in regard to their *in vivo* application [111]. Another indirect approach to determine ROS is based on **chemiluminescene**, which needs highly specialised equipment. The consequence of electron transfer reactions, initiated by a probe is the emission of light (in the wavelengths of near-infrared and infrared regions). The two major probes used in human samples are lucingenin, which detects superoxide anions and luminol, which is a more general detector for ROS [112,113]. Intracellular ROS can also be determined fluorometrically, with the **dichlorofluorescein (DCFH-DA) assay** using a flow cytometric analysis system. DCFH-DA is deacetylated by cytosolic esterases, this metabolite is trapped within the cytoplasm where it is oxidised in the presence of ROS to 2,7 dichlorofluorescein (DCF). Furthermore also a number of colorimetric and fluorometric assays exist to determine H_2O_2, which are rather simple to perform, but the instability of the molecule remains problematic [114,115].

2.3.2 Measurement of oxidised biomolecules: DNA, proteins, lipids

With these approaches not the quantity of antioxidants or ROS is measured, but the damage, which is caused by ROS. Biomarkers of oxidative damage are important in human trials since alterations of biologically relevant molecules have an impact on the health status.

The oxidation products are used to determine oxidative stress; therefore they can provide information about the antioxidant capacity of different compartments.

DNA is highly susceptible to radical-induced damage. Oxidative alterations are measured in many laboratories since it is assumed that such damage is involved in the etiology of many diseases, including cancer. The incidence of most cancers rises with the fourth of fifth power of age in animals and about 35% of humans have cancer by the age of 85. Since the beginning of the 80ties it has been hypothesized that aging is due to a lifetime of attack by ROS [116]. It is notable, that oxidative adducts occur at a frequency of 1 or more orders of magnitude higher than non-oxidative adducts [37]. However, so far it has been not proven that oxidised DNA is a valid biomarker for cancer risks [117]. In the following section, several measurements are described which are used in dietary human intervention studies to determine the oxidative state of the DNA.

The most important oxidation product formed as a consequence of ROS mediated damage is **8-hydroxydeoxyguanosine (8-OHdG)**, which can be monitored with different methods in urine and peripheral lymphocytes of humans. Most frequently used methods are based on chromatography such as gas chromatography coupled with mass spectrometry (GC/MS), liquid chromatography prepurification followed by gas chromatography coupled with mass spectrometry (LC-GC/MS), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and others [2]. Recently also attempts have been made to develop enzyme linked immunosorbent (ELISA) kits [118]. It is known that guanine is prone to oxidation if precautions are not taken during the preparation of samples for analysis [119], therefore also artefacts might be measured. There are also large discrepancies between results obtained with different methods for the determination of 8ohdG (for details see [2]). For example, GC/MS estimates of DNA oxidation are consistently higher than HPLC estimates by a factor of 10 [120]. The cause of these differences is artificial oxidation with GC-MS vs. an underestimate due to inefficient enzymatic digestion with HPLC-ECD [121]. Also the results obtained with ELISA are different to those of chromatographic methods. Attempts have been made by the European Standards Committee on Oxidative DNA Damage (ESCODD) to compare different groups in several countries to clarify methodological questions and to develop optimal protocols [122-124]. So far, more than 20 studies have been published, in which the impact of dietary factors on 8-OHdG was measured [125,126].

The **single cell gel electrophoresis (SCGE) assay** is based on the determination of the migration of DNA in an electric field. DNA damage leads to comet shaped images, therefore this technique is also called “Comet assay” (see Figure 11). The first experiments were carried

out under neutral conditions, which allowed only the detection of double strand breaks [127], subsequently a modified protocol was developed by Singh et al. [128] and Tice et al. [129] in which the DNA is unwinded under alkaline conditions (pH>13). This version enables also the determination of single strand breaks, DNA protein crosslinks and apurinic sites. The target cells used in most human studies are peripheral lymphocytes, only few investigations were carried out with exfoliated epithelial cells from the buccal cavity [130-133]. The use of epithelial cells is problematic due to their low viability. Recently, a human intervention trial was published in which bioptic material from the colon was used to investigate the influence of fried meat consumption on DNA stability [134]. More information concerning human intervention trial and the application of the SCGE assay is provided in a review which is part of the present thesis (page 135) [135].

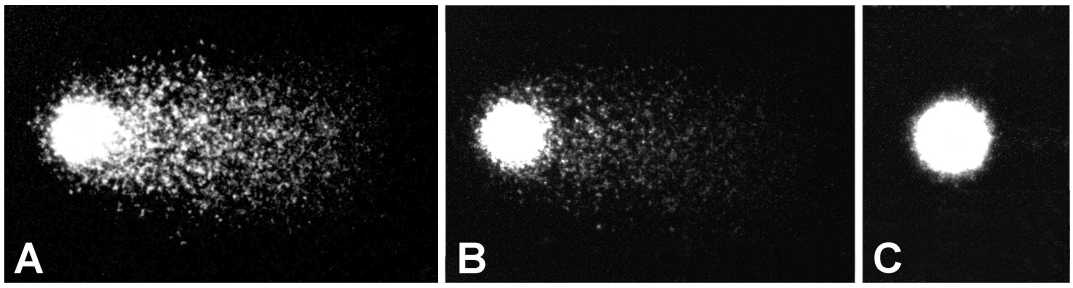

Figure 11: High (A), moderate (B) and undamaged (C) cell nuclei (examined with the SCGE technique)

The **cytokinesis block micronucleus (CBMN) method** developed by Fenech et al. [136,137] allows to monitor micronucleus (MN) formation in peripheral blood cells *in vitro* and can be also used in dietary human studies with lymphocytes. This assay is one of the most important techniques to assess structural and numerical chromosome damage and has widely replaced conventional chromosomal aberration (CA) analyses as the MN experiments are less time consuming and laborious. The procedure is based on the use of the mitogen phytohaemagglutintin (which stimulates nuclear division), in combination with cytochalasin B (which stops cytokinesis). Treatment of the cells with these chemicals leads to formation of binucleated cells, which are a signal of replication. The CBMN assay can be evaluated using simple morphological criteria. Additionally other endpoints of genotoxicity and cytotoxicity can be determined, such as chromosome breakage, chromosome loss, chromosome rearrangement (nucleoplasmic bridges), cell division inhibition, necrosis and apoptosis (see Figure 12). Only few studies have been performed to investigate the impact of nutritional factors in humans with the MN assay. Examples are interventions with red wine [138-140]

and vitamin C [141], a comparative study of vegetarians and non-vegetarians [142] and experiments concerning the role of folic acid and vitamin B12 [143].

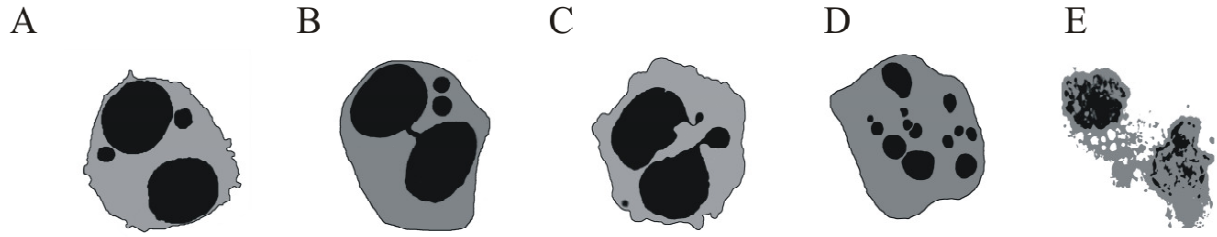

Figure 12: Examples of different endpoints, which can be evaluated with the cytokinesis block micronucleu (CBMN). A: cell with two micronuclei, B: nucleoplasmatic bridges, C: nuclear buds, D: apoptotic cell, E: necrotic cell

Cell membranes are highly susceptible to **lipid peroxidation (LP)** due to their specific composition, which is characterized by their high content of polyunsaturated fatty acids (PUFAs). PUFAs are sensible to oxidation, as the presence of a double bond weakens the C-H bonds on the adjacent carbon atoms, facilitating H^+ removal. Free radicals (e.g., hydroxyl radical- OH^-, alkoxyl radical- RO^- and peroxyl radical- ROO^-) initiate LP in mitochondrial and microsomal membranes rich in PUFAs. LP oxidation is a chain reaction and leads to alterations of the membrane properties, which include inactivation of membrane-bound receptors and enzymes, disturbance of the membrane fluidity, increase of the permeability and also rupture the membranes, which leads to a release of the cell organelle contents [81]. Oxidative damage of membrane PUFAs leads to formation of numerous products, most of them are unstable and degrade rapidly (e.g., shortchain alkanes and aldehydes). Some of the intermediates or endproducts are used for *in vivo* measurements of LP, for example conjugated dienes, malondialdehyde (MDA), F2-isoprostanes, exhalation of the alkanes pentane and ethane gases and cytotoxic aldehydes [144]. The most important parameters are described below.

Determination of **malondialdehyde (MDA) and thiobarbituric acid-reacting substances (TBARS)** in body fluids is one important and widely used parameter of PUFA peroxidation [2]. The derivatisation of MDA with thiobarbituric acid (TBA) results in formation of a pink MDA-TBA adduct, which is quantified using a spectrophotometer [111]. There are several concerns regarding the validity and specificity of this method [111,145]. It has been stressed, that the presence of iron in the assay reagents may lead to false results. Other sources of uncertainties are the rapid metabolism of MDA and the fact that MDA

represents less than 1% of lipid peroxide [111]. Also heating of the samples with TBA at low pH is far from physiological conditions and not only free MDA in the original sample is measured but also the aldehyde generated during this procedure by decomposition of lipid peroxides [146]. Also other compounds like haemoglobin, sugars or amino acids present in the sample can interact with TBA. HPLC separation of these compounds before to the acid heating can increase the sensitivity of the assay. The harsh conditions of the sample preparation and the cross reactivity with other aldehydes might be an explanation of the overestimation of MDA levels by commonly used TBAR measurements (more than 10-fold) [147]. Furthermore, MDA is not only formed during LP but is also a byproduct of cyclooxygenase activity in platelets [148]. Attempts are made to refine the techniques for the measurement of MDA, but the clinical relevance of this approach as an index of LP is questionable. Nevertheless it is notable that it has been shown by a large number of studies that increased levels are found in patients with ROS related diseases, such as cancer, diabetes and cardiovascular disease [149].

Isoprostanes are isomers of enzymatically formed prostaglandins [150]. The advantages of their use as marker of isoprostanes are that they are structurally stable, they are produced *in vivo* and are present in relatively high amounts [151]. Morrow et al. [152] discovered in the 1990s the formation of prostaglandin F2-like compounds in humans by non-enzymatic free radical-induced peroxidation of arachidonic acid. At present, the measurement of isoprostanes is regarded as one of the most reliable markers of oxidative stress. It has been postulated that the measurement of 8-iso-PGF-2α is 20 times more sensitive than the determination of thiobarbituric acid-reactive substance (TBARS) [151]. A large number of endproducts are generated but interest has focused mainly on F-2-Isoprostanes in particular, on 8-iso-prostaglandin F-2α (PGF-2α) (sixty-four different F-2-isprostanes exist) [13]. One important consideration to ensure reliable isoprostane measurements is to prevent the autooxidation of the samples, therefore the probes should be stored immediately after collection at –80°C and addition of antioxidants (e.g., butylated hydroxytoluene (BHT) and triphenylphosphine (TPP)) during sample processing is recommended [81,153]. Several approaches are available for the detection of F-2 isoprostanes in biological fluids (e.g., urine, plasma) including radioimmunoassay (RIA), enzyme immunoassay (EIA), high performance-liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-tandem MS), gas chromatography-mass spectrometry (GC-MS) and GC-tandem MS. The first results were obtained by use of the MS technique, which allows the structural identification of several isoprostanes, isoprostane esters and isoprostane metabolites [153]. Mass spectrometric

methods are expensive and time consuming, but they are highly specific and sensitive. More recently also radio- and enzyme immunoassays have been developed for isoprostane measurement [154,155]. The results obtained with these techniques correlate quite well with the findings of GC-MS measurements [155], however some discrepancies can occur due to the cross reactivity of the antibodies with other prostaglandin metabolites, especially in plasma samples [156]. Therefore purification methods before using immunoassays should be applied to improve specificity. Although the measurement of F-2-Isoprostanes by mass spectrometry is regarded as the most reliable method [157], the use of immunoassays might be appropriate in intervention studies with various samplings from the same subject, if the results are not focused on the absolute levels but on the relative change [2]. Several human intervention studies showed that intake of antioxidants can suppress the levels of isoprostane formation. For example, in an intervention study with high doses of antioxidants (including vitamin C, vitamin E and ß-carotene) the participants had a reduced level of isoprostane by 25-55% after 2 weeks of supplementation and also the application of vitamin E for 2 weeks in a human trial resulted in a decrease of isoprostane formation by 37% [81].

After peroxidation of ω-6 and ω-3 polyunsaturated fatty acids (PUFAs), the relatively unstable fatty acid hydroperoxides are converted into more stable aldehydes. The main aldehydes formed after peroxidation of ω-3 PUFAs are propanol and 4-hydroxyhexenal and whereas the peroxidation of ω-6 leads to the formation of **4-hydroxynonenal (4-HNE)** and hexanal [158,159]. These LP products are the most frequent formed aldehydes in biological tissues [160]. The most common used method to determine the aldehyde concentrations are based on GC-MS [161]. Experiments with mammalian cells indicate that 4-HNE causes acute cytotoxicicity [159], furthermore it induces also rapid depletion of GSH, inhibition of DNA, RNA and protein synthesis, LP and inhibition of respiration and glycolysis which precede the cell death [160]. The *in vivo* concentrations of "free" aldehydes are low, because of their conjugation to proteins. This fact, makes the assessment of their true levels based on HPLC or GC methods difficult. A wide range of antibody-based methods for the determination of aldehyde-protein conjugates are available [13]. The antibodies which are available have variable specificity; for example the preferred antigen recognized by monoclonal anti-HNE antibody Ig4 is an HNE histidine adduct [162].

The measurement of **breath hydrocarbons** is a non-invasive method which is based on the determination of LP through exhaled breath by determination of trace volatile hydrocarbons [163]. The end products of ω-6 fatty acid peroxidation are pentane and its isomer isopentane while from ω-3 fatty acid peroxidation ethane is formed. ω-6 PUFAs

predominate in the human body, so that pentane would be formed predominantly if all PUFAs would peroxidize equally. Both gases are measured by use of GC [164]. The expired breath is passed through an adsorbent, which concentrates the hydrocarbons, which are then desorbed and assayed. These approaches have some disadvantages; the hydrocarbons are minor endproducts of peroxidation and their formation depends on the presence of transition metals and oxygen concentrations [165], also bacteria present in the skin, colon and/or cavity produce hydrocarbons in significant amounts. Furthermore, the air in large cities is contaminated with hydrocarbons due to combustion processes (e.g., motor vehicle, environmental smoke) [13] and analytical problems were encountered in the past to separate pentane from isoprene, resulting in high levels of breath pentane. As a consequence, attempts have been made to improve these techniques. Some human studies indicate increased levels of hydrocarbon exhalation with age, smoking, hyperoxia, scleroderma, diabetes and some lung disease [13,166-169].

The **oxidation of lipoproteins**, in particular of low density lipoproteins (LDL) cholesterol plays an important role in the development of artheriosclerosis [170]. Beside of analytical methods (GC/MS, HPLC/MS) an enzyme-linked immunosorbent assay (ELISAs) kit is available to detect oxidised LDLs (oxLDL) in a quick and simple manner. The antibodies are directed against the oxidative modifications of LDL. This technique provides an *in vivo* marker of protein or lipid peroxidation.

Proteins can be damaged directly via ROS or via LP products (e.g., 4-HNE). The oxidative damage leads to alterations of the function of biomolecules such as transport proteins, receptors and enzymes. Modification of membrane proteins might lead to a disturbance of the ionic balance. Markers of protein oxidation are in general less frequently used than LP parameters and are mainly used in combination with them [2]. **Protein carbonyls** are formed as a consequence of the oxidation of amino acid side chains can be detected with the "carbonyl assay". To determine protein carbonyls a conventional colorimetric assay with 2,4-dinitrophenylhydrazine is used [171]. Other methods to determine protein carbonyls are atomic absorption spectroscopy, fluorescence spectroscopy, HPLC [81] and spectrophotometrically by ELISA techniques. The detection of protein carbonyls is widely used in different laboratories. However, there are considerable variation reported in the baseline levels of protein carbonyls, depending on the assay performance [13].

The attack of tyrosine leads to formation of **3-nitrotyrosin (3-NT)**, which can be measured by GC/MS [172], HPLC [173] and also with immunological methods [174].

2.4 Conclusions and outlook

The assumption that there is no individual biomarker, which can be used for the characterisation of oxidative stress, is due to the complexicity of the reactions of different ROS. Dotan et al. found in a recent evaluation only weak correlations of commonly used parameters of oxidative stress [50]. The authors analysed the results of studies in which two or more methods were used under identical conditions and conclude that relationships between peroxidation products such as malondialdehyde, F-2 isoprostans, and carbonyls exist but not with concentrations of antioxidants and DNA migration. Inconsistent findings were also seen in human intervention trials where multiple endpoints were determined. For example in a study where the participants (n=18) were asked to consume anthocyanin/polyphenolic rich fruit juices the DNA migration in lymphocytes was decreased and increased levels of reduced GSH was observed, while other parameters of oxidative stress (e.g., MDA in plasma, excretion of isoprostanes in urine) were not affected. –Omics based approaches (genomics, proteomics, metabolomics) are increasingly used to study the impact of dietary antioxidants on human health. A detailed review about the advantages and shortcomings of the different methods has been published recently by Knasmüller et al. [2]. The authors conclude that the new high throughput techniques do not allow to establish antioxidant specific patterns of gene transcription and protein alterations, but stress that these approaches can provide evidence for protective effects of antioxidants in specific experimental settings and reversions of gene and protein expression patterns caused by ROS related diseases.

CHAPTER 3

ANTIOXIDANT EFFECTS OF COFFEE CONSUMPTION

3.1 Introduction

Coffee is one of the most widely consumed beverages worldwide. Its production (about 70 % Arabica and 30 % Robusta) varies annually and has increased by about 15-20% in the last 20 years from 5.2 million tons per year in 1984 to 5.9-6.4 million nowadays [175]. Besides of caffeine, which is well known for its stimulant effects, coffee contains numerous other bioactive substances. These compounds include, purine derivatives, polyphenolics including chlorogenic acid and its degradation product caffeic acid, Maillard reaction products and diterpenes such as cafestol and kahweol (C+K) [176]. Pellegrini et al. [177] demonstrated that coffee itself possesses a higher antioxidant activity compared to other beverages (e.g., 5 fold higher than green tea and 3 fold higher than red wine). Besides of antioxidant properties seen in *in vitro* studies there is also evidence from animal experiments and human studies. The consequences of coffee consumption on human health have been studied during the last decades and a series of epidemiological studies have associated coffee consumption with beneficial health effects, such as reduction of the risks of certain forms of cancer [178], diabetes type 2 [179], Parkinson and Alzheimer diseases [180] and it is conceivable that these protective effects are partly due to inactivation of ROS by coffee components.

This chapter concerns the antioxidant properties of coffee and its constituents with particular emphasis on human intervention trials, which have been conducted so far including studies, which have been carried out in course of the present thesis (see publications).

3.2 Bioactive constituents of coffee

Coffee is a complex mixture and contains a broad variety of substances [176]. In the volatile fraction more than 1400 different substances, which are responsible for its smell and flavour. The most intensely studied substances in the volativle fraction are shown in Figure 1. Some of them possess antioxidant properties and/or alter drug metabolizing or DNA repair enzymes (for details see [181]).

Caffeine (1,3,7, trimethylxanthine) is a purine alkaloid and antagonizes the adenosine receptor [182,183]. This results in stimulatory effects, such as elevation of blood pressure, increased metabolic rate, diuresis and stimulation of the central nervous system [184]. The alkaloid is almost completely and rapidly absorbed in the stomach and in the small intestine, thereafter it is distributed to all tissues including the brain [185]. A number of low dose *in vitro* experiments provide evidence that caffeine may contribute substantially to the antioxidant effects of coffee [186-188].

The coffee specific diterpenoids **cafestol and kahweol** (C+K) are responsible for the cholesterol raising effects of coffee [189]. They are present in unfiltered coffee, therefore Scandinavian boiled coffee, Turkish coffee and French press coffee contain high levels of C+K, whereas filtered coffee and instant coffee contain low levels [189,190]. Animal studies indicate that C+K induce phase II enzymes and inhibit phase I enzymes which activate carcinogens [191] and recent findings of Higgins et al. [192] indicate that they are responsible for the induction of the transcription factor Nrf2, which regulates enzymes involved in the antioxidant defence system.

A number of investigations indicate that **hydrocinnamic acids**, e.g. chlorogenic acid (CA) and caffeic acid, which are the major phenolic compounds found in coffee, play a key role in the antioxidant effects of coffee [7-9]. It has been shown that CA and caffeic acid are well absorbed in humans, but so far it is still unclear if the effects of seen in *in vitro* studies are relevant for humans, because they are extensively metabolized and metabolites of phenolics have often lower antioxidant capacities [4].

During the roasting process **trigonelline** in coffee beans is demethylated and forms nicotinic acid (niacin). It has been reported that coffee contains 1-3 mg of niacin per cup [193]. Niacin is an important watersoluble vitamin and is converted to nicotinamide and then to NAD and NADP *in vivo*, which play essential roles in living cells. Another degradation product of trigonelline is N-methylpyridinium, which possess antioxidative properties (induction of GST, elevated TAC levels in an animal feeding study) [194].

The bioactive substances in coffee, which possess antioxidant properties, are listed in Table 3.

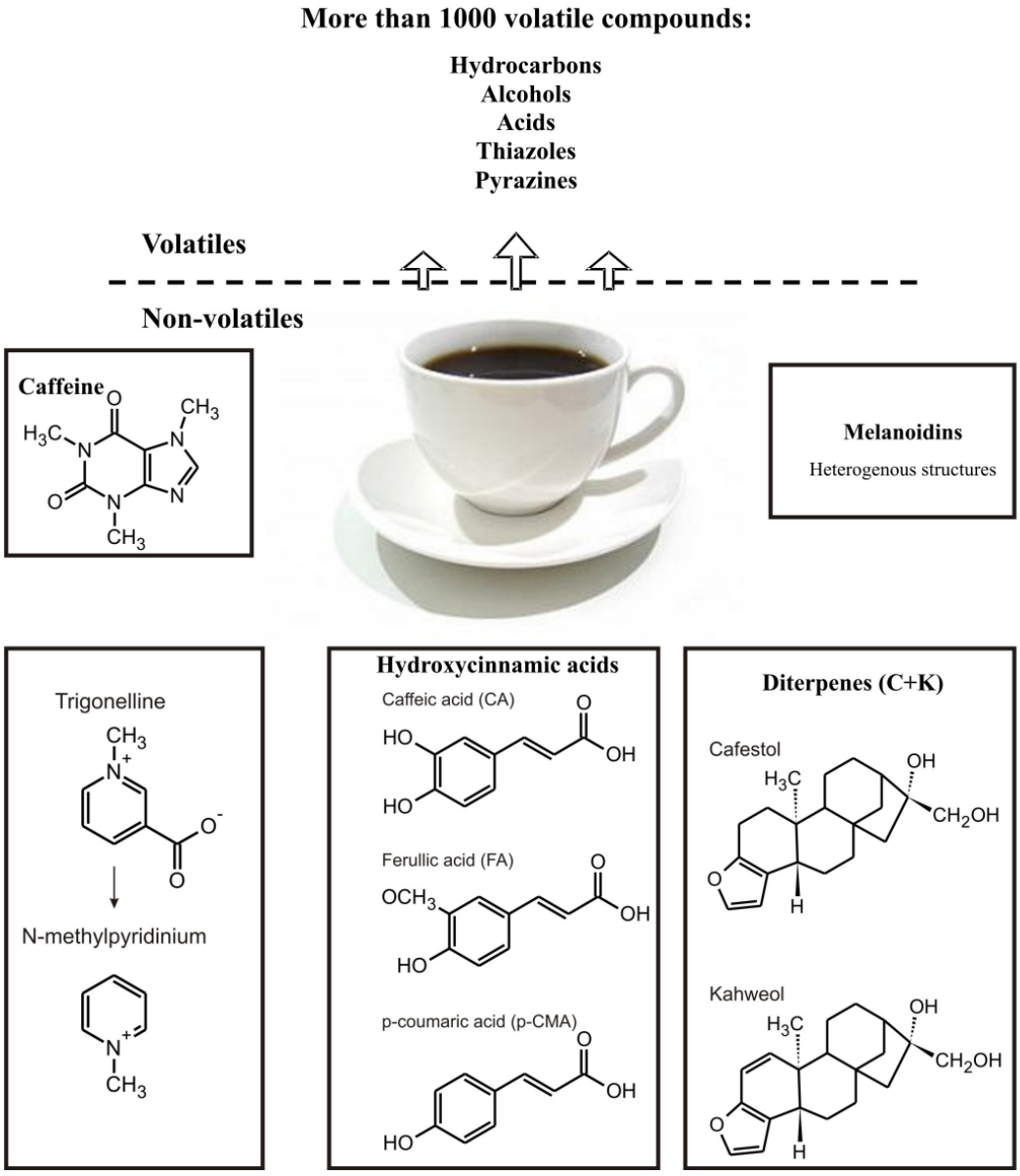

Figure 13: Structures of bioactive constituents of coffee (adapted from Faustmann et al. [181])

The antioxidant capacity of instant coffees (e.g., [177,195-198]) and also for filtered coffees (e.g., [199-201]) has been demonstrated in a number of *in vitro* studies. In this context it is notable that also adverse effects have been observed, for example induction of mutations in the Salmonella/microsome assay, which were attributed to formation of hydrogen peroxide. However, addition of a metabolic activation mix (S9) diminished these effects [202].

Also animal studies indicate antioxidative effects of coffee (for details see [203]). For example, in a study with instant coffee (8 days, 0.146-0.351 g coffee) which was given to CCl_4 treated Sprague-Dawley rats a increase of the TAC levels and a decrease in MDA levels was detected [204]. Furthermore also induction of GST levels attributable to coffee

consumption was observed in the study of Huber et al. (treatment with Turkish coffee) [205] and Somoza et al. (treatment with filter coffee) [194].

Table 3: Antioxidant properties of individual coffee components *in vitro* (adapted from [181])

Test compound	Results and comments
Caffeine	↓ formation of $OH^{\bullet}$ and $^1O_2$; protection against oxidative damage of calf thymus DNA and of radical induced migration in human lymphocytes
Hydroxycinnamic acids CGAs, CA, FA & p-CMA	numerous investigations showed inactivation of peroxides (t-BOOH, H_2O_2), scavenging of DPPH-radicals, inhibition of LP and reduction of radical induced DNA damage in bacterial and mammalian cells; the antioxidant activity of CGA and CA was higher than of Trolox
Cafestol & Kahweol	no antioxidant effects were found in *in vitro* experiments with human lymphocytes, Induction of Nrf2 and antioxidant enzymes in mice
Maillard products Melanoidins	most studies were conducted with "model mixtures", only in one, melanoidins from coffees with different degrees of roasting were tested, antiradical effects were found to ↓ with roasting time but prevention of LA peroxidation ↑ with roasting
Guaiacol & derivatives	fat-soluble phenolic compounds were found to possess high antioxidant activity in MDA experiments
Trigonelline & niacin	were found to possess lower antioxidant properties than CGA and caffeine (measurement of deoxyribose degradation)
Caffeoyltryptophan & protocatechuic acid	caused similar effects as CA and CGA (DPPH-radical scavenging)
Pyrolysis products of caffeic acid	different components were found protective at low doses
Heterocyclic compounds	strongest effects were seen with 2-acetylpyrrole and 1-methylpyrrole (measurement of conversion of hexanal to hexanoic acid)

Abbreviations: CA; caffeic acid, CGA; chlorogenic acid, DPPH; diphenyl-1-picrylhydrazyl, FA; ferulic acid, LA; linoleic acid, LP; lipid peroxidation, MDA; malondialdehyde, $^1O_2$; singlet oxygen, $OH^{\bullet}$; hydroxylradical, p-CMA; p-coumaric acid, t-BOOH; t-butylhydroperoxide

3.3 Epidemiological studies concerning health effects of coffee

In the recent article of Higdon and Frei [4] the results of epidemiological studies were summarized and the authors conclude:

Epidemiological studies indicate a decreased risk of **hepatic injury, cirrhosis and hepatocellular carcinoma** related to coffee consumption (e.g., [206-208]). In this context it is notable, that oxidative damage plays a crucial role in the etiology of these diseases and numerous studies indicated that dietary antioxidants are preventive against the devolopment of these injuries [209,210]. Furthermore it is well documented that cirrhosis is associated with increased incidences of hepatocellular cancer. It is also assumed that ROS play a crucial role in this disease [211,212]. In this context it is notable that the acitivites of serum aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) were decreased in two Japanese case control studies due to coffee consumption.

Furthermore, case control studies indicate inverse associations between coffee and **colorectal cancer** risk, but this effect has not generally been confirmed in prospective cohort studies [213,214]. Beside of other mechanism (e.g., insulin resistance), oxidative stress (e.g., via inflammation) may also play a role in the development of colorectal cancer and thus might be prevented via antioxidants [215].

In several large prospective studies an inverse association between the risk of **Parkinson disease** and the caffeine consumption from coffee has been found (e.g., [216,217]). In the case of neurological disorders it is less clear if the antioxidant effects of coffee play a role as other modes of action (e.g., ability of caffeine to block adenosine A_{2A}-receptors) may account for its protective properties.

Prospective cohort studies indicate a dose dependent relationship between coffee consumption and reduced risk of **type 2 diabetes**. (e.g., [179,218]). It is also unclear if antioxidant effects play a role in diabetes. Recent studies indicate that the impairment of glucose uptake by coffee components for example chlorogenic acid account for this effect [219]. It is notable that this phenomenon may lead to a hypocaloric state, which leads to changes of the mitochondria functions and as a consequence to lower release of reactive oxygen species.

It is also interesting that prospective studies have not found an association between coffee consumption and an increased risk of **cardiovascular disease or stroke** [220-223]. But randomised controlled trials (up to 12 weeks) found an increase in cardiovascular disease risk factors, such as blood pressure and plasma homocystein levels [224].

It has been suggested that for adults consuming moderate amounts of coffee (3-4 cups/d), little evidence exists for health risks, but some evidence of health benefits. Some groups, including people with hypertension, children, pregnant women and elderly may be more vulnerable to the effects of caffeine.

3.4 Human intervention studies with coffee

Several human intervention studies have been carried out to investigate the effect of coffee consumption on different markers of oxidative stress and/or on detoxifying enzymes. The main results of these human intervention trials are summarised in Table 4.

3.4.1 Impact of coffee consumption on DNA stability

Hoelzl et al. [225] and Bichler et al. [226] (see publications, page 79 and 91) measured the influence of coffee consumption on the DNA stability in human intervention trials with the single cell gel electrophoresis (SCGE) technique. The SCGE assay is based on the determination of the migration of DNA in an electric field. Undamaged DNA retains with matrix proteins in the nucleus, wherease damaged cells migrate and thus form comet like structures (viewed by fluorescence microscopy).

In the pilot study of Bichler et al. [226] (n=8, 200 ml French press + 400 ml paperfiltered coffee/d, 5 days) a significant reduction on the formation of oxidised purines and pyrimidines was observed after coffee intake, furthermore the sensitivity towards H_2O_2 induced DNA damage was reduced (for details see paper I, page 79).

On the contrary in the study of Hoelzl et al. [225] (n=29, 800 ml instant coffee/d, 5ds) no significant alterations of oxidised purines or pyrimidines and no reduction of H_2O_2 induced DNA damage was observed after the coffee drinking period. However, a slight decrease of these parameters was detectable but reached only in female volunteers in the case of FPG significance (detection of oxidised purines) after coffee consumption (for details see paper II, page 91).

The article of Steinkellner et al. [227] (for details see paper III, page 121) reports on a reduced sensitivity of peripheral human lymphocytes against (±)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE) induced DNA damage attributable to coffee cosumption which might be due to the induction of GSTP an detoxifying enzyme of polycyclic aromatic hydrocarbons (PAHs).

In an Italian questionnaire-based study (n=71) oxidised purines were determined (modified SCGE with formamidopyrimidine DNA glycosylase) [228] and a positive association with coffee consumption was detected, whereas in another questionnaire based

study (n=102) an inverse (but not significant) correlation between coffee consumption and formation of 8-OHdG in leukocytes was observed [229].

The urinary mutagenicity of coffee drinkers (n=32, 12g instant coffee/d for 12 ds) was investigated by Aeschbacher and Chappuis [230] in the Salmonella/microsome assay, but no significant alterations were detected.

3.4.2 Determination of lipid peroxidation products in human intervention trials

Natella et al. [231] found in a recent study that coffee consumption (n=10, 200 ml/P, once) induces incorporation of phenolic acids into LDL and increases the resistance of LDL to *ex vivo* oxidation. This observation is in agreement with the findings of an earlier study of Yukawa et al. [232], who conducted a human intervention study with instant coffee (n=11, 8 g/150 ml, 7 ds) and also reported on increased resistance of LDL to *ex vivo* oxidation, furthermore, a reduction of total cholesterol, LDL-cholesterol and MDA was observed after coffee intervention.

A large clinical trial in which 43 healthy non-smoking men were included no effects on LP parameters (serum LDL conjugated dienes, plasma hydroxy fatty acids, plasma F2-isoprostanes, serum cholesterol and triacylglycerol, serum LP resistance, LDL and HDL cholesterol) were detected. The participants (all males) of this study drank either 3 (450 ml) or 6 (900 ml) cups of paper-filtered coffee for 3 weeks. Also the single dose study 1 (150 ml), 2 (300 ml) cups of the same coffee brand did not cause any changes.

In the clinical trial described in paper II (page 91) the participants (n=29) were asked to drink 800 ml instant coffee for 5 days and different LP parameters such as MDA, oxLDL and F2-Isoprostane levels were determined. No alterations of the MDA or oxLDL levels were detected, but a significant decrease of F2-isoprostane levels was detected in the urine samples of the participants after coffee consumption.

3.4.3 Impact of coffee consumption on the activites of detoxifying enzymes

Several studies investigated the impact of coffee consumption on detoxifying enzymes. Bichler et al. [233] (paper I, page 79) observed an increase of superoxide dismutase (SOD) after the coffee consumption period, whereas no increase in the activity of glutathione peroxidase (GPx) was detected in human peripheral lymphocytes. On the contrary, no changes in the levels of SOD and GPx were observed after coffee intake (800 ml/p/day, 5 ds) in the study of Hoelzl et al. [225] (paper II, page 91), in which the parcitipants consumed instant coffee. Also Mursu et al. [234] measured the GPx activities in the plasma of the participants before and after coffee consumption and found no significant changes.

Steinkellner et al. [227] (paper III, page 121) found higher levels of GST (glutathione-S-transferase π) after coffee drinking, this phenomena was observed with both filtered and unfiltered coffee. An earlier human observation by Sreerama et al. [235] indicated elevated levels of GST (α, μ and π), aldehyde dehydrogenase (ALDH) and DT-diaphorase in salvia and plasma of coffee drinkers (>150 ml/d). These enzymes were also increased in an intervention trial with one volunteer (22 days, 1-2 l/d). In an investigation with (metal filtered) French press coffee no alteration of GST activity in colorectal mucosa cells was seen [236].

3.4.4 Impact of coffee consumption on endogenous antioxidants, total antioxidant capacity, H_2O_2 formation and protein nitrosation

In two earlier studies an increase of the endogenous formed antioxidant glutathione (GSH) was reported. In one of them, plasma GSH was determined [237], in the other GSH levels were determined in colorectal mucosa cells [236].

Also the total antioxidant capacity (TAC) levels measured with TRAP and the crocin test were elevated after coffee consumption (200 ml, n=10, once) in the study of Natella et al. [238]. Comparison with tea consumption carried out in the same trial indicated that coffee has higher antioxidant potency. These measurements were carried out in the plasma of the participants [238]. In another study by Garsetti et al. [239] the antioxidant activity in feces of the volunteers was determined (with the $ABTS^{\cdot}$ decolorisation assay), the results indicate that coffee increase the antioxidant activity.

Elevated urinary levels of H_2O_2 were detected after coffee consumption in two intervention trials, one trial was conducted with instant coffee the other study was conducted with brewed or canned coffee [240,241]. Also the results of a questionnaire-based study (n=33) indicate that the H_2O_2 levels in the urine of coffee drinkers are increased [242].

In the clinical trial described in paper II (see page 91) GSH levels were monitored in erythrocytes of the volunteers and no alterations due to coffee consumption were detected, also the TAC in plasma and the intracellular ROS levels (lymphocytes) were not altered. Due to our knowledge the study described in paper II is the first were protein nitrosation has been determined in a human intervention trial with coffee and we observed a significant decrease due to coffee consumption of this parameter.

Table 4: Human intervention studies

Design/coffee type/dose	Measurement	Results and comments	Ref.
Studies indicating pro-oxidative effects of coffee consumption			
n=71 questionnaire based	SCGE with restriction enzyme (FPG)	↑ DNA damage (low vs. high coffee consumers)	[228]
n=10 1 cup of brewed (20 g/140 ml, without sugar and milk) or canned coffee (187 ml, with sugar and milk), once	H_2O_2 concentration in urine by FOX assay for 200 min after coffee consumption	↑ 3-10-fold of H_2O_2 levels after consumption; peak between 50 and 100 min. possible H_2O_2 generating component is 1,2,4-benzenetriol	[240]
n=4 1 cup of instant coffee (2.5 g/200 ml, with sugar and creamer), once	H_2O_2 concentration in urine by FOX assay for 200 min. after coffee consumption	↑ of H_2O_2 levels for up to 2 h. after coffee consumption; ≥3-fold ↑ after ~30 min.;no effect of sugar and creamer	[241]
n=33 questionnaire based	urine was tested for clastogenicity in CHO-cells and for H_2O_2-formation (spectrophotometrically, λ=575 nm)	↑ of clastogenic effects in urine of coffee drinkers; ↑ H_2O_2-formation (at high pH)	[242]
Studies indicating no effect of coffee consumption			
n=43 0, 450 and 900 ml of paper filtered coffee, 3 wks n=35 0, 150 or 300 ml, once	Lipid peroxidation parameters (such as conjugated dienes, plasma hydroxy fatty acids, F_2-isoprostanes) Plasma total homocysteine Antioxidant enzymes GPx; SOD, PON	↔ neither short- nor long-term effects on lipid peroxidation or on the activity of measured AO enzymes	[234]
n=32 12g instant coffee/d, 4 ds or 12 g/d, once	urinary mutagenicity test in TA98 and TA100 strains (± ß-glucuronidase)	↔ urinary mutagenicity	[230]
Studies indicating antioxidative effects of coffee consumption			
n=29 800 ml/d (Instantcoffee), 5 ds, cross-over design	SCGE under standard conditions, with restriction enzymes (ENDO III, FPG) and H_2O_2 sensitivity; oxLDL, MDA, 2F-isoprostane, SOD, GPx, GST, intracellular ROS, TAC, GSH, 3-NT RT-PCR	↔ of DNA migration under standard conditions, oxidised purines and pyrimidines, H_2O_2-sensitivity ↓ oxidised purines in female volunteers ↔ oxLDL, MDA, SOD, GPx, GST, intracellular ROS, TAC, GSH, RT-PCR ↓ F2-isoprostane, 3-NT	[225][1]
n=8 600 ml/d (400 ml paper filtered and 200 ml French press coffee), 5 ds	SCGE under standard conditions, with restriction enzymes (ENDOIII, FPG) and H_2O_2-sensitivity; GPx and SOD activity in plasma	↔ of DNA migration under standard conditions; ↓ DNA migration due to oxidised purines and pyrimidines; ↓ H_2O_2-sensitivity ↑ SOD- ↔ GPx-activity	[233][1]
n=10 200 ml of filtered coffee (60 g/1000 ml), once	LDL oxidation before, 30 and 60 min. after coffee intake; *ex vivo* determination of incorporation of HCA into LDL	↑ of lag time 30 and 60 min after coffee consumption by 11 and 20%; ↑ of CA, FA and p-CA after coffee consumption in LDL	[231]
First trial: n=10 1l boiled coffee/d, 5 ds Second trial: n=7 1l coffee/d, 3 ds (paper-filtered vs. unfiltered coffee) Third trial: n=7 1l boiled coffee/d, 5 ds	GST activity in saliva and plasma; GST isozymes in plasma (ELISA) SCGE assay, comet formation induced by BPDE	first trial: ↑ GST in saliva and blood, sign. only in plasma samples; ↑ of GSTπ-levels; ↔GSTα, second trial: ↑ GSTπ with filtered and unfiltered coffee third trial: ↓ DNA migration due to BPDE	[227][1]

Table 4: continued

Design/coffee type/dose	Measurement	Results and comments	Ref.
n=11 24 g instant coffee/d (8 g/150 ml), 7 ds	LDL oxidation TBARS in blood and urine levels of caffeine in serum and CGA in urine by HPLC	↑ of LDL-oxidation lag time (8%); ↓ of TBARS	[232]
n=23; 5 cups/d (preparation n.s.), 7 ds	GSH-plasma levels by HPLC H_2O_2- plasma levels (by D-ROMS test), homocysteine-plasma levels	↑ GSH levels ↔ no significant increase of plasma-homocysteine levels ↔ no effect on the oxidative status	[237]
n=10 200 ml drip filtered coffee once	plasma AO capacity 0,1 and 2h after coffee intake with TRAP and crocin test	↑ AO capacity with both tests by ≥ 4%; stronger effects with coffee than with tea	[238]
n=64, cross-over design 1l (39g coffee/l) French press coffee/d, 2 wks;	proliferation of colorectal cells; GST activity (spectro-photometrically), GST- α, μ, π by Western blot; GSH levels (by HPLC) in mucosa cells	↔ cell proliferation and GST-activities; ↑ of GSH concentrations by 8%	[236]
n=14 participants recorded their food intake three times for a period of 2 d	48 h dietary weighing protocol, subsequent 24 h collection of faeces; TAA of faeces by $ABTS^{\bullet+}$-decolorization assay	↑ TAA (r=0.758/p=0.002) and increase of faecal weight with coffee consumption;	[239]
n=102 questionnaire based	8-OHdG measured in leukocytes by HPLC	inverse relation with coffee consumption (not sign.)	[229]
n=25, coffee drinkers (>150 ml/d) vs. abstainers n=1, intervention with coffee 1-2 l/d, 22 ds	GST isozymes (α, μ, π) 3 aldehyde dehydrogenase DT-diaphorase in saliva and plasma	↑ plasma and salivary content of all 3 enzymes ↑ salivary enzyme levels ↓ to basal when coffee was removed from the diet (tested in one subject)	[235]

Abbreviations: $ABTS^{\bullet+}$; 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, AO; antioxidant, CA; caffeic acid, CGA; chlorogenic acid, CHO; chinese hamster ovary cells, ds; days, DT-diaphorase; NAD(P)H:(quinone-acceptor) oxidoreductase, ENDOIII; endonuclease III, FA; ferulic acid, FOX; ferrous oxidation xylenol orange method, FPG; formamidopyrimidine DNA glycosylase, GPx; glutathione peroxidase, GSH; glutathione, GST; glutathione S transferase, HCA; hydroxycinnamic acids, LDL; low density lipoproteins, MDA; malondialdehyde, n.s.; not specified, oxLDL; oxidised low density lipoprotein, p-CA; p-cumaric acid, PON; paraoxonase, SCGE; single cell gel electrophoresis, SOD; superoxide dismutase, TAA; total antioxidant activity, TAC; total antioxidant capacity, TBARS; thiobarbituric acid reactive substances, TRAP; total radical trapping AO parameter, 3-NT; (±)-anti-B[a]P-7,8-dihydrodiol-9,10-epoxide, BPDE; 3-nitrotyrosine, 8-OHdG; 8-hydroxydeoxyguanosine, ↑; increase, ↔; no change, ↓; decrease, [1] results presented in the present thesis

3.5 Conclusion and outlook

Taken together, the currently available data indicate, that coffee intake may protect humans against oxidative DNA damage and its consequences. There is some evidence that high coffee levels can generate hydrogen peroxide under *in vitro* conditions (for details see [243]), but it seems that these findings are not relevant for the situation in humans as the effects were seen under unphysiological conditions [244]. It is also notable that the detection of ROS in the urine (see Table 4) is not directly indicative for DNA damage in inner organs; and that in many of the human trials direct or indicrect evidence for antioxidant properties of coffee were observed. As mentioned above, the responses of the individual endpoints differed strongly, but this may be due to the fact that the mechanism which underly the antioxidant

activities are quite complex (i.e. direct scavenging, induction of tanscription factors, indirect mechanisms caused by reduced glucose uptake in the gastrointestinal tract).

It has been stressed in a meta-analyses by Dotan et al. [50], that antioxidant properties can be not described by the measurement of individual parameters and that strong differences exist in the sensitivity of different endpoints. Furthermore, comparisons of the results of coffee studies are difficult, as the volunteers consumed different types of coffees and also the time schedule (duration of coffee intake) varied, which is an important for the outcome of the studies.

The concentrations of putative active compounds in coffee can be modified via selection of the cultivars used, but also by the roasting and brewing procedures and specific production strategies (e.g. addition of clorogenic acid or decaffeination). This will enable to design coffees with increased beneficial health effects in the future.

4 References

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PUBLICATIONS

The present thesis is based on orginal articles and a review. Paper I describes a pilot human intervention trial with a mix of paper and metalfiltered coffee. The main part of the thesis is a clinical human intervention trial with instant coffee (Paper II). Paper III describes a human intervention trail in which GST measurements were performed and also a modified version of the SCGE assay. The review article describes the use of single cell gel electrophoresis (SCGE) assays in human intervention trials. This technique is used to determine the DNA stability in peripheral lymphocytes of the volunteers as one of the primary outcomes of the studies described in paper I, II and III.

Paper I Bichler J, Cavin C, Simic T, Chakraborty A, Ferk F, **Hoelzl C**, Schulte-Hermann R, Kundi M, Haidinger G, Angelis K, Knasmüller S. Coffee consumption protects human lymphocytes against oxidative and 3-amino-1-methyl-5H-pyrido[4,3-b]indole acetate (Trp-P-2) induced DNA-damage: results of an experimental study with human volunteers. *Food Chem Toxicol. 2007; 45(8):1428-36*

Author contribution: Christine Hölzl was involved in the conception of the human intervention trail and helped with the performance of the SCGE assays.

Paper II **Hoelzl C**, Cavin C, Kundi M, Wagner KH, Neubauer O, Simič T, Elbling L, Huber W, Kager N, Ferk F, Ehrlich V, Nersesyan A, Knasmüller S. Instant coffee reduces DNA damage and affects biochemical antioxidant parameters: results of a clinical intervention trial. *American Journal of Clinical Nutrition 2009, prior to submission*

Paper III Steinkellner H, **Hoelzl C**[1], Uhl M., Cavin C, Haidinger G, Gsur A, Schmid R, Kundi M, Bichler J, Knasmüller S. Coffee consumption induces GSTP in plasma and protects lymphocytes against(±)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide induced DNA-damage: Results of controlled human intervention trials. *Mutation Research 2005; 591, 264–275,*

[1]contributed equally

Review **Hoelzl C**, Knasmüller S, Mišík M, Collins A, Dušinská M, Nersesyan A. Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: Recent results and trends. *Mutation Research/Reviews in Mutation Research 2009; 681(1):68-7*

PAPER I

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Coffee consumption protects human lymphocytes against oxidative and 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole acetate (Trp-P-2) induced DNA-damage: Results of an experimental study with human volunteers

J. Bichler [a], C. Cavin [b], T. Simic [c], A. Chakraborty [a], F. Ferk [a], C. Hoelzl [a], R. Schulte-Hermann [a], M. Kundi [d], G. Haidinger [a], K. Angelis [e], S. Knasmüller [a,*]

[a] *Institute of Cancer Research, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria*
[b] *Nestlé Research Centre, Food Safety Group, P.O. Box 44, CH-1000 Lausanne 26, Switzerland*
[c] *Institute of Biochemistry, School of Medicine, Pasterova 2, 11000 Belgrade, Serbia and Montenegro*
[d] *Institute of Environmental Health, University of Vienna, Kinderspitalgasse 15, A-1090 Vienna, Austria*
[e] *Laboratory of DNA Repair, Institute of Experimental Botany, Rozvojavá 135, Prague, Czech Republic*

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Abstract

Aim of the study was to investigate the impact of coffee on DNA-stability in humans. DNA-damage was monitored in lymphocytes of eight individuals with single cell gel electrophoresis assays before and after consumption of 600 ml coffee (400 ml paper filtered and 200 ml metal filtered/d) for five days. Under standard conditions, no alteration of DNA-migration was seen, but a strong reduction of DNA-migration attributable to endogenous formation of oxidised purines and pyrimidines was detected with restriction enzymes; furthermore DNA-damage caused by reactive oxygen radicals (H_2O_2 treatment) and by the heterocyclic aromatic amine 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole-acetate was significantly reduced after coffee consumption by 17% and 35%, respectively. Also in *in vitro* experiments, inhibition of H_2O_2 induced DNA-damage was observed with coffee at low concentrations ($\leqslant$25 μl/ml) whereas the diterpenoids cafestol and kahweol caused only marginal effects indicating that the effects of coffee are due to scavenging effects of other constituents. Enzyme measurements showed that additionally induction of antioxidant enzymes may play a role: while the activity of glutathione peroxidase was only marginally increased after coffee consumption, a significant (38%) increase of superoxide dismutase activity was detected. Comparisons with results of earlier studies suggest that coffee consumption may prevent oxidative DNA-damage to a higher extent as diets enriched in fruits and vegetables.

Keywords: Coffee; Comet assay; DNA-damage; Human intervention study; Trp-P-2

1. Introduction

Coffee is one of the most widely consumed beverages worldwide. The production (about 70% Arabica and 30% Robusta) shows annual variations due to recurring calamities but has generally increased by about 15–20% in the last 20 years from 5.2 million tons per year in 1984 to 5.9–6.4 million nowadays (Clarke and Vitzthum, 2001).

Abbreviations: C + K, cafestol and kahweol; DMSO, dimethyl sulfoxide; FPG, formamidopyrimidine glycosylase; GPx, glutathione peroxidase; HA, heterocyclic aromatic amines; HCC, hepatocellular carcinoma; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis assay; SOD, superoxide dismutase; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole acetate.

* Corresponding author. Tel.: +43 1 4277 65142; fax: +43 1 4277 65159.
E-mail address: siegfried.knasmueller@meduniwien.ac.at (S. Knasmüller).

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Coffee contains a variety of bioactive compounds including caffeine and other purine derivatives, polyphenolics including chlorogenic acid derivatives and its degradation product caffeic acid, Maillard reaction products and specific diterpenes such as cafestol and kahweol (C + K) (IARC, 1991). The consequences of coffee consumption on human health have been studied intensely during the last decades (for reviews see for example Stavric (1992)) and a number of epidemiological studies indicate that coffee drinking is inversely related to the incidence of liver and colon cancer in humans (Gallus et al., 2002a; Gelatti et al., 2005; Giovannucci, 1998; Inoue et al., 2005; Kurozawa et al., 2004; Shimazu et al., 2005).

Aim of the present investigation was to elucidate if these protective effects are causally related to prevention of DNA-damage. It is well documented that reactive oxygen species (ROS) play a key role in the aetiology of liver cirrhosis and hepatocellular carcinoma (Gebhardt, 2002; Ichiba et al., 2003; Szuster-Ciesielska et al., 2002) and a number of *in vitro* and animal studies indicate that coffee and many of its constituents are protective towards ROS (Daglia et al., 2000,2004; Devasagayam et al., 1996; Iwai et al., 2004; Stadler et al., 1995,1996b), while results of human studies based on food questionnaires are scarce and controversial (Giovannelli et al., 2002; Pellegrini et al., 2003; Svilaas et al., 2004). ROS may also play a role in the aetiology of other forms of cancer as well as in degenerative diseases and ageing (for reviews see Harman, 1981; Hoelzl et al., 2005; Squier, 2001).

Another potential cancer risk factor are heterocyclic aromatic amines (HAs) which are formed during cooking of meats. It has been shown in animal studies that the coffee specific diterpenoids cafestol and kahweol (C + K) reduce the formation of HA DNA-adducts in colonic tissue (Huber et al., 1997), also *in vitro* experiments with human derived cells provided evidence for protective effects (Majer et al., 2005), which were attributed to induction of detoxifying enzymes (Cavin et al., 1998; Huber et al., 1997; Majer et al., 2005). Over the last three decades, intense efforts have been made to identify dietary constituents which protect against HAs but evidence for effects in humans are restricted to indirect approaches, i.e. chemical analyses of urinary metabolites and urinary mutagenicity tests (for reviews see (Dashwood, 2002; Schwab et al., 2000). Recently, we developed a protocol for single cell gel electrophoresis (SCGE) experiments with lymphocytes which can be used to study alterations of HA induced DNA-damage induced by dietary factors in humans (Hölzl, 2004).

To investigate the potential DNA protective effects of coffee in humans, we conducted an experiment in which we monitored the effects of coffee consumption on endogenous formation of single strand breaks, oxidised purines and pyrimidines and ROS sensitivity in single cell gel electrophoresis (SCGE) assays with peripheral lymphocytes. In addition, experiments were conducted to find out if the impact of coffee on oxidative DNA-damage is due to direct scavenging of ROS or to induction of the antioxidant enzymes superoxide-dismutase (SOD) and glutathione peroxidase (GPx).

To elucidate if coffee drinking affects DNA-damage caused by HAs we also monitored DNA-migration caused by 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole acetate (Trp-P-2) in peripheral lymphocytes of the participants of the intervention trial before and at the end of the study. We showed earlier that lymphocytes are able to convert HAs to DNA reactive metabolites and used the tryptophan pyrolyzate in the present study as it is a more potent inducer of DNA-damage as other amines (Hölzl, 2004). This compound was the first HA detected in fried meats (Sugimura et al., 1977) and is a potent carcinogen in rodents (IARC, 1993).

2. Materials and methods

2.1. Chemicals

Trp-P-2 was purchased from the Nard Institute (Nishinagasu Amagasaki, Japan); hydrogen peroxide (H_2O_2) and dimethyl sulfoxide (DMSO) were from Merck (Darmstadt, Germany). RPMI was from Sigma–Aldrich (St. Louis, USA). Cafestol and kahweol (a mix of 52.5:47.5 C + K, purity >98%) were a gift from Nestlé (Lausanne, Switzerland). The RANSOD kit used to monitor superoxide dismutase (SOD) activity was purchased from Randox Laboratories Ltd. (Ardmore, UK); agarose from Invitrogen Life Technologies Ltd. (Paisley, Scotland). Endonuclease III (ENDO III) and formamidopyrimidine glycosylase (FPG) were provided by the laboratory of DNA Repair (Prague, Czech Republic).

2.2. In vitro experiments with peripheral lymphocytes

Lymphocytes were isolated from blood of a healthy donor by centrifugation (Collins and Dusinska, 2002), washed twice with PBS (pH 7.4) and transferred into RPMI medium. The cells were treated in culture flasks (1.5 ml, Eppendorf, Hamburg, Germany) either with different amounts of coffee, or with the coffee diterpenoids C + K (dissolved in DMSO) for 30 min. The coffee used in *in vitro* experiments was prepared with the French Press Method (see below). In combination experiments with hydrogen peroxide, 50 μM H_2O_2 were added to the cells on ice for 5 min and coffee concentrations were used which did not cause acute toxic and genotoxic effects. To terminate the exposure, the cells were centrifuged and washed twice with PBS (pH 7.4).

2.3. Design of the human study

In total, eight healthy, non smoking volunteers (age 20–50 years) participated in the study. From all individuals, written consent was obtained and the study was approved by the Austrian Ethical Commission. One week before and during the intervention, the participants consumed a restricted diet (i.e. they refrained from consumption of more than 200 g of the following foods: citrus fruits, fruit juices, cabbage, onions, whole meal products and alcoholic beverages) and did not consume additional coffee, tea, cola and energy drinks. During the intervention, each of them drank in total 600 ml coffee (200 ml metal filtered and 400 ml paper filtered coffee, Brand: Brasil sanft) per day over a period of five days. The metal filtered coffee was prepared with the French Press Method (Original French Press, Bodum, Triengen, Switzerland). Per liter, 50 g of ground coffee were used for both preparations. At the beginning and on the last day of the study, blood (10 ml) was aspired by venipuncture and collected in heparinised tubes (10 ml, BD Vacutainer Systems, Plymouth,

UK). Lymphocytes were isolated using Histopaque-1077 (Sigma–Aldrich, St. Louis, USA) according to the instructions of the manufacturer.

2.4. Single cell gel electrophoresis assays

The SCGE experiments were conducted according to the guidelines of Tice et al. (2000). In the *in vitro* experiments, the survival of the cells was determined with trypan blue (Lindl and Bauer, 1994), only cultures with a viability $\geqslant$80% were analysed for comet formation. To compare DNA-migration before and after coffee intervention, the cells were either analysed without pretreatment under standard conditions (25 V, 300 mA, and 20 min electrophoresis time), additionally nuclei were treated either with FPG or ENDO III according to the protocol of Collins et al. (1997,1993). To monitor alterations of the chemical sensitivity of the cells, lymphocyte cultures were exposed either for 5 min on ice to H_2O_2 (50 μM) or to Trp-P-2 (200 μM) for 30 min in PBS. The exposure concentrations of the chemicals were chosen on the basis of earlier experiments (Hölzl, 2004). After the treatment, the cells were washed and transferred to agarose coated slides for comet analysis.

From each participant, three slides were prepared for each experimental point and from each slide 50 cells were evaluated. Tail lengths and tail moments were measured with a computer aided image analysis system (Helma and Uhl, 2000).

2.5. Enzyme measurements

GPx activity was measured in cytosols of peripheral lymphocytes according to the protocol of Gunzler et al. (1974) which is based on the spectrophotometrical determination of reduction of NADPH ($\lambda =$ 340 nm). SOD activity was determined in cytosols with the RANSOD test kit (Randox Laboratories Ltd., Ardmore, UK). The inactivation of superoxide by SOD was determined by monitoring the formation of a red formazan dye ($\lambda = 505$ nm). Each measurement was carried out in triplicate.

2.6. Statistics

Differences in the median tail lengths were tested by analysis of variance (ANOVA). For *in vitro* experiments, in case of a significant ($p \leqslant 0.05$) main effect of experimental conditions, Dunnett's tests were performed to compare the different test conditions with the control condition. The results of the experiments with human volunteers were analysed with two-factor ANOVAs with data before/after coffee consumption as the experimental factor and subjects as a random factor. In all tests, a two-sided p-value $\leqslant 0.05$ was considered significant.

3. Results

3.1. In vitro SCGE experiments

The results of comet assays in which the effects of coffee on induction of DNA-migration and cell survival were measured in human lymphocytes are depicted in Fig. 1a and b. It can be seen that coffee caused a dose dependent decline of the viability of cells in the dose range tested (25–600 μl coffee/ml medium). In the same experiment also induction of DNA-migration was observed which was statistically significant at exposure concentrations $\geqslant$50 μl coffee/ml medium. On the contrary, no DNA-damaging effect was detectable with the coffee diterpenoids in the SCGE assay and the viability of the cells was not significantly affected (Fig. 2a and b).

The results of combination experiments with coffee and H_2O_2 are shown in Fig. 3a and b. In this experiment subtoxic concentrations of coffee were used which caused neither acute toxic nor genotoxic effects. Over the entire dose range, pronounced protective effects were observed, which were significant at dose levels which caused slight cytotoxic effects whereas in parallel experiments with C + K only moderate (27–38%) inhibition of H_2O_2 induced DNA-migration was observed (Fig. 4a and b).

3.2. Effects of coffee consumption on DNA-migration in humans

The results of the SCGE measurements in peripheral lymphocytes before and after coffee consumption are shown in Fig. 5a–e. DNA-migration was significantly increased after treatment of the nuclei with the restriction enzymes and also after exposure of the cells to H_2O_2 and Trp-P-2.

When the comet assays were carried out under standard conditions (20 min electrophoresis time, 25 V, 300 mA), no significant impact of coffee consumption on DNA-damage

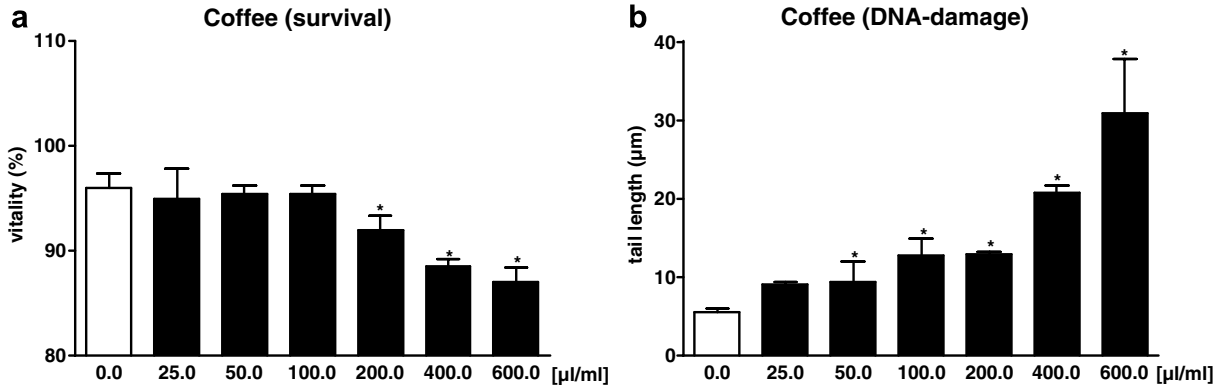

Fig. 1. Effect of coffee on the viability (a) and DNA-migration (b) of peripheral human lymphocytes. Values on the x-axis indicate the amount of coffee. The lymphocytes were exposed to the coffee for 30 min. Subsequently, the cell viability was determined with trypan blue and comet formation was monitored. Per experimental point, three cultures were prepared in parallel. *Indicates statistical significance (p-value $\leqslant 0.05$, analysis of variance ANOVA). Bars indicate means ± SD results obtained with three slides (per slide 50 cells were evaluated).

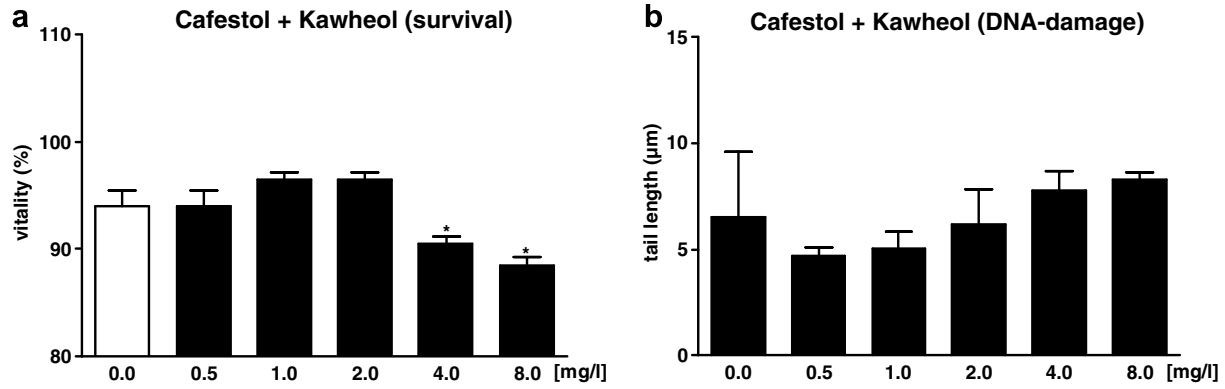

Fig. 2. Effect of the coffee specific diterpenoids cafestol and kahweol on the viability (a) and on DNA-migration (b) of human lymphocytes. The diterpenoids were dissolved in DMSO and different concentrations were added to the cell suspensions for 30 min. Controls were exposed to the solvent only. Subsequently, cell viability and comet formation were determined. Per experimental point, three cultures were prepared in parallel. *Indicates statistical significance (p-value $\leqslant 0.05$, analysis of variance ANOVA). Bars indicate means ± SD results obtained with three slides (per slide 50 cells were evaluated).

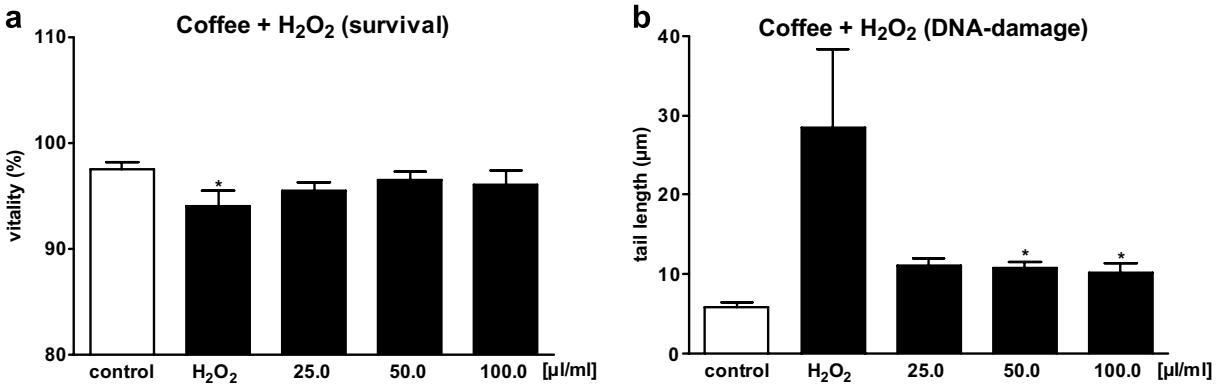

Fig. 3. Effect of coffee on the acute toxicity (a) and on DNA-damage (b) caused by H_2O_2 in peripheral human lymphocytes. The experiment was carried out as described in the legend of Fig. 1, but after exposure of the cells to subtoxic concentrations coffee, H_2O_2 (50 µM) was added to the cells for 5 min on ice, controls were exposed to the solvent only. Per experimental point three, cultures were prepared in parallel. *Indicates statistical significance (p-value $\leqslant 0.05$, analysis of variance ANOVA). Bars indicate means ± SD results obtained with three slides (per slide 50 cells were evaluated).

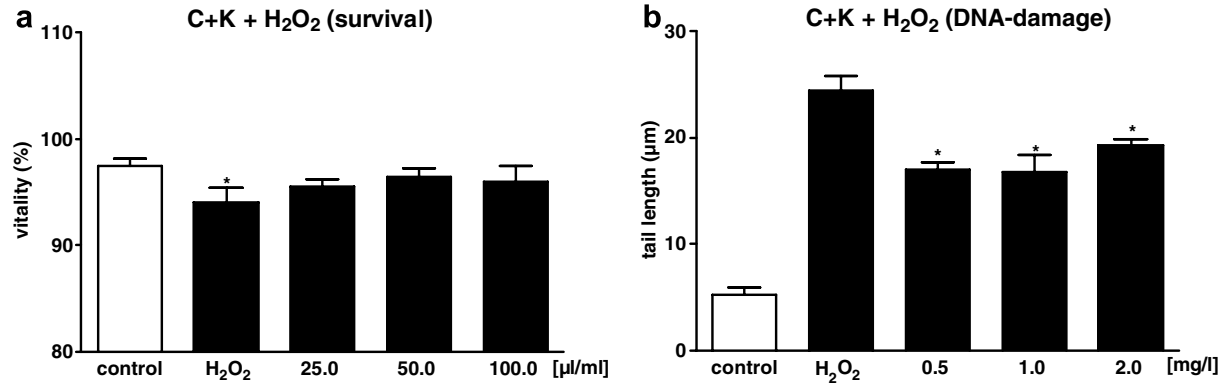

Fig. 4. Effect of cafestol and kahweol on the acute toxicity (a) and on DNA-damage (b) caused by H_2O_2 in peripheral human lymphocytes. The experiment was carried out as described in the legend of Fig. 2, but after treatment with the diterpenoids, H_2O_2 (50 µM) was added to the cells for 5 min. Controls were exposed to the solvent only. Per experimental point three cultures were prepared in parallel. *Indicates statistical significance (p-value $\leqslant 0.05$, analysis of variance ANOVA). Bars indicate means ± SD results obtained with three slides (per slide 50 cells were evaluated).

was observed. However, when DNA-migration was determined after addition of the restriction enzymes (FPG, ENDO III), significant alterations were detected (Fig. 5b and c). The bars of the figures depict both, DNA-migration due to formation of endogenous single and double strand breaks and additionally also migration attributable to

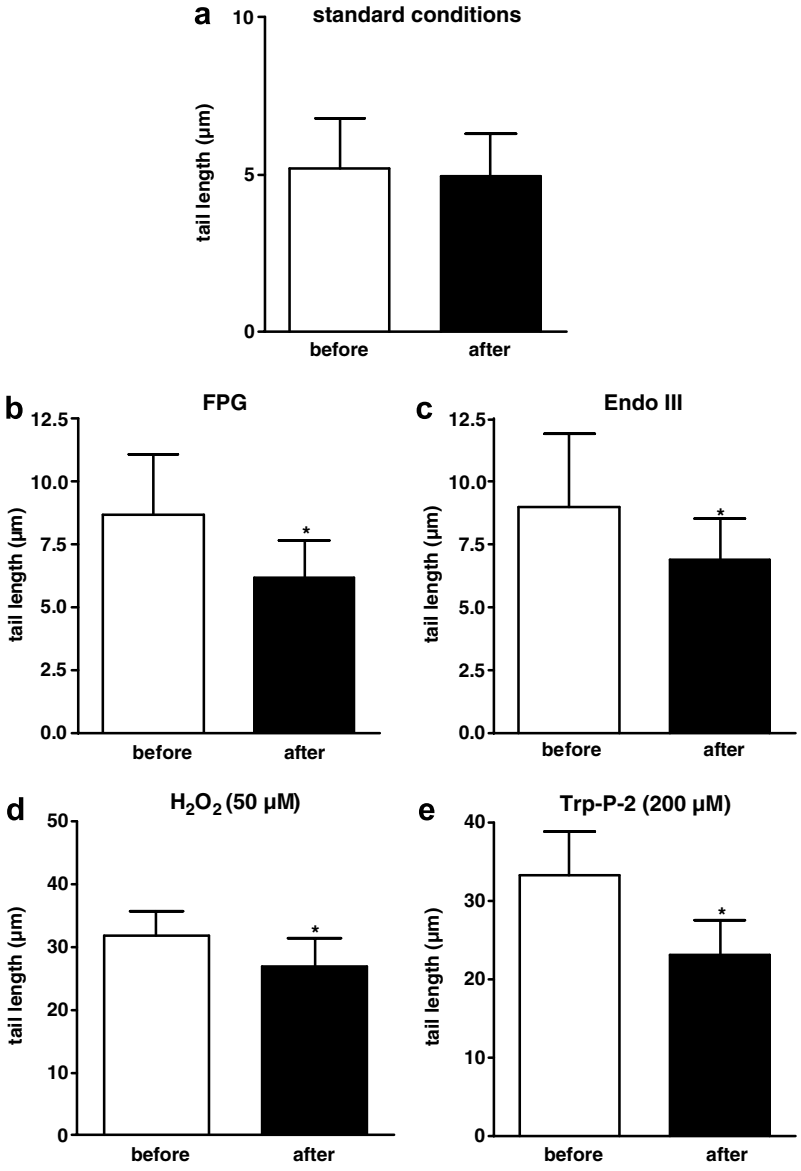

Fig. 5. Effect of coffee consumption on endogenous and chemically induced DNA-damage. Eight individuals participated in the intervention trial. Each of them consumed 600 ml coffee consecutively over a period of five days. Before and after the intervention lymphocytes were isolated from blood and analysed for DNA-migration in SCGE experiments. (a) Shows the results obtained with standard electrophoresis conditions. In experiments with H_2O_2 (d) and Trp-P-2 (e), three cultures per participant were prepared in PBS and the cells were treated on ice with H_2O_2 (50 μM) for 5 min or 30 min with Trp-P-2 (200 μM). To monitor endogenous formation of oxidised bases, FPG (b) and ENDO III (c) were added to the slides with the nuclei. From each participant and time point three cultures were prepared and 50 cells per slide were evaluated for comet formation from each culture. Bars indicate means ± SD of results obtained with the eight participants. Black bars: DNA-migration after coffee consumption, white bars: before coffee consumption. *Indicates statistical significance (p-value $\leqslant 0.05$, analysis of variance ANOVA).

formation of oxidised purines and pyrimidines. The extent of migration attributable solely to oxidised purines before the intervention was on average 3.5 μm and after intervention 1.2 μm, the corresponding values for DNA-migration due to oxidised pyrimidines are 3.8 μm and 1.9 μm (data were calculated on the basis of the differences of migration seen in absence and presence of the restriction enzymes). Also when DNA-migration was induced by treatment of the cells with H_2O_2 (Fig. 5d), a significant protective effect (17% reduction) was observed. The results obtained with Trp-P-2 are shown in Fig. 5e; also with the tryptophan pyrolyzate a significant decrease of the tail lengths (by 35%) was observed at the end of the intervention (Fig. 5e).

In all experiments the tail moments were monitored in addition to the tail lengths (data not show) and the evaluation of this parameter led to the same conclusions.

3.3. Effects of coffee consumption on the activities of antioxidant enzymes

The results of the enzyme measurements with cytosolic fractions of lymphocytes are shown in Fig. 6a and b. It

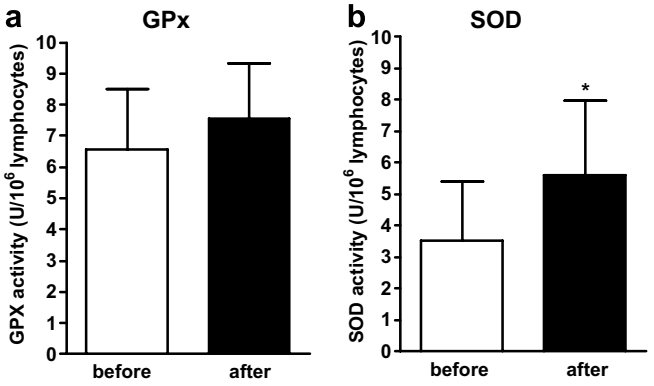

Fig. 6. Effect of coffee consumption on the activity of antioxidant enzymes. The measurements were carried out with cytosolic fractions of lymphocytes of the participants of the intervention trial. The intervention was carried out as described in the legend of Fig. 5. GPx (a) was measured according to the method of Gunzler et al. (1974), SOD (b) was determined with the RANSOD test kit. Each measurement was made in triplicate. Bars indicate means ± SD of results obtained with eight participants. Black bars: enzyme activity after coffee intervention, white bars: enzyme activity before coffee consumption. *Indicates statistical significance (p-value $\leqslant 0.05$, ANOVA).

can be seen that the activity of GPx was not altered significantly after coffee consumption (Fig. 6a), whereas the activity of SOD (Fig. 6b) was increased by 38%.

4. Discussion

Aim of the present study was the investigation of potential DNA protective effects of coffee consumption in humans, in addition also *in vitro* experiments with lymphocytes were carried out.

The findings of the *in vitro* experiments show that exposure of the cells to high concentrations of coffee, but not to coffee specific diterpenoids (C + K), causes induction of DNA-migration (Fig. 1). This observation was not unexpected; also in several other *in vitro* experiments with bacterial and mammalian indicator cells positive results were obtained (IARC, 1991; Stadler et al., 1994). A number of earlier investigations indicate that generation of H_2O_2 accounts for the genotoxic effects of coffee (Fujita et al., 1985; Nagao et al., 1986; Wakabayashi et al., 1989) and it was reported more recently that chlorogenic and caffeic acids and their pyrolysis products which are contained in coffee cause formation of ROS (Iwahashi et al., 1990; Tsuji et al., 1991; Yamanaka et al., 1997), also caffeine was shown to possess prooxidant properties under specific conditions (Azam et al., 2003). As described above (Fig. 2), no genotoxic effects were observed with the coffee specific diterpenoids C + K.

In contrast to the results obtained with coffee in the first experimental series (Fig. 1), pronounced protective effects were observed in experiments with low coffee concentrations (Fig. 3) which caused no acute toxic and genotoxic effects in combination with H_2O_2. This observation is in agreement with the results of a number of earlier *in vitro* experiments and with *in vivo* studies with rats (Daglia et al., 2000,2004; Pellegrini et al., 2003; Somoza et al., 2003; Stadler et al., 1995,1996a,b). Also specific constituents of coffee such as caffeine and phenolic acids (i.e. chlorogenic-, ferulic- and caffeic acid) and Maillard reaction products are known to act as antioxidants (Azam et al., 2003; Devasagayam et al., 1996; Iwai et al., 2004; Khan et al., 2000; Kono et al., 1997; Nardini et al., 1995,1997,1998; Stadler et al., 1995,1996a,b). The ROS protective effects of the coffee diterpenoids C + K have not been investigated earlier and the results of our experiments indicate that they possess only weak antioxidant activity.

In the human study, no indication for induction of genotoxic effects by coffee consumption was found. Neither with the standard protocol (which enables the detection of single and double strand breaks), nor with the restriction enzymes increased DNA-migration was seen (Figs. 5 and 6). The lack of DNA-damaging properties of coffee is in line with data from animal experiments in which consistently negative results were obtained (Aeschbacher et al., 1984; Shimizu and Yano, 1987). In this context it is notable that Rinkus and Taylor (1990) emphasised that H_2O_2, which causes the genotoxic effects of coffee under *in vitro* conditions (Stadler et al., 1994) is probably not formed after ingestion and that it may be a confounding factor in *in vitro* tests. Only a few other studies are available in which the effects of coffee consumption on genomic stability in humans were investigated. Smith et al. (1990) reported a 2-fold increase of micronuclei levels in erythrocytes of spleenectomized humans who consumed five or more cups of coffee and/or tea per day but the results of this study do not provide information which of the two beverages caused the effect. Also the reports of Chen et al. (1989) and Reidy et al. (1988) in which increased frequencies of chromosomal aberrations and sister chromatid exchanges were found in peripheral lymphocytes of coffee drinkers do not allow to draw firm conclusions. The scorer effects exceeded in both studies the effects seen with coffee and the authors stress that the results should be interpreted with caution due to uncertainties in the quantification of coffee consumption.

The results of the present experiments with H_2O_2 and with restriction enzymes show clearly that coffee protects human lymphocytes against oxidative DNA-damage (Fig. 6). The formation of oxidised purines was reduced by 64%, and damage attributable to oxidised pyrimidines was decreased by 48%; furthermore, the extent of DNA-migration caused by H_2O_2 declined by 17% (Fig. 5b–d). Only data from few other investigations on antioxidative/oxidative effects of coffee consumption in humans are available. Van Zeeland et al. (1999) found decreased formation of 8-OHdG in DNA isolated from lymphocytes of coffee drinkers. In this context it is notable that a good correlation was observed between 8-OHdG formation and FPG induced DNA-migration monitored with the SCGE assay in human lymphocytes (ESCODD, 2000). Further support

for the assumption that coffee protects against DNA-damage caused by ROS comes from a human study (Natella et al., 2002) in which a pronounced increase of the antioxidant capacity of plasma was found after consumption of 200 ml coffee; likewise also in experiments with rats an increase of the antioxidant status (i.e. of TROLOX-equivalents) was seen after administration of coffee extract (Somoza et al., 2003). In contrast to these findings, a positive association between coffee consumption and FPG DNA-migration was observed in an Italian study, which was based on intake assessment with questionnaires (Giovannelli et al., 2002).

As mentioned above, coffee contains a variety of constituents, which inactivate oxygen radicals. It is likely that the effects seen in the *in vitro* experiments (Fig. 3) are due to direct scavenging whereas under *in vivo* conditions additionally enzymatic effects may be involved. As described in Section 3, a significant (38%) increase of SOD activity was found after coffee consumption (Fig. 6b). According to our knowledge, our coffee study is the first investigation in which induction of SOD by a dietary factor in humans was found. Another indirect mechanism, which may account for protection against oxidative DNA-damage is the increase of plasma glutathione levels caused by coffee drinking, which was found in an earlier study by Esposito et al. (2003).

At present, results of 51 human trials with diets and individual food components are available in which the SCGE-technique was used (for reviews see Moller and Loft, 2002,2004). In approximately 50% of the studies protective effects were detected. Comparisons of the results of the present study with data from earlier trials show that coffee consumption causes effects, which are similar to those seen after intake of antioxidant vitamins. For example, a 20% reduction of H_2O_2 induced DNA migration was detected after continuous supplementation with a combination of 100 mg vitamin C, 280 mg vitamin A and 25 mg β-carotin (Duthie et al., 1996); DNA-migration due to formation of oxidised pyrimidines (FPG) was reduced by 75% in the same experiment. It is notable, that no protection was seen in a recent intervention trial after consumption of large amounts of mixed fruit and vegetables (600 g/P/d for 24 days) (Moller et al., 2003). Also in another study in which 500 g of fruit and vegetable juice were given daily over three weeks, no effects were observed (van den Berg et al., 2001). These comparisons suggest that coffee drinking may contribute to a higher extent to prevention of oxidative DNA-damage in humans than consumption of fruits and vegetables. This assumption is also supported by the findings of Svilaas et al., (2004) who postulated on the basis of an assessment of the antioxidant properties of different foods and beverages that coffee is a greater contributor to the total antioxidant intake in man than plant derived foods.

The findings of the present study are of particular interest in the light of recent observations which indicate an inverse relationship between coffee consumption and the incidence of liver cirrhosis (Corrao et al., 2001; Gallus et al., 2002b) and hepatocellular carcinoma (Gallus et al., 2002a; Kurozawa et al., 2004; Shimazu et al., 2005). It is known that cirrhosis and other inflammatory liver diseases are associated with increased incidences of HCC, and it is assumed that ROS play a causal role (Gebhardt, 2002; Ichiba et al., 2003; Szuster-Ciesielska et al., 2002). It can be tentatively assumed that the protection against oxidative DNA-damage, which we detected in the present study may account for the prevention of these diseases. Attempts to elucidate if coffee protects cirrhotic liver cells against ROS mediated DNA damage are currently under progress.

As described in Section 3, we also observed pronounced inhibition of Trp-P-2 induced DNA-damage by coffee (Fig. 5e). We included this parameter, since several earlier investigations indicated that coffee specific diterpenoids are protective towards DNA-damage caused by HAs in animals (Huber et al., 1997) and human cells (Majer et al., 2005). It was postulated that the prevention of DNA-damage is due to inhibition of activation (acetylation) and induction of detoxifying enzymes (glutathione-*S*-transferase and UDP-glucuronosyl transferase) (Huber et al., 2002) and it is known that these enzymes are also induced by coffee in rats (Somoza et al., 2003); furthermore, recent findings provide evidence that also in man a pronounced increase of GST takes place after coffee consumption (Steinkellner et al., 2005). Our findings suggest that coffee consumption may protect also humans against the DNA-damaging effects of heterocyclic aromatic amines.

Taken together, the results of this study support the assumption that the inverse relationship between certain forms of cancer and coffee consumption, which was found in epidemiological studies, is causally related to prevention of DNA-damage.

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PAPER II

Instant coffee prevents oxidative DNA damage, reduces F2-isoprostane and nitrosation of proteins: results of a clinical trial

Christine Hoelzl, Christophe Cavin, Michael Kundi, Karl-Heinz Wagner, Leonilla Elbling, Wolfgang W. Huber, Tatjana Simic, Oliver Neubauer, Nina Kager, Franziska Ferk, Veronika Ehrlich, Armen Nersesyan, Siegfried Knasmüller S.[1-3]

[1] From the Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Austria (CH, LE, WH, NK, FF, VE, AN, SK), Nestlé Research Center, Quality and Safety Department, Lausanne, Switzerland (CC), Institute of Environmental Health, Medical University of Vienna, Austria (MK), Department of Nutritional Science, University of Vienna, Austria (KW, ON), Institute of Biochemistry, Faculty of Medicine, University of Belgrade, Belgrade, Serbia (TS)

[3] Address reprint requests to S Knasmüller, Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria. E-mail: siegfried.knasmueller@meduniwien.ac.at

Running title: Effect of coffee consumption on the redox status

KEY WORDS: Instant coffee, antioxidants, SCGE, humans, DNA-damage

ABSTRACT

Background: Coffee is among the most frequently consumed beverages worldwide. Epidemiological studies indicate that its consumption is inversely related to the incidence of reactive oxygen species (ROS) related diseases (liver cirrhosis, specific forms of cancer and neurodegenerative disorders) and it was postulated that these effects are due to antioxidant properties.

Objectives: We investigated the impact of a new instant coffee which contains high levels of chlorogenic acids on DNA damage and on markers of the antioxidative status.

Design: The impact of coffee consumption (800 ml/P/d, 5 days) on DNA damage was studied in single cell gel electrophoresis assays under standard conditions and with lesion specific enzymes in an intervention trial with a cross-over design (n = 29).

Results: DNA migration due to formamidopyrimidine-DNA glycosylase (FPG) sensitive sites (reflecting oxidised purines) was reduced by 6.7 % and damage due to endonuclease III sensitive sites (oxidised pyrimidines) was lowered by 9.8 %. The effect was only significant with FPG in females (reduction by 23.2 %). Also, F2-isoprostane levels in urine and 3-nitrotyrosine levels in plasma were clearly decreased (by 14.9 % and 17.2 %, respectively). Again the effects were more pronounced in females. Other markers (superoxide dismutase, glutathion-S-transferase and glutathione peroxidase activity, glutathione levels, total antioxidant capacity, oxidised LDL, malondialdehyde and intracellular ROS) were not significantly altered.

Conclusions: Intake of the coffee reduced endogenous oxidative DNA damage, lipid peroxidation and protein nitration in women but not in men. These biomarkers are associated with pathophysiological processes, therefore the results are indicative of beneficial health effects.

INTRODUCTION

Numerous studies indicate that cellular damage caused by reactive oxygen species (ROS) is causally involved in the etiology of a variety of diseases (1-3). Therefore, strong attempts have been made over the last decades to identify dietary components with antioxidant properties (4).

Recently, we reported the results of a small intervention trial (n=8) in which we found that consumption of a mix of metal and paper filtered coffee prevents the endogenous formation of oxidised DNA bases in peripheral human lymphocytes (5). This is an interesting observation, as coffee is the second most traded commodity after petrol and among the most frequently consumed beverages. Evidence of its antioxidant properties is also supported by results of *in vitro* experiments, which showed that it inactivates ROS by direct scavenging (6) and by findings showing induction of antioxidant enzymes in rodents (7, 8). *In vitro* analyses demonstrated that coffee possesses a higher antioxidant capacity than other beverages, e.g. 5-fold higher than green tea and 3-fold higher than red wine (9). It has been stressed that coffee is one of the most important sources of phenolic compounds and that its consumption contributes to a higher extent to the intake of antioxidants than consumption of fruits and vegetables (10). In this context, it is notable that increasing evidence suggests coffee that intake is inversely related to the incidence of liver and colon cancer, liver cirrhosis, Alzheimer's and Parkinson's diseases, and diabetes (6, 11-13). It is assumed that ROS plays a crucial role in the etiology of all these diseases (1, 2).

Aim of the present study was the investigation of the antioxidant properties of a new brand of instant coffee whose antioxidant properties were improved by addition of dried material of green beans containing chlorogenic acids (CAs). CAs are assumed to contribute substantially to the antioxidant properties of the brew (14-16).

The impact of coffee consumption on the formation of endogenous oxidised DNA bases was monitored in single cell gel electrophoresis (SCGE or comet) assays in peripheral human lymphocytes. Furthermore, we analysed alterations of the sensitivity of the lymphocytes towards ROS (H_2O_2) induced DNA-damage, as well as DNA-damage due to formation of single and double strand breaks. These parameters were monitored in SCGE experiments, which are based on the determination of DNA migration in an electric field and are increasingly used in dietary human intervention trials (for review see (17, 18)).

Additionally, a number of other endpoints were monitored which are indicative of antioxidant effects, including oxidised low density lipoproteins (oxLDL) and malondialdehyde (MDA) levels in plasma, as well as F2-isoprostane levels in urines, which are regarded as a reliable marker for lipid peroxidation (4). Other biochemical endpoints were glutathione (GSH) concentrations in erythrocytes, 3-nitrotyrosine (3-NT) levels and the total antioxidant capacity (TAC) in plasma. Since recent findings indicate that coffee might also affect the expression of genes controlled by the transcription factor Nrf2 (7) we additionally measured the impact of coffee consumption on the expression of genes encoding for antioxidant defence mechanisms by use of real time PCR, as well as the activities of a panel of antioxidant enzymes (glutathione peroxidise (GPx), superoxide dismutase (SOD) and glutathione-S-transferase (GST)).

SUBJECTS AND METHODS

Subjects

The study was approved by the Ethical Commission of the Medical University of Vienna and informed consent was obtained from the participants, which were recruited via a placard. Only individuals who fulfilled the inclusion criteria (no overweight, non-smokers, no intake of pharmaceutical drugs, no intake of food supplements 4 weeks prior and during the

study, no participation in another clinical trial, no pregnancy, compliance with the protocol, no blood withdrawal 3 weeks before the study) were included (n=36). At the beginning and at the end of the trial, blood pressure and the pulse frequencies were monitored. Seven participants were excluded from the data analysis because of illness or other relevant reasons. In total, samples from 13 men and 16 women were analyzed. The characteristics of the participants are summarised in **Table 1**.

Table 1: Baseline characteristics of the participants by gender

Gender	male (n=13)			female (n=16)		
	mean ± SD	min	max	mean ± SD	min	max
Age (y)	25.2 ± 5.6	20	41	29.3 ± 10.9	20	55
Weight (kg)	76.2 ± 8.6	64	92	61.8 ± 8.1	48	74
Height (cm)	181.7 ± 7.4	173	201	168.2 ± 0.1	157	184
BMI (kg/m2)	23.0 ± 1.7	20	26	21.8 ± 2.4	19	27
Blood pressure dia (mmHg)	78.8 ± 6.4[1] 74.5 ± 14.8[2] 81.1 ± 8.9[3]	65[1] 46[2] 67[3]	90[1] 98[2] 97[3]	72.9 ± 10.6[1] 73.7 ± 8.2[2] 75.4 ± 7.2[3]	50[1] 64[2] 67[3]	97[1] 92[2] 90[3]
Blood pressure sys (mmHg)	132.3 ± 11.4[1] 130.2 ± 17.8[2] 127.1 ±14.6[3]	115[1] 104[2] 99[3]	154[1] 165[2] 158[3]	118.8 ± 11.4[1] 116.9 ± 13.0[2] 115.6 ± 13.1[3]	105[1] 98[2] 95[3]	154[1] 150[2] 143[3]
Pulse frequency (P/min)	67.5 ± 12.8[1] 71.7 ± 16.5[2] 73.3 ± 20.2[3]	42[1] 50[2] 50[3]	89[1] 99[2] 109[3]	76.8 ± 9.9[1] 80.7 ± 20.2[2] 78.1 ± 13.2[3]	56[1] 63[2] 63[3]	96[1] 142[2] 120[3]

[1]baseline, [2]water period, [3]coffee period

Study design

Figure 1 shows the experimental design of the study. One week before the start of the intervention phase (coffee/water), the participants were asked to control their consumption of fruit juices and other dietary factors such as citrus fruits or cruciferous vegetables, which may have an impact on the outcome of the study (for details see (17)). Furthermore, they were asked to avoid excessive physical exercise, since it is known that it may cause alteration of DNA migration (19).

The participants were allocated into 2 groups (18 coffee/water, 18 water/coffee). The individuals in the coffee/water group consumed coffee (800 ml/d) after a 7-day run-in phase. Following a wash-out period (5 weeks) and a restriction phase (1 week), they drank 800 ml water/d instead of coffee. The participants in the water/coffee group followed the protocol in the reversed order.

Additionally, all participants were asked to limit their coffee consumption to 1 cup per day 3 weeks before the begin of the run-in phase of the study and during the first five weeks of the wash-out period.

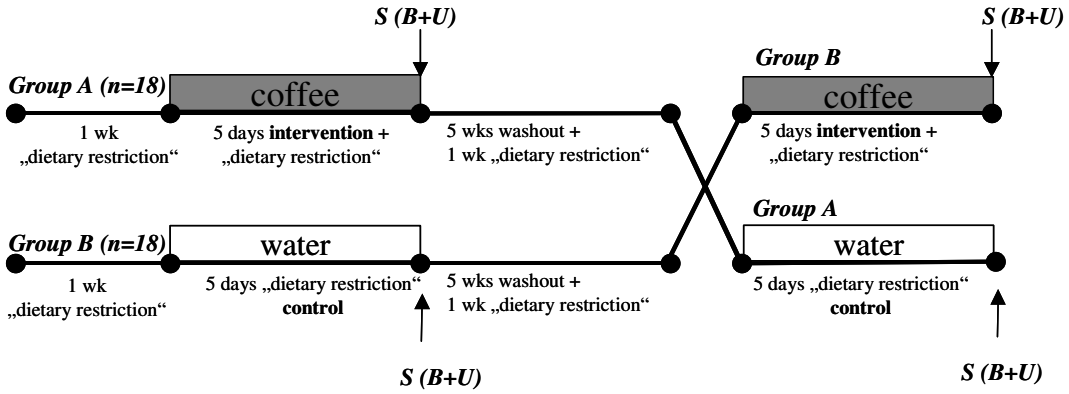

Fig. 1 Schematic overview of the crossover design

Study product

The coffee was provided by the Nestlé Research Center (Lausanne, Switzerland) and distributed in pre-weighed portions (3.4 g/package) at the beginning of the intervention. The participants consumed 4 x 200 ml of 1.7 % (w/w) coffee/d without a fixed schedule.

The product used in the study was a mix of green and roasted coffee. Since a large proportion of green coffee antioxidants, in particular chlorogenic acids (CAs) are destroyed during the roasting process (20), the concentration of CAs was increased by addition of dried material from green (unroasted) beans.

Sample preparation

After the intervention phase (coffee or water), blood (80 ml/P) was collected in heparinized tubes (Becton-Dickinson, Plymouth, UK) by venipuncture immediately after isolation with Histopaque-1077 (Sigma-Aldrich, Steinheim, Germany) according to the instructions of the manufacturer. Lymphocytes were analyzed immediately after collection in SCGE assays or stored in liquid nitrogen. Plasma was aliquoted in 500 µl portions and stored deep-frozen at -80°C. Furthermore, 24 hr urine samples were collected; the total amounts were recorded and subsequently the samples were aliquoted and stored at –80°C.

Single-cell gel electrophoresis assays (comet assays)

The SCGE experiments were carried out according to international guidelines (21, 22).

To monitor single and double strand breaks and apurinic sites, the experiments were conducted under standard conditions (20 min alkaline unwinding ph > 13, 20 min electrophoresis, 25 V, 300 mA, in presence of autologous plasma (10 %)).

Experiments with the DNA lesion-specific enzymes endonuclease III (Endo III) and formamidopyrimidine–DNA glycosylase (FPG), which enable the detection of endogenous formation of oxidised pyrimidines and purines were carried out as described by Collins et al. (23, 24). Lymphocytes (0.025×10^6) were transferred to agarose-coated slides, lysed (4 ˚C, 1 hr) and washed (3 times with enzyme buffer) prior to incubation with 50 µl of Endo III (for 45 min) or FPG (for 30 min) at 37˚C. Subsequently, the slides were placed for 40 min in a horizontal electrophoresis unit (C.B.S. Scientific Company, Del Mar, CA) filled with alkaline buffer (pH>13), subsequently electrophoresis was conducted (25 V, 300 mA, 30 min).

To monitor alterations of the sensitivity of the lymphocytes towards ROS, the cells were exposed to H_2O_2 (15 min at 4°C, 25 µM) according to the protocol of Collins et al. (25).

After treatment, cells were mixed with low melting agarose (0.5 %, Gibco, Paisley, UK) and transferred to agarose coated slides. After lysis (4 °C, 1 hr) and unwinding in alkaline buffer (20 min, pH > 13), electrophoresis was performed (25 V, 300 mA, 20 min).

DNA was stained with 40 µl ethidium bromide (20 µg/ml). For each experimental point, three slides were prepared in parallel and from each, 50 cells were analyzed by a blinded observer with a computer aided image analysis system (Comet Assay IV Perceptive Instruments Ltd., Haverhill, UK). As endpoint the percentage of DNA in tail was recorded, which is an internationally accepted parameter (26, 27).

Acute toxic effects were monitored with the trypan blue exclusion technique (28) and only cells from cultures in which the viability was ≥ 80 % were analyzed for comet formation as an excess of dead cells may cause false positive results (22, 29).

Determination of lipid peroxidation products

Determination of malondialdehyde (MDA)

MDA levels were determined according to the method of Ramel et al. (30) by use of high performance liquid chromatography (HPLC). Briefly, the samples were hydrolyzed after heating (60 min, 100˚C) with phosphoric acid. This leads to formation of a complex with thiobarbituric acid, which was determined fluorometrically after deproteinisation (excitation: λ=532nm, emission: λ=563nm). Each sample was measured in duplicate.

Measurement of oxidized low density lipoproteins (oxLDL)

oxLDL concentrations were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Mercodia AB, Uppsala, Sweden). Absorbance of samples and standards was determined with a fluorimeter (BMG Lab Technologies, Offenburg, Germany).

Determination of 8-epi-$PGF_{2\alpha}$ with LCMSMS

8-epi-$PGF_{2\alpha}$ was determined in plasma. 8-epi-$PGF_{2\alpha}$ was extracted with commercially available immunoaffinity columns (Cayman Chemical Co., Ellsworth Ann Arbor, MI) prepared from an 8-iso PGF selective antibody. The procedure was carried out according to the instructions of the manufacturer. After centrifugation, the samples were spiked with 5.0 ng of deuterated standard of the title compound, acidified and passed through a pre-washed immunoaffinity column. After elution with 5.0 ml buffer (95 % ethanol/ 5 % water), the eluates were collected and the solvent was evaporated.

8-Iso-$PGF_{2\alpha}$ was determined with LC/MS/MS (Sciex API 4000 Triple Quad, Applied Biosystems, Van Allen Way Carlsbad, CA), which involves a coupled HPLC (Agilent 1100, Santa Clara, CA) separation of the sample prior to MS analysis. MSMS was operated in ESI mode (negative ionisation, -4500 electron volts). In all experiments, a stable deuterated internal was used.

Measurement of antioxidant enzymes

CuZn-SOD activity was determined in the cytosolic fractions of lymphocytes with the RANSOD test kit (Randox Laboratories Ltd., Ardmore, UK). The test is based on the use of the xanthine/xanthine oxidase reaction to generate O_2**-** radicals (31). The activity of the enzyme was measured spectrophotometrically (λ=505 nm) by determination of the degree of inhibition of O_2 induced formation of a red formazan dye. Each measurement was carried out in triplicate.

GPx activity was determined spectrophotometrically (λ=340 nm); the assay is based on the method developed by Gunzler and Flohe (32) The results were standardized on the

basis of the protein concentrations which were measured with the Bradford method (33). Each measurement was carried out in duplicate.

Intracellular ROS (DCFH-DA assay)

Intracellular ROS levels were measured by flow cytometric analysis of 7-dichlorofluorescein (DCFH) oxidation using DCFH-DA (Fluka, Buchs, Switzerland) as described previously (34). Isolated cells were stained with PerCP labeled anti CD-45 (pan leukocytic marker) and APC labeled anti CD-14 (monocyte/macrophage specific) antibodies (BD Phorminogen, San Jose, San Francisco) for 15 min. Afterwards, DCFH-DA (10 µM) was added to the cell suspensions in presence or absence of autologous plasma (1.0 %).

Additionally lymphocyte suspensions were exposed to 50 µM H_2O_2 (60 min at 37°C). After treatment, the cells were analyzed by multiparametric analysis using a FACS Calibur TM system (Becton-Dickinson, San Jose, CA) with excitation and emission settings of 500 ± 15 and 535 ± 15 nm, respectively.

Lymphocytes, monocytes and granulocytes were discriminated by scatter gating strategy (low, moderate and high FSC/SSC, respectively) and CD staining (CD45+/CD14-, CD45+/CD14+ and CD45dim/CD14dim).

Background fluorescence was determined in DCFH-delabeled cells without treatment. Each sample was measured in duplicate. Data are given as mean fluorescent peak heights in arbitrary units.

Total Antioxidant Capacity (TAC)

TAC in plasma was analyzed spectrophotometrically (UV/VIS Spectrometer Lamda2; Perkin Elmer; λ=734nm) after iron induced oxidation. The assay is based on the ability of an antioxidant to scavenge blue-green colored 2,2´-azinobis(3-ehtylbenzthiazoline-6-sulfonic

acid) radicals (ABTS*) which are generated by the peroxidase activity of metmyoglobin in presence of the sample (35).

Glutathione (GSH)

GSH levels were determined photometrically in erythrocytes. The tripeptide was released from the cells by hemolysis (36), which leads to removal of protein and blood pigments. The remaining suspensions were mixed with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and absorbance read at 412 nm (UV/VIS Photometer ATI-Unicam UV 4, Perkin Elmer, Waltham, MA). The measurements were carried out in duplicate.

Glutathion-S-transferase (GST)

GST activity was spectrophotometrically (λ=340nm) determined in the cytosols of lymphocytes according to the standard method developed by Habig et al. (37) at 37 °C, using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The protein contents were determined with the BIORAD/Bradford assay (33). All measurements were carried out in triplicate.

3-Nitrotyrosine (3-NT)

The levels of 3-NT in plasma were determined by liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). After extraction of the free and protein associated form of nitrotyrosine (for details see (38)), the eluates were injected in a HPLC system (Hewlett-Packard, Pali-Alto, CA). Detection was performed with positive electrospray ionisation tandem mass spectrometry (Finnigan MAT TSQ 7000 with API interface, San Jose, CA).

Gene expression

The lymphocytes for RNA preparation were separated with Vacutainer® CPT cell preparation tubes (bd, city, land), subsequently the pellets were treated with TRIZOL® Reagent (Invitrogen, city, land) according to the manufacturer´s instructions. Samples were stored at –80°C.

RNA was converted to cDNA using the TaqMan Reverse transcription Kit system (Applied Biosystem, Foster City, CA). Reverse transcription was performed according to the manufacturer's instructions (Applied Biosystems, Foster City, CA), using the random hexamers primer and 2.0 µg of pooled RNA samples. cDNA synthesis was performed in a thermal cycler (Applied Biosystems, Foster City, CA) under the following conditions: 10 min at 25 °C, 30 min at 48 °C and a final inactivation step of 5 min at 95 °C. Samples were amplified in Taqman Low Density custom Array with the ABI Prism ® 7900HT Sequence Detection System using 1.0 µl of cDNA template and the TaqMan PCR master mix 2 x (Applied Biosystems, Foster City, CA). The thermocycler conditions included two incubations (2 min at 50 °C and 10 min at 95 °C), followed by 40 cycles, each consisting of a denaturation step for 15 s at 95 °C, and a second annealing and extension step for 1 min at 60 °C. Quantification of amplified PCR products was performed using ABI Prism®7900HT Sequence Detection System software Version 2.2. (Applied Biosystems, Foster City, CA), normalized to the GAPDH gene as an internal control. All rat PCR probes and primers used in this study were obtained from the Low Density custom array of Applied Biosystems.

Statistical analyses

Statistical analyses of the comet assay data were performed with the following procedures. From each slide, 50 cells were evaluated and the medians were calculated, which were arcsine transformed in order to obtain homogeneity of variance. Outliers among

triplicates were removed with the Dixon's test. The means of triplicates within each condition were computed, and these values submitted to Grubb's test. Outliers among subjects within conditions were removed with this procedure. Analysis of variance was conducted by application of a mixed model with subjects as random factor, gender as between subjects factor, period (first/second) and condition (coffee/water) as within subjects factors.

In addition, interactions between gender and condition as well as sequence and conditions were evaluated. By this procedure, a potential seasonal effect was removed from the other effects. Main effects and interactions were considered significant if p values were $\leq$ 0.05. Homogeneity of variances was tested by Levene's test. Residuals were assessed for normality by Lilliefors tests. For these tests, the significance level was set to 0.01. The statistical analyses were performed using the Statistica 8.0 (StatSoft GmbH, Hamburg, Germany) software. The gene expression data were log transformed and analyzed by Wilcoxon sign-rank tests. Statistical significance of all other parameters was analyzed by use of the Wilcoxon signed ranks test (significance level $P \leq 0.05$).

RESULTS

The impact of coffee consumption on different parameters of DNA migration, which was measured with the SCGE technique, is summarized in **Table 2**. Comet formation under standard conditions (which reflects single and double strand breaks and apurinic sites) was reduced after coffee intake, but the effect did not reach statistical significance. Treatment of the cells with the enzymes lead to a strong (ca. 3-fold) increase of DNA-migration attributable to FPG-sensitve sites (reflecting oxidised purines) and Endo III-sensitve sites (reflecting oxidised pyrimidines). The results obtained with the enzymes are also shown graphically in **Figure 2**. In the case of Endo III a borderline effect was detected in the entire study group (P = 0.050019), while in the case of FPG a significant decrease of DNA-migration was found only in females.

Table 2: Effects of coffee consumption on different endpoints carried out with the SCGE (Comet) assay [1, 2]

SCGE assay (% DNA in tail)	water period	coffee period	Δ (%)[3]	p-value[4]
Treatment with lesions specific enzymes				
Endonuclease III	9.79±3.43	8.82±4.14	-9.88	0.05
Formamido pyrimidine glycosylase	14.96±5.95	13.95±5.38	-6.74	0.62
Hydrogen peroxide induced DNA damage	17.94±11.08	16.84±9.05	-6.09	0.77
Endogenous DNA damage	1.60±1.67	1.13±0.77	-29.09	0.13

[1]All values represent means ± SD. SCGE; single cell gel electrophoresis. DNA damage was measured as percentage DNA in the tail. All measurements were carried out in triplicate in peripheral human lymphocytes.
[2]n=29, expect for Endo III n=27
[3]Δ (%) values were calculated from the raw data;
4p-values were calculated with ANOVA and linear contrast

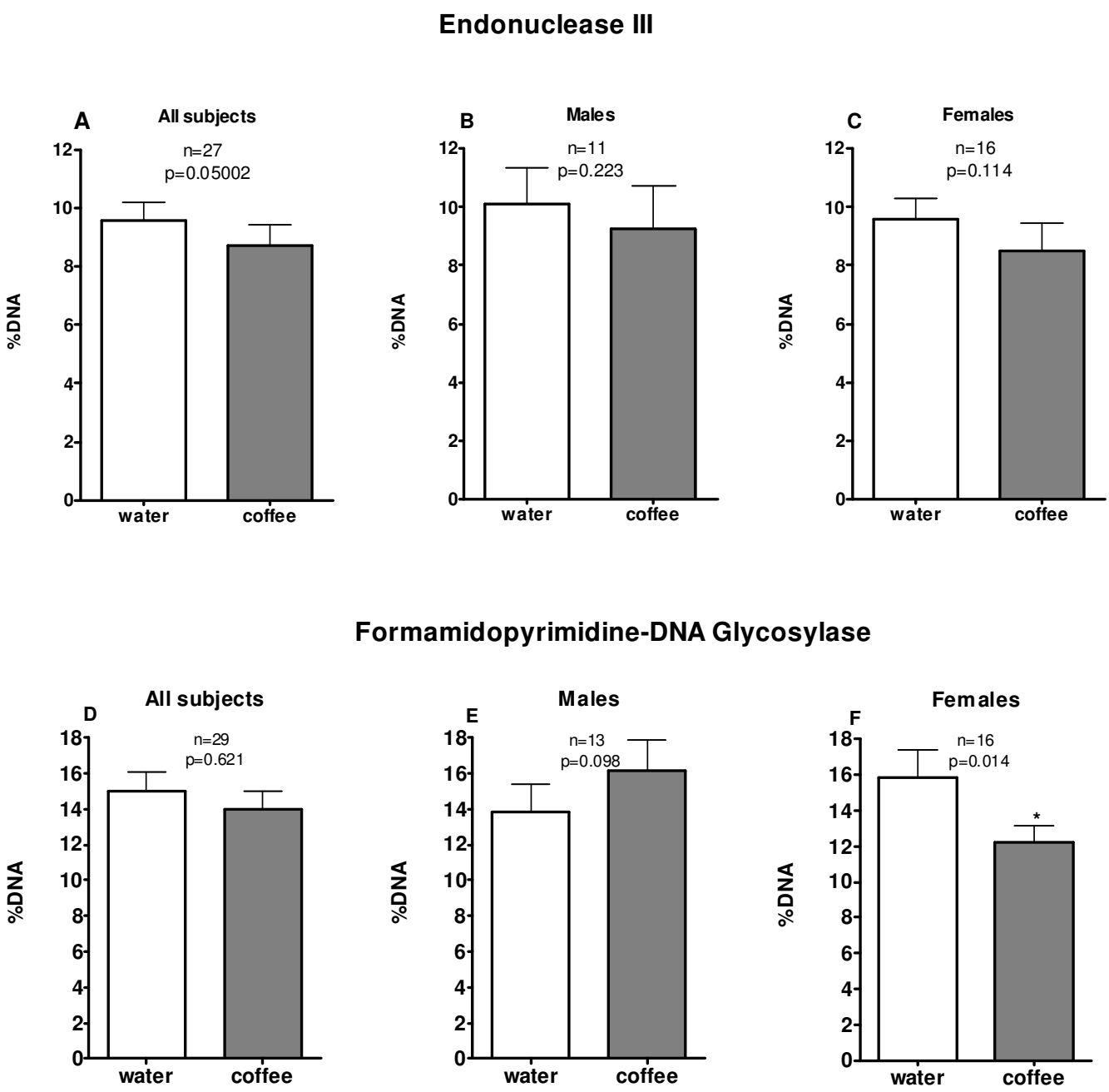

Fig. 2 A-F Impact of coffee consumption on DNA migration after treatment of nuclei with the lesion specific enzyme endonuclease III (1 A-C, treated for 45 min at 37˚C) and with formamido pyrimidine glycosylase (1 D-E, treated for 30 min at 37°C). From each participant three slides were made per experimental point in parallel and from each 50 cells were analysed for the comet formation as described above. Bars represent the mean ± SEM. Asterisks indicate statistical significance, $P \leq 0.05$ (ANOVA and linear contrast).

The results of the measurements of different biochemical parameters are summarized in **Table 3**. All endpoints, which are indicative for lipid peroxidation were reduced after intake of coffee, but the effect reached significance only in the case of the F2-isoprostanes. **Figure 3** depicts the gender specificity of the effect and it can be seen that the overall decrease is due to the strong effect in females.

Table 3: Effects of coffee consumption on different biochemical parameters[1]

Parameters (unit)	**water period**	**coffee period**	Δ (%)[2]	**p-value**[3]
Lipidperoxidation Products				
oxLDL (U/L)[4]	40.05 ± 11.41	38.05 ± 13.48	-4.98	0.421
MDA (µM/L)[4]	1.16 ± 0.68	1.07 ± 0.59	-7.57	0.365
8-epi-PGF2α (ng/mg Cr)[5]	0.09 ± 0.04	0.07 ± 0.03	-14.93	0.001*
Enzymes[6]				
SOD (U/mg/protein)	7.60±2.66	7.32 ± 2.40	-3.68	0.585
GPx (U/mg protein)	0.56±0.19	0.58 ± 0.26	+4.17	0.513
GST (nmol/min/mg protein)	277.58±86.90	288.36 ± 78.38	+3.88	0.233
Intracellular ROS[6]				
without challenge (- plasma)	10.66±4.19	10.25 ± 2.61	-3.76	0.693
without challenge (+ plasma)	9.00±3.25	9.21 ± 2.27	+2.33	0.758
with H_2O_2 (- plasma)	151.89±92.74	166.92 ± 99.84	+9.90	0.384
with H_2O_2 (+ plasma)	57.10±42.91	42.93 ± 30.39	-24.81	0.291
Other parameters				
TAC (mM TE)[4]	0.71 ± 0.16	0.76 ± 0.15	+6,61	0.253
GSH (mg/dl)[7]	72.52 ± 9.41	72.43 ± 11.28	-0.12	0.772
3-NT pg/µg tyrosine[4]	8.47 ± 3.08	7.01 ± 2.28	-17.24	0.011*

[1]All values are means ± SD. 8-epi-PGF2α, 8-epi-prostaglandin F2α; GSH; glutathione, GPx; glutathione peroxidase, GST; glutathione-S-transferase, MDA, malondialdehyde; oxoLDL, oxidised low density lipoprotein; ROS; reactive oxygen species; SOD; superoxide dismutase, TAC; total antioxidant capacity, TE; trolox equivalent,

[2]Δ (%) values were calculated from raw data

[3] Statistical analyses were performed with the Wilcoxon signed-ranks test;

[*] stars indicate statistical significance ($p \leq 0.05$)

[4] Measurements were performed in plasma, [5] in urine, [6] in lymphocytes, [7] in erythrocytes

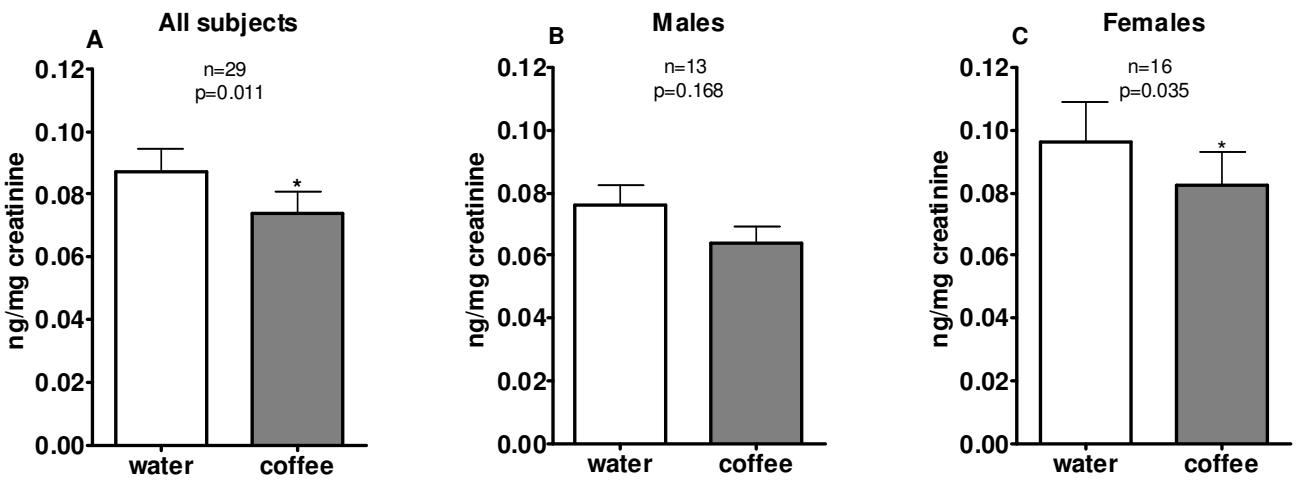

Fig. 3 A-C Impact of coffee consumption on the levels of 8-iso PGFα concentrations in the urines measured with LCMSMS. Bars represent the mean ± SEM. Asterisks indicate statistical significance, $P \leq 0.05$ (Wilcoxon signed-rank test).

The overall 3-NT levels were reduced by 17.2 % after coffee intake and it can be seen in **Figure 4**, that the protective effect was again due to the strong effect in females. None of the other endpoints except 3-nitrotyrosine (3-NT) was affected by coffee consumption.

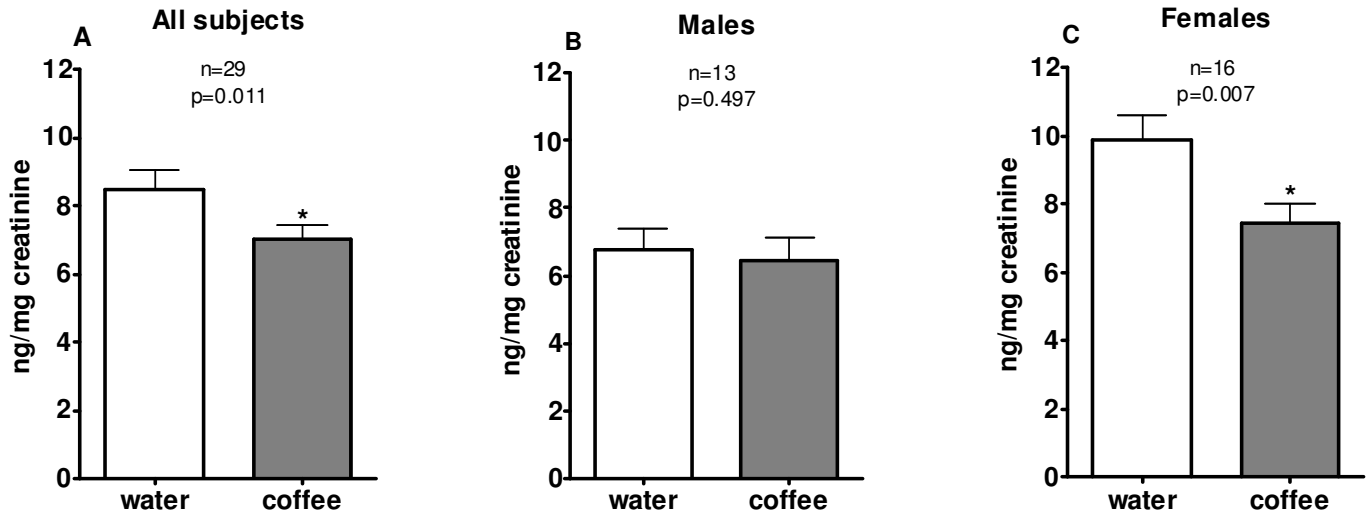

Fig. 4 A-C Impact of coffee consumption on the levels of 3-nitrotyrosine determined by LC-ESI-MS/MS. Bars represent the mean ± SEM. * Asterisks indicate statistical significance $P \leq 0.05$ (Wilcoxon signed-rank test).

Intracellular ROS levels were increased after treatment of the cells with H_2O_2 and the levels were in general lower when the cells were cultivated in plasma, which contains a variety of antioxidants. No significant changes were seen after coffee consumption in these experiments.

The expression of a variety of genes was analysed by RT-PCR; some of them *NQO1* (7.8 %), *SOD1* (22.2 %), *UGT2A1* (125.4 %), *UGT2A3* (14.4 %), *UGT2B15* (20.6 %), *UGT2B17* (15.0 %), *GSTA1* (24.8 %), *GSTA2* (45 %), *GSTA5* (19.5 %) were increased after coffee consumption. (Numbers in parenthesis indicate the increase in percent). Also the expression of Nrf2 was increased (by 9.9 %). However, none of these effects reached statistical significance.

DISCUSSION

Overall, the results of the present study show, that consumption of instant coffee rich in chlorogenic acids (CAs) resulted in a significant protection towards DNA migration attributable to formation of oxidized bases, reduced the 3-NT levels in plasma and decreased the concentrations of F2-isoprostanes in urine, while other biomarkers of oxidative stress were not altered.

The protection of oxidative DNA damage was not unexpected as we observed (in a small (n=8) recent pilot study with coffee) a reduction of FPG and Endo III sensitive lesions (5). In this earlier trial, the participants consumed a mix of paper and metal filtered coffee, while, in the present study, we used instant coffee containing high levels of hydroxycinnamic acids. This may explain why the effects we observed were less pronounced. However, as described above, the observation of a significant decrease of endogenous formation of FPG-sensitive sites was restricted to female participants. Furthermore, it is notable that comet formation was decreased in the present study also under standard conditions (which reflect single and double strand breaks), however this effect did not reach significance.

Also a number of earlier investigations are indicative of DNA protective effects of coffee. For example, van Zeeland et al. (39) reported lower levels of 8-OHdG in peripheral leukocytes of coffee drinkers. Furthermore, a large number of *in vitro* experiments with different types of coffee and with coffee constitutents such as CAs, caffeine and Maillard products provided evidence of antimutagenic properties (for review see (6)).

Recently we reviewed the results of human intervention trails in which the SCGE technique was used to monitor the effects of dietary components on DNA stability (17). It is notable that in a large study no significant protection was observed after consumption of high levels of fruits and vegetables (600g/P/d) (40), while strong effects were seen with specific items, such as Brussels sprouts (41), watercress (42) and also with the common spice sumach

(43). These findings indicate that only specific food items protect against oxidative DNA damage.

Another relevant marker of oxidative damage, which was decreased in the present trial are the F2-isoprostane concentration in urines. On the contrary, no such effect was seen in a study with paper filtered ground coffee (n=43, uptake of 450/900 ml for 3 weeks or n=35, uptake of 150/300 ml single dose) by Mursu et al. (44). The reason for this discrepancy may be that another type of coffee was used and/or that they monitored the isoprostanes in plasma and not in urine. F2-Isoprostanes are regarded as a reliable marker for lipid peroxidation (4) and high levels are associated with increased risk of a number of diseases, including neurological disorders, coronary heart and lung diseases, and renal dysfunction, to name only a few (for review see (45)). It has been shown in human intervention trails that dietary factors such as vitamin C (in smokers) (46), genistein (47) as well as specific foods such as legumes (48) and yam (49) decrease F2-isoprostane levels, while no such effects were seen with other dietary components, such as chocolate (50), tomato based drink (51) and blueberries (52).

Another endpoint, which was affected by coffee intake are the 3-NT levels in plasma, which are indicative of pathological conditions, such as inflammation, neurodegenerative diseases and cardiovascular disorders (for review see (53)). Protein alterations, which are characterised by higher NT levels, are also characteristic for artheriosssclerotic lesions (54) and it is known that they are associated with a number of functional consequences, such as modifications of the activities of specific enzymes (53). Weight loss in overweighted Caucasian women (55) and also the glycemic index of food items (56) affect human NT levels but no results from intervention trials with specific dietary factors have been published according to our knowledge. However, a number of *in vitro* studies showed that a citrus flavone (hesperitin) (57), tomato extracts (58) and hydroxycinnamic acids inactivate peroxynitrite (59, 60), which is responsible for the nitration of tyrosine (61). The latter

observation is of particular importance since the coffee that we used was rich in CAs. In this context, it is notable that chlorogenic acid (5-caffeoylquinic acid) reduced the NT levels in the aorta of rats. This effect was paralleled by the attenuation of hypertension and improvement of endothelial function (62).

In the RT-PCR we detected alterations of the activities of some enzymes involved in antioxidant defence such as NQO, SOD1 and specific forms of UGT and GST. None of these changes was significant, but nevertheless they suggest a trend towards a specific pattern of transcriptional changes. Interestingly, Cavin et al. (7) found that coffee and coffee diterpenoids activate the transcription factor Nrf2 in the liver of rats, which controls the aforementioned genes and we detected also in the present study a moderate activation of this transcription factor in the lymphocytes (which was statistically not significant).

None of the other markers, which we monitored in the present study, such as GSH, MDA, oxoLDL, SOD, GPx and intracellular ROS formation was altered after coffee intake. These findings are partly in contrast to results of other coffee studies. For example, Esposito et al (63). found increased GSH levels after intake of espresso. Bichler et al. (5) detected an increase of SOD activities with a mix of metal and paper-filtered coffee and Steinkellner et al. (64) found a strong enhancement of the levels of GST isoenzymes after intake of metal filtered coffee. On the contrary, no changes of most of these markers were found by Mursu et al. (44), who conducted a clinical trial with paper filtered ground coffee containing low amounts of cafestol and kahweol. These diterpenoids are known to interact with cell signalling pathways (65, 66).

Dotan et al. (67) compared the association between several markers of oxidative stress used in human trials and/or in animal studies. The authors found that no or only weak correlations exist between different groups of endpoints, for example between markers of

lipid peroxidation and oxidative DNA damage and emphasize that oxidative stress and protection against it can not be defined in universal terms.

In the case of the instant coffee we used, it is possible that indirect effects may account for its antioxidant properties. It was shown recently that green tea increased the uptake of glucose in the gastrointestinal tract which leads to a hypocaloric status associated with decreased ROS production in mitochondria. This effect was not paralleled by changes in the TAC (68). Also with coffee reduced glucose uptake was found in a recent human study and the authors suggest an antagonistic effect of CAs on glucose transport (69).

The most pronounced antioxidant effects were seen in the present study consistently in females. Overall the gender specificity of the oxidant status in humans is a controversial issue. A number of comet studies indicate that the extent of DNA damage is lower in females than in males (70, 71). However, Moller et al. (72) found no clear sex differences in a recent meta-analysis.

According to our knowledge, no gender specific differences were reported in earlier dietary intervention trails (17). In this context, it is notable that the lack of an effect of coffee on a panel of different lipid peroxidation parameters reported by Mursu et al. (44) may be due to the fact that only males participated in this trial while in the SCGE study of Bichler et al. (5, 73), who found pronounced reduction of DNA damage, only females were enrolled. Interestingly, the results of some epidemiological studies indicate that coffee consumption is more protective in women. For example, a stronger inverse association between coffee intake and the incidence of liver cirrhosis was found in an Italian case control study (in males OR 0.6 and in females OR 0.2) (74). Also a recent meta-analysis concerning the correlation between coffee and colorectal cancer showed stronger effects in females (75) and in a large prospective Japanese study (76), a significant risk was only seen in females. It is also notable

that the inverse association of caffeine intake and diabetes type 2, which was described by Iso and coworkers in a Japenese study was less pronounced in males (77).

Take together our results suggest that coffee consumption may contribute substantially to the improvement of the antioxidant status in humans and to the prevention of ROS-related diseases.

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CH, CC and SK were the principle investigators, who designed the study and were in charge for all overall operations and responsible for the manuscript preparation. MK was responsible for the statistical evaluation of the data. KHW and ON determined the TAC, oxLDL and MDA. TS was responsible for the GPx and SOD measurements, LE for the DCFDHA assay, WWH for the GST experiments and CC for the 3-NT and F2-isoprostane measurements. CH, VE and NK were responsible for recruitment of the participants and the CRFs. CH, FF, NK, VE carried out the SCGE experiments. AN, WWH reviewed the manuscript. None of the authors have financial or personal conflicts of interest.

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PAPER III

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Coffee consumption induces GSTP in plasma and protects lymphocytes against (±)-anti-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide induced DNA-damage: Results of controlled human intervention trials

Hans Steinkellner[a,1], Christine Hoelzl[a,2], Maria Uhl[a], Christophe Cavin[b], Gerald Haidinger[a], Andrea Gsur[a], Rainer Schmid[c], Michael Kundi[d], Julia Bichler[a], Siegfried Knasmüller[a,*]

[a] *Institute of Cancer Research, Medical University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria*
[b] *Food Safety Group, Nestlé Research Center, Switzerland*
[c] *Department of Blood Chemistry, General Hospital, University of Vienna, Austria*
[d] *Institute of Environmental Hygiene, University of Vienna, Austria*

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Abstract

A number of animal studies indicate that coffee protects against chemical induction of cancer; also human studies suggest that coffee consumption is inversely related with the incidence of different forms of cancer. The protective effects were attributed to induction of glutathione-*S*-transferases (GSTs) and aim of the present human study was to find out if coffee causes induction of GSTs and protects against DNA-damage caused by (±)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE), the DNA-reactive metabolite of benzo(*a*)pyrene. Ten participants consumed 1 L unfiltered coffee/d over 5 days. Before and after the intervention, saliva and blood were collected and the overall GST activity was measured with 1-chloro-2,4-dinitrobenzene (CDNB). Additionally, GSTP and GSTA were determined in plasma with immunoassays. In blood, only weak ($p = 0.042$) induction of GST (CDNB) was found. Furthermore, pronounced (three-fold) induction of GSTP was observed in blood, whereas GSTA was not altered. No correlations were seen between induction of GST (CDNB) and GSTP activities and the *GSTP1* genotypes of the participants. Also clinical parameters (creatinine, alanine, aminotransferase, aspartate aminotransferase, alkaline phosphatase), which are markers for organ damage, were monitored. None of them was altered by coffee, but serum cholesterol levels were slightly (not significantly) enhanced. In a second trial ($n = 7$), GSTP induction by unfiltered and paper filtered coffees, differing

* Corresponding author. Tel.: +43 1 4277 65142; fax: +43 1 4277 65103.
E-mail address: siegfried.knasmueller@meduniwien.ac.at (S. Knasmüller).
[1] Present address: European Commission, Joint Research Centre, Institute for Health and Consumer Protection, European Chemicals Bureau, Via E. Fermi, I-21020 Ispra, VA, Italy.
[2] Contributed equally.

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in cafestol and kahweol contents, were compared. The participants consumed 1 L coffee/d over 3 days. Again significant (threefold) induction of GSTP was observed. The effects seen with the two coffees were identical, indicating that the diterpenoid concentrations are not responsible for the effects. In a further trial ($n = 7$), the effect of coffee (unfiltered, 1 L/d, 5 days) on BPDE induced DNA-migration was studied in comet assays. A 45% reduction effect was observed. Our findings show that coffee induces GSTP in humans and indicate that consumption may lead to protection towards polycyclic aromatic hydrocarbons.

Keywords: Coffee; Glutathione-*S*-transferase; Human intervention; SCGE; BPDE; DNA protection

1. Introduction

Coffee is among the most widely consumed beverages and its annual production is increasing [1]. As coffee contains a broad variety of potentially bioactive compounds, strong efforts have been made to study its effects on human health. A number of epidemiological studies indicate, that the consumption of coffee is inversely related to the incidence of colon cancer [2–5], additionally also protection towards other forms of cancer has been reported [6,7].

These observations are supported by data from animal studies which showed that coffee protects against dietary carcinogens such as nitrosamines and polycyclic aromatic hydrocarbons (PAHs) [8,9]. One of the most important mechanisms by which coffee and the coffee specific dipterpenoids cafestol and kahweol (C + K) protect against cancer appears to be induction of glutathione-*S*-transferases (GSTs) [10]. GSTs are a family of phase II enzymes which inactivate a broad variety of environmental and dietary toxins [11,12] including food carcinogens such as PAHs, aflatoxins and heterocylic aromatic amines (HAAs). Recent studies indicate that the isozyme GSTP is highly protective towards (±)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE) which is the most important DNA reactive metabolite formed from benzo[*a*]pyrene (BaP) [13–15]. In a number of animal studies as well as in experiments with human derived cells [16], induction of GSTs by coffee and C + K was found and these effects were paralleled by protection towards chemical induction of tumors and DNA-damage [16–18]. Further evidence for the important role of GSTs in human cancer comes from a number of studies which show that functional polymorphisms of GST isozymes play a role in the aetiology of different forms of cancer [19,20].

Only a few studies are available in which the induction of GSTs by dietary factors was investigated in humans. It was shown that consumption of Brassica vegetables causes induction of overall GST and of specific isozymes in plasma of humans [21–23]. Only one comprehensive study on the effect of coffee is available: Grubben et al. [24] monitored the effect of unfiltered coffee on GST levels in colon mucosa cells. Neither the overall GST activity nor any of the isozymes GSTA, GSTP, GSTM were significantly induced, but a slight increase in the glutathione levels in the colorectal mucosa (8%) and plasma (15%) was found. Sreerama et al. [25] reported on induction of GST activity in saliva after coffee consumption but only one individual was monitored, therefore no firm conclusions can be drawn from this observation.

In the present study we investigated the effect of unfiltered coffee on the GST levels in serum and saliva in a controlled study. The activity of the enzyme was measured with 1-chloro-2,4-dinitrobenzene (CDNB) which is a substrate for several isozymes [26], and provides information on the overall GST status. Furthermore, we also measured GSTA and GSTP with ELISAs, two important isozymes which can be induced by vegetables [23,27,28]. To elucidate if the inducibility of GST depends on the genetic background, the *GSTM1* and *GSTP1* genotypes of the participants were determined by PCR, furthermore several clinical parameters were measured in the serum to exclude that the induction effects are a consequence of toxicity and organ damage. In an additional trial we compared the effects of unfiltered and paper filtered coffee on GSTP induction to find out if the effects depend on the diterpenoid contents. With paper filtered coffee, the amounts of lipids are normally below 0.2% (<0.6 mg total diterpenes/cup), whereas levels up to 20% of oil (6–20 mg diterpenes/cup) have been reported for boiled (Scandinavian), French Press and metal filtered coffees [29–33].

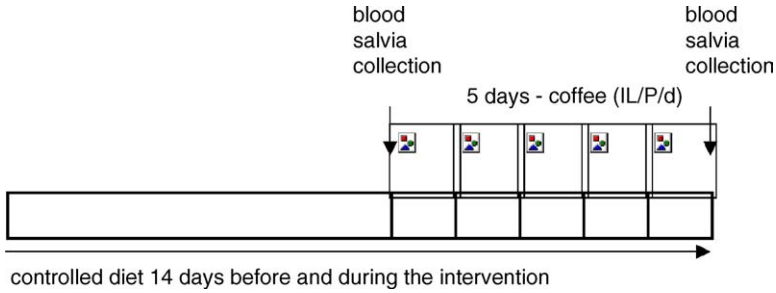

Fig. 1. Design of the first intervention study.

As mentioned above, it is known that GSTs play a key role in the detoxification of PAHs which are an important class of DNA-reactive carcinogens. Therefore, we conducted a further trial in which the impact of coffee consumption on the sensitivity of peripheral lymphocytes towards BPDE induced DNA-damage was monitored in single cell gel electrophoresis assays (SCGE, comet assay).

2. Materials and methods

2.1. Design of the study

Ten healthy, non-smoking, non-vegetarians (three males, seven females) participated in the first trial. The design of the study is depicted in Fig. 1. Fourteen days before the start and during the study, the volunteers (mean age: 26 ±4 years, b.w.: 75 ± 9 kg) had to refrain from consumption of cruciferous vegetables, coffee and tea. The participants were also asked to reduce the consumption of alcoholic beverages and fruit juices (not more than 200 mL/d), to consume limited amounts of fruits and vegetables (not more than 200 g/d) and not to perform physical exercises. 500 g of ground coffee ("Brasil sanft", Eduscho-Kaffee GmbH, Vienna, Austria) were boiled in 10.0 L tap water for 5 min, subsequently, the coffee (designated as "unfiltered coffee") was pressed through a metal mesh (Phillips, Vienna, Austria). All individuals consumed seven cups (in total 1.0 L) of coffee daily over 5 consecutive days. The design of the second intervention was identical as that of the first. Fourteen individuals (mean age: 25 ± 6 year, b.w.: 74 ± 10 kg) different from those involved in the first trial participated. Seven consumed unfiltered coffee (prepared as described above) and seven consumed coffee which had been filtered through a paper filter ("Melitta Aromapor", Melitta Haushaltsprodukte GmbH&Co. KG, Minden, Germany) for three days. Before and at the end of the interventions, blood and saliva were collected. The study was approved by the Austrian Ethical Commission and informed consent was obtained from all participants.

The third trial was conducted under identical conditions as the first study but peripheral lymphocytes were used in this experiment for single cell gel electrophoresis assay and other participants were involved. The average age of the participants was 26 ± 6 years and their average body weight was 72 ± 8 kg.

2.2. Preparation of samples

A 4.0 mg DTT (Sigma–Aldrich, St. Louis, MO, USA) were added to 5.0 mL of sputum in plastic vials which were centrifuged at 9000 × *g* for 15 min. A 10.0 mL of blood were aspired by venipuncture and centrifuged in heparinised glass vials (Becton-Dickinson, Plymouth, UK) at 760 × *g* for 10 min. The saliva supernatants and the plasma samples were stored in liquid nitrogen. For single cell gel electrophoresis assays, the lymphocytes were isolated by Ficoll centrifugation [35].

2.3. Enzyme measurements

Protein contents of saliva and plasma were determined spectrophotometrically according to Bradford [36] with the BIO-RAD® Protein Assay (BIO-RAD®, Munich, Germany). GST activity in saliva and plasma was determined according to Habig et al. [37] with CDNB as substrate. The chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA). Spectrophoto-

metrical measurements were carried out in triplicate (Beckman DU 640 spectrophotometer, Fullerton, CA, USA). GSTA and GSTP contents were determined in plasma with quantitative immunoassays (HEPKIT^{TM}-Alpha and HEPKIT^{TM}-Pi, Biotrin International Ltd., Dublin, Ireland).

2.4. Determination of GSTM1- and GSTP1 genotypes

DNA was isolated from buccal cells with the QIAGEN blood kit (QIAGEN, Hilden, Germany) and stored in elution buffer (Perkin Elmer Cetus, Norwalk, USA) at −20 °C. Polymorphisms in the GSTM1 gene locus were determined with PCR as described by Bell et al. [38] in which β-globin was co-amplified as an internal standard. Amplification products (β-globin 268 bp, *GSTM1* 215 bp) were resolved on 2% ethidium bromide-stained gel with minor modifications [39]. For the determination of the *GSTP1* polymorphism PCR/FLP assays, with separation of PCR products on a 3% ethidium bromide-stained gel were carried out according to Harries et al. [40] with minor modifications [39].

2.5. Blood parameters

Spectrophotometrical determinations of creatinine-, cholesterol-, alanine aminotransferase-, aspartate aminotransferase- and alkaline phosphatase contents were performed with an automated spectrophotometer (SYS 3 BM/Hitachi 747/737, Boehringer Mannheim, Germany) according to standard protocols [41–43].

2.6. Single cell gel electrophoresis assays

The lymphocytes were resuspended in phosphate buffered saline (pH = 7.4) and exposed to 0.4 μM (±)-anti-B[*a*]P-7,8-dihydrodiol-9,10-epoxide (BPDE, obtained from Biochemisches Institut für Umweltkarzinogene, Germany, CAS: 58917-67-2) dissolved in DMSO (containing 0.1% trietylamine, Sigma, St. Louis) at 37 °C for 30 min. Subsequently the cells were washed with PBS, centrifuged (8 min, 110 × *g*) and transferred to agarose coated slides, which were prepared and further processed according to the protocol of Collins et al. [44]. Comet formation was monitored with a computer aided image analysis system (for details see Helma and Uhl [45]). Per experimental point, three cultures were prepared and from each 50 cells were analysed. Only cultures were evaluated in which the viability of the cells was ≥80% compared to untreated controls. The viability of the cells was determined with trypan blue [46].

2.7. Statistical analyses

To determine statistical differences of the enzyme activities before and after the interventions, analyses of variance (ANOVA) and Wilcoxon tests were carried out. Correlations between gender, genotype, blood parameters, body weight and GST induction were analysed with Spearman's rank correlation test.

Comet tests in vitro with BPDE were tested by analysis of covariance, comparisons of doses against control were done by Dunett's test. For statistical analyses of the SCGE intervention study, the median and 90th percentiles of the distribution of the tail lengths were calculated and subjected to an analyses of variance with control versus BPDE and time points (before coffee consumption and after consumption) as experimental factors and subjects of random factor. Although the data showed no significant deviation from normality they were log-transformed to reach homogeneity of variances. Post hoc tests of means of experimental conditions were done by Tukey's HSD tests. For all comparisons a *p*-value below 0.05 was considered significant.

3. Results

3.1. GST (CDNB) measurements in blood and saliva

The results of GST measurements in saliva are shown in Fig. 2a. Before the intervention, the average activity was 29.47 ± 14.00 mIU/mg. At the end of the study a 1.13-fold (not significant, $p = 0.290$) increase was detected.

The results obtained with the plasma samples are depicted in Fig. 2b. The enzyme activities were approximately three orders of magnitude lower than in saliva. The background activity before the intervention was 0.037 ± 0.011 mIU/mg, after the intervention,

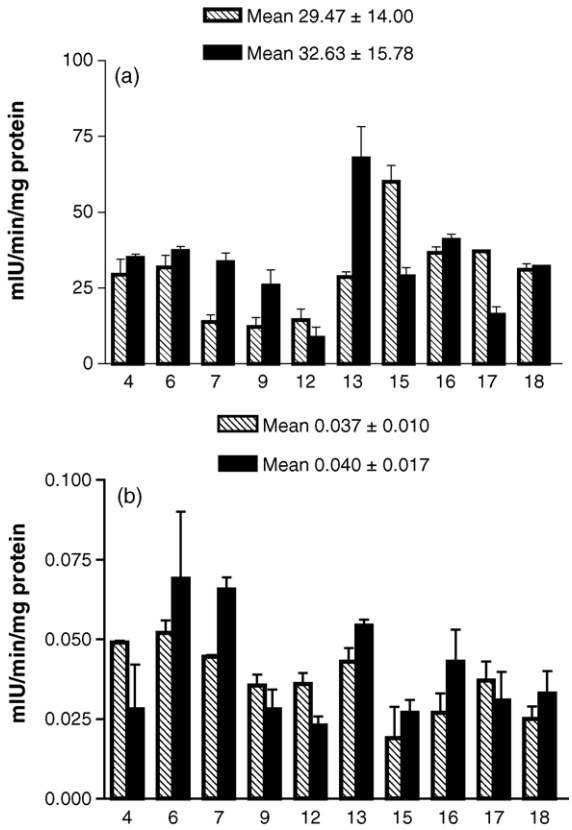

Fig. 2. (a) and (b) Effect of consumption of unfiltered coffee (1 L/P/d) on GST activities (mIU/min/mg) in saliva (a) and plasma (b). The enzyme activities were determined according to Habig et al. [37], protein contents were determined according to Bradford. [36]. Numbers on the *x*-axis are codes of the different participants. Striped bars: GST activity before coffee consumption, black bars: activity after consumption, Bars indicate mean ± S.D. of three measurements in parallel, numbers indicate codes of the different participants.

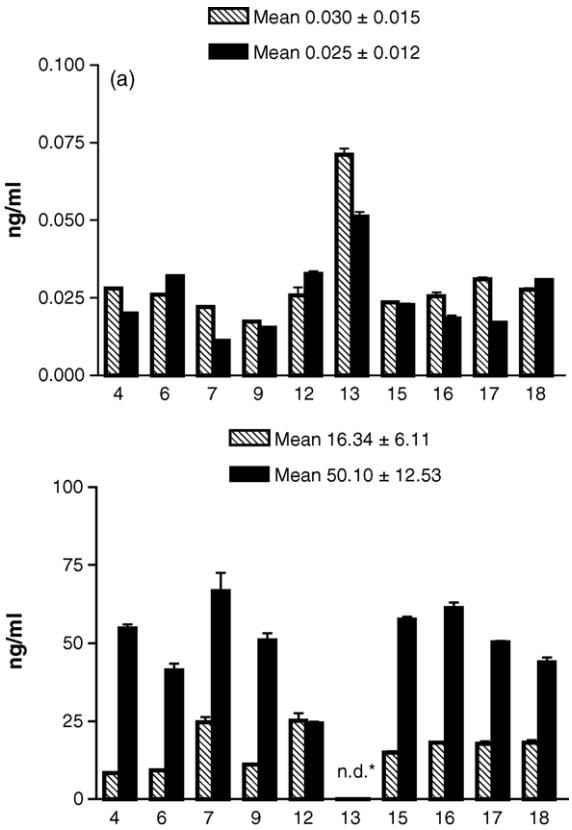

Fig. 3. (a) and (b) Effect of consumption of unfiltered coffee (l L/P/d) on GSTA (a) and GSTP (b) in plasma over three days. The isozymes were determined with ELISA. Striped bars: GSTA and GSTP activity before coffee consumption, black bars: activity after consumption, Bars indicate mean ± S.D. of three measurements in parallel, numbers indicate codes of the different participants.

it was significantly increased (0.040 ± 0.017 mIU/mg, $p = 0.042$). It is notable that the inter-individual fluctuations were smaller than in saliva.

3.2. Measurement of GSTA and GSTP

To find out which GST isozymes were induced, immunoassays for GSTA and GSTP were conducted with the plasma samples. It can be seen in Fig. 3a that GSTA was not affected ($p = 0.440$) whereas a pronounced, highly significant ($p = 0.005$) increase in GSTP was observed (Fig. 3b). Before the intervention, the mean GSTP content was 16.34 ± 6.11 ng/mL, at the end of the study it was 50.06 ± 12.53 ng/mL.

3.3. Genotyping of the participants

From the ten participants of the first study, four had an intact *GSTM1* gene (participant numbers 4, 9, 12, 13), six were *GSTM*0* (6, 7, 15, 16, 17, 18), four (12, 13, 16, 17) had the wildtype *GSTP1* gene, the others (4, 6, 7, 9, 15, 18) were heterozygous.

The results of Spearman's rank correlation tests showed no significant correlations between induction of GST activity in plasma and increased GSTP contents ($p = 1.0$) and also no relation between induction of overall GST, GSTP and GSTA and the different genotypes

(the p values for induction of GST and *GSTP1* genotype was 0.046, for GSTP and *GSTP1* the value was 0.51).

3.4. Clinical blood parameters

Data of the measurements of the blood parameters (creatinine-, cholesterol-, alanine aminotransferase, aspartate aminotransferase and alkaline phospatase) are not shown in detail. With exception of serum cholesterol, none of them was enhanced after coffee consumption. The cholesterol level was 195.2 ± 37.7 mg/100 mL before the begin of the study and was increased by 6.5% after the intervention (mean 207.3 ± 25.7 mg/100 mL). However, this effect was not statistically significant ($p = 0.06$).

3.5. Comparison of GSTP induction by filtered and unfiltered coffee

The results of the second trial in which the effects of unfiltered and paper filtered coffee on GSTP induction were compared are depicted in Fig. 4a and b. It is known that the bioactive coffee compounds C + K are retained by paper filtration [33,34]. Both types of coffee were prepared from the same brand and boiled for 5 min. As mentioned above, the C + K values are substantially lower in paper filtered coffee. Nevertheless, identical GSTP induction effects were seen with both preparations.

3.6. Results of SCGE experiments

In order to define the optimal concentration of BPDE for the intervention trial, a dose response experiment was carried out with lymphocytes from one individual. The results are depicted in Fig. 5. It can be seen that the diol induced DNA-migration over the entire range tested; already at the lowest dose (0.25 μM) a significant effect was observed.

The results of the intervention trial are summarised in Fig. 6. In all participants the DNA-damage induced by BPDE was substantially reduced after coffee consumption. Overall the protection effect caused by coffee was 45% and highly significant ($p = 0.0001$). In control cultures (i.e. in cells which were not treated with the diol), no difference in DNA migration was seen before and after the invention ($p = 0.84$).

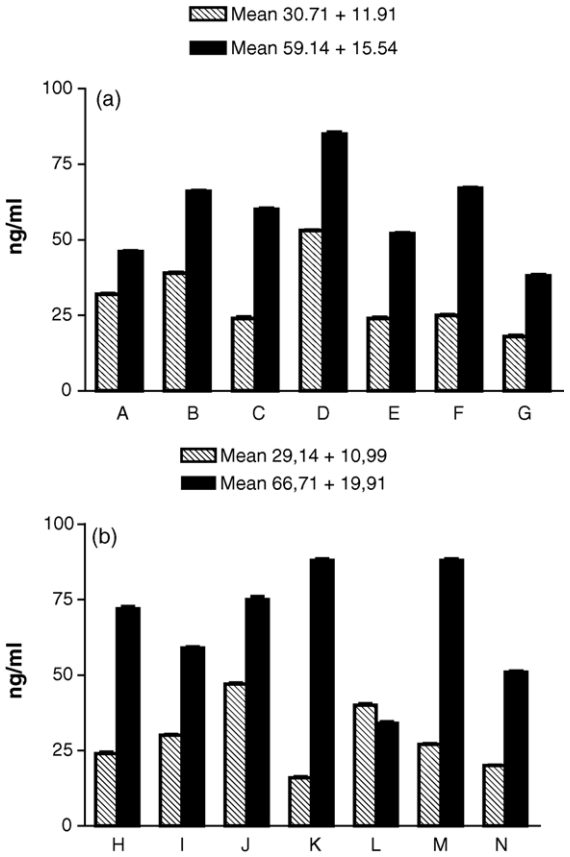

Fig. 4. (a) and (b) Effect of consumption of paper filtered (a) and unfiltered (b) coffee (1 L/P/d) on the induction of GSTP in plasma. The measurements were carried out with ELISA. The bars show mean ± S.D. of results obtained with seven individuals per group. Striped bars: GSTP activity before coffee consumption, black bars: activity after consumption, Bars indicate mean ± S.D. of three measurements in parallel, letters indicate the codes of the participants.

4. Discussion

More than 20 years ago Wattenberg [9] showed that feeding of coffee beans to rodents leads to an increase of the GST activity in different organs and protects against tumor induction by PAHs [9]. Also for green tea which is rich in phenolics, protective effects towards dietary carcinogens via this mechanism is well documented [47]. The results of the present study show that consumption of coffee leads also in humans to a pronounced increase in the activity of this detoxifying enzyme. Furthermore, we demonstrated that this effect is paralleled by substantial protection

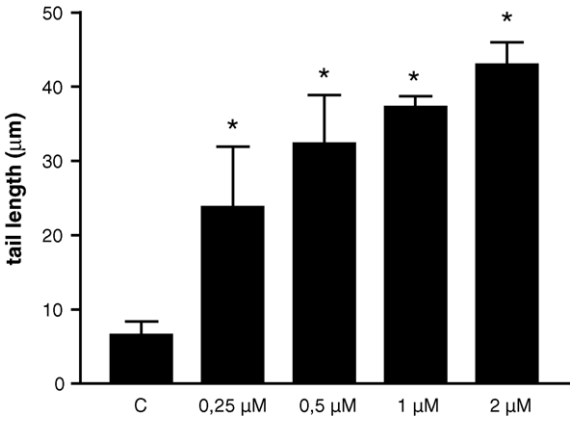

Fig. 5. Induction of DNA migration in human lymphocytes by BPDE. The cells were exposed for 30 min (at 37 °C) to different concentrations of BPDE. Per experimental point three cultures were prepared and from each 50 cells were analysed. Stars indicate statistical significance ($p \leq 0.05$).

towards DNA-damage caused by BPDE in peripheral lymphocytes.

The present study was conducted under controlled dietary conditions. This has the advantage that individual fluctuations can be minimized. We found in earlier investigations that GST levels as well as DNA migration vary strongly when the measurements are conducted without dietary control. Due to the reduction of these fluctuations and the design of the study as an intervention trial it is possible to get meaningful results with a lower number of participants as those required in studies which are based on comparisons of different groups. Also in earlier human studies in which the impact of dietary factors on GST levels and on DNA-damage in lymphocytes were measured, similar or even smaller group sizes as in the present study were used [27,28,44,48–51]. The induction of GSTP as well as the protective effect towards DNA-damage caused by BPDE was seen in ≥90% of the participants and are statistically highly significant. For the negative results (lack of GST (CDNB) induction in saliva and lack of GSTA induction in plasma) we calculated the statistical power of our study to detect a 25% alteration (increase or decrease) of these parameters; for GST (CDNB) it was 84% and for GSTA it was 88%, respectively. This shows that the number of participants in our experiments was sufficient to detect relevant alterations.

Only a few earlier studies on the induction of GSTs in humans by dietary factors are available. Apart from

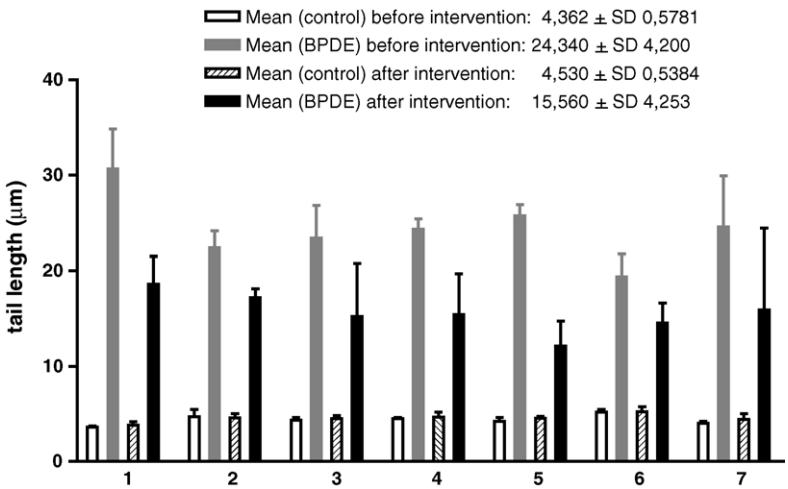

Fig. 6. Impact of coffee consumption on induction of DNA migration by BPDE in human lymphocytes. The participants consumed 1 L of unfiltered coffee per day over a period of 5 days, before and after the intervention, the cells were isolated and treated with BPDE (0.4 μM) or with the solvent (DMSO) only for 30 min. Subsequently, the viability and DNA migration were monitored. The survival of the cells was in all cases ≥80% (data not shown). Bars indicate mean ± S.D. of three cultures in parallel. From each culture, 50 cells were analysed for DNA migration. Numbers on the *x*-axis indicate individual participants. White bars: DNA migration before coffee consumption in control cultures, shaded bars: DNA migration in control cultures after coffee consumption, grey bars: DNA migration induced by BPDE before coffee consumption, black bars: DNA migration induced by BPDE after coffee consumption.

the coffee study by Grubben et al. [19] who did not find an effect on GSTs in colon mucosa after coffee consumption, all other investigations concerned the effects of vegetables. For example Nijhoff et al. [27,28] reported induction of GSTA with Brussels sprouts in males but not in females, more recently Lampe et al. [52] found induction effects by Brassica and Allium diets. Also with these vegetables, cancer protective effects were seen in animal experiments which were attributed to induction of GSTs [53].

The human glutathione biotransformation system consists of four major classes of GSTs namely GSTA, GSTM, GSTP and GSTT which differ in their tissue distribution and also in their substrate specificity [54]. In the aforementioned vegetable studies, the increase in overall GST was found in spectrophotometric assays with CDNB which is a substrate for different isozymes except GSTT [54]. Immunoassays showed induction of GSTA in the plasma [28,52] whereas GSTP levels were not altered [28], only in an experiment with intestinal biopsies, a significant induction of both isozymes was observed in rectal cells after consumption of Brussels sprouts [27]. In the present study, a different induction pattern was observed, i.e. the GSTA levels were not affected whereas a drastic increase in GSTP was observed.

GSTP is the most widely distributed enzyme of all GSTs and the most abundant form in many tissues except in the liver [55]. It is dominating for example in the lungs [56] and in the rectum [27], also in lymphocytes its activity is higher as that of other GSTs [54]. Approximately 50% of the Caucasians carry a mutation in the *GSTP* gene [40] and it is known that polymorphisms exist in humans in exons 5 and 6 which have functional effects on the gene product resulting in decreased enzyme activity [57–60]. A number of studies indicate that these polymorphisms are associated with increased risks for different forms of cancer in humans for example in the lung [61–64], breast [65,66], oesophagus [67], prostate [40,68], bladder [40], testes [40] and colon [69]. It is notable that some other studies failed to find correlations between GSTP polymorphisms and specific forms of cancer, for example no associations were seen in two other studies on colon cancer [61,70] and two Swedish investigations did not detect associations with prostate cancer incidence. Nevertheless, the overall evidence strongly suggests that the GSTP status has an impact on cancer risks in humans.

The observation that coffee consumption reduces BPDE induced DNA-damage was not unexpected. The protective effect of GSTP towards chemical carcinogens was shown earlier in a number of genotoxicity studies which indicated that this enzyme detoxifies representatives of different classes of DNA-reactive carcinogens. Ryberg et al. [62] found increased hydrophobic DNA-adducts in lung tissues of cancer patients (smokers) who had the GG phenotype. The GG phenotype (*GSTP1*-valin variant) causes formation of an altered enzyme protein which has lower affinity to CDNB, but a greater affinity towards other substrates (bromosulfophtalein and ethacrynic acid) [71]. In another study with smokers, no effect of *GSTP1* mutations on adduct formation in peripheral white blood cells was found, but in combination with *GSTM1* null genotypes a significant increase was detected [72]. No such combination effects were seen in a similar study in lung cells, but again in blood cells the adduct levels were increased in smokers with the *GSTM1* null genotype and *GSTP1* (Ile105/Val105 or Val105/Val105). These findings were explained by the fact that GSTP catalyses the detoxification of BPDE (the ultimate DNA-reactive metabolite of 3,4-benzo[*a*]pyrene (B(*a*)P) and other diols [73–75]). Consistent with these biochemical data it was shown that GSTP transfected cells are more resistant towards BPDE induced DNA-damage than normal cells [13–15] and enhanced skin tumorigenesis by 7,12-dimethylbenz[*a*]anthracene (DMBA) was found in knockout mice lacking class GSTP [76]. GSTP plays also an important role in protection towards other DNA-reactive carcinogens. In workers exposed to styrene oxide, a higher number of HPRT mutations was found in individuals with heterozygosity of the *GSTP1* gene [77]. These effects towards styrene oxide were further confirmed in in vitro micronucleus experiments with human lymphocytes [78]. Protective effects of GSTP were also reported against the heterocylic aromatic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in experiments with human liver and prostate cells [79,80], towards the UV mimetic compound 4-nitroquinoline-1-oxide (4-NQO), alkylating agents [81–84] and against different DNA-reactive anticancer drugs such as *cis*-platin, carbo-platin and thiotepa [85].

The results described in the last paragraphs indicate that GSTP plays an important role in the protection towards DNA-reactive carcinogens (in particular towards PAHs) and that altered activity (due to polymorphisms) is associated with increased cancer risks. The strong increase in the activity of this isozyme (two- to six-fold) seen in the present study supports the assumption that coffee consumption has beneficial effects in humans in terms of reducing their cancer risks and is supported by the results of the SCGE experiments which clearly show a protective effect of coffee consumption towards BPDE induced DNA-damage in peripheral human lymphocytes. It is notable, that we observed also pronounced enzyme induction effects in individuals who were heterozygous in the *GSTP1* gene. This indicates that the upregulation is not affected by base mutations in the *GSTP1* allele.

In the present study we were interested initially in overall GSTP induction effects in the body and not in the alterations of this enzyme in the lymphocytes. However, it is not possible to rule out on the basis of the present findings in which cells/organs induction effects take place. This question will be clarified in further animal studies which are in progress. It is known that extracellular GSTP induction may be due to toxic effects but since no alterations in the clinical parameters were seen and no changes of the viability of the lymphocytes before and after coffee consumption were observed, this possibility can be excluded. As mentioned above, strong protective effects towards BPDE were seen in the lymphocytes. Since it is known that GSTP protects against DNA-damage and that lymphocytes posses this enzyme, this can be taken as indication that GSTP induction takes also place intracellularly in the blood cells.

One of the mechanisms which may account for the GST induction is the increase in the glutathione concentrations by coffee consumption in plasma which was seen in two human intervention studies [19,86]. It is conceivable that higher co-substrate levels lead to an enhanced activity of the enzyme.

The results of the second intervention trial in which we compared the effects of unfiltered and paper filtered coffee on GSTP induction were unexpected. As shown in Fig. 3 no significant difference in GSTA induction was seen although the two types of coffee differed substantially in their diterpenoid contents due to different preparation procedures [29–33]. In many earlier studies, it was claimed that the DNA- and cancer protective effects of coffee as well as the induction of GST and other enzymes might be due to the coffee specific diterpenoids C + K [17]. However, it was also shown by Wattenberg and Lam [18] that C + K account only for 40% of GST (CDNB) inducing activity of coffee in the mouse liver and Esposito et al. [86] found induction of glutathione after consumption of coffees with low C + K contents. Since C + K leads to hypercholesteraemic effects [29,30] it is important that the increase in the cancer protective enzyme system was also seen after consumption of a coffee preparation which had low diterpenoid levels. In this context, it is notable that it has been postulated that, apart from C + K other coffee components, in particular polyphenols such as chlorogenic acids and melanoidins produced during the roasting process, may also account for GSH and GST induction [86–88].

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Review

Use of single cell gel electrophoresis assays for the detection of DNA-protective effects of dietary factors in humans: Recent results and trends

Christine Hoelzl [a], Siegfried Knasmüller [a,*], Miroslav Mišík [a], Andrew Collins [b], Maria Dušinská [c,d], Armen Nersesyan [a]

[a] Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Borschkegasse 8a, 1090 Vienna, Austria
[b] Department of Nutrition, Faculty of Medicine, University of Oslo, PB 1046 Blindern, 0316 Oslo, Norway
[c] Norwegian Institute of Air Research (NILU), Centre for Ecological Economics, P.O. Box 100, NO-2027 Kjeller, Norway
[d] Slovak Medical University, Department of Experimental and Applied Genetics, Limbová 12, 833 03 Bratislava, Slovakia

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ABSTRACT

This article summarises the results of human dietary intervention trials employing the comet assay (single cell gel electrophoresis, SCGE), which have been published in the last few years (i.e., between 2005 and 2008) and describes new trends and developments as well as current problems concerning the design of intervention trials and the interpretation of the results. Most new studies were carried out with complex plant derived foods and juices; only a few were conducted with individual food constituents. With specific vegetables, for example with water cress and Brussels sprouts, potent antioxidant effects were observed; also coffee caused a protective effect and it is notable that it was more effective than consumption of a diet containing increased levels of fruits and vegetables. Interesting recent developments include the development of protocols which enable us to monitor protection towards genotoxic chemicals contained in the human diet, and it was shown in preliminary studies that alterations of the activities of drug metabolising enzymes by dietary factors lead to altered sensitivity of lymphocytes against DNA damage caused by certain dietary carcinogens. Another novel approach is the development of methods to monitor the effects of dietary factors on DNA repair. The development of protocols for experiments with exfoliated buccal cells is another potentially valuable innovation. The adequate experimental design of SCGE trials is still a matter of debate and the evaluation of the available data shows that there is an urgent need to develop guidelines concerning the number of participants, sampling periods, duration of trials, use of placebos, and definition of adequate run-in and wash-out phases. Recent studies showed that the results of dietary studies could be biased by factors such as age, sex, body mass index and life style habits and by seasonal effects. Another still unsolved problem is the interpretation of the results of SCGE trials in regard to potential beneficial health effects. The use of –omics techniques may contribute to provide mechanistic explanations in addition to conventional approaches (such as enzyme measurements). Information on health effects of dietary factors and on prevention of diseases related to DNA damage can also be obtained in experiments with animals, using SCGE to detect decreases in DNA damage in inner organs.

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* Corresponding author. Tel.: +43 1 4277 65142; fax: +43 1 4277 9651.
E-mail address: siegfried.knasmueller@meduniwien.ac.at (S. Knasmüller).

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1. Introduction

Since the first use of the single cell gel electrophoresis (SCGE, or comet assay) technique in an intervention trial with vitamins in 1996 by Duthie and co-workers [1], this method has increasingly been employed to investigate the impact of dietary factors on DNA stability in humans as it is less labour-intensive and costly than other methods such as chromosomal aberration analysis or the micronucleus assay. Briefly, cells (such as peripheral lymphocytes) are embedded in agarose on a microscope slide, and lysed with detergent and 2.5 M NaCl to remove membranes and soluble cytoplasmic and nuclear constituents, including most histones. This leaves nucleoids, in which the DNA is still attached to the nuclear matrix and supercoiled. Electrophoresis at high pH (>13) draws the DNA towards the anode – but only if its supercoiling has been relaxed by DNA breaks. Thus comet like structures (viewed by fluorescence microscopy) are formed, in which the relative intensity of the comet tail reflects the number of relaxed DNA loops and therefore the number of strand breaks (including alkali-labile sites) in the DNA.

To date, results of 84 intervention trials using SCGE have been published. As this technique may be useful for the justification of health claims made on foods that are requested by authorities in the USA and in Europe it has gained importance in the last years [2–4]. A recent comprehensive review evaluating methods which are currently used to monitor antioxidant effects of dietary factors led us to the conclusion that the SCGE assay and isoprostane measurements are among the most reliable approaches [5].

Møller and Loft [6–8] evaluated and summarised in several reviews the results of earlier human SCGE studies. In the present article, we give a short overview on recent investigations (not included in their papers) and describe new developments, i.e., protocols which enable the identification of food components that interact with repair processes, attempts to use indicator cells other than peripheral blood lymphocytes and the design of modified versions of the SCGE assay which provide information on protective effects of dietary factors towards representatives of different classes of food-related DNA-reactive carcinogens. The last paragraphs concern the experimental design of SCGE trials and describe further perspectives of their use in food research.

2. Conventional SCGE trials with complex foods and individual food constituents—an update

The majority of investigations have been carried out according to protocols developed by Collins and co-authors (for reviews see [9,10]) either with untreated peripheral lymphocytes under standard electrophoresis conditions, after exposure of the cells to reactive oxygen species (ROS), or after treatment of nuclei with lesion-specific enzymes. Comets which are formed under standard conditions reflect endogenous DNA damage (single and double strand breaks, apurinic/apyrimidinic sites) whereas experiments with the lesion-specific enzymes endonuclease III (EndoIII) and formamidopyrimidine DNA glycosylase (FPG) provide information on endogenous formation of oxidised pyrimidines and purines, respectively. The yield of DNA breaks after exposure of intact cells to ROS (either by treatment with hydrogen peroxide or other ROS-forming chemicals or by exposure to ionizing radiation) is indicative for changes of the sensitivity of the cells towards exogenous oxidative DNA damage [11] and thus gives information on antioxidant status.

In approximately half of the studies conducted so far, a significant decrease in the formation of oxidised bases and/or protection towards ROS-mediated DNA damage was seen, while only in one third a decrease in DNA migration was found under standard conditions (i.e., measuring endogenous DNA breaks alone, Fig. 1).

It can be seen in Fig. 2 that the overlap of the different endpoints is quite poor, i.e., only in about 10% of the studies a significant protective effect was detected in all parameters.

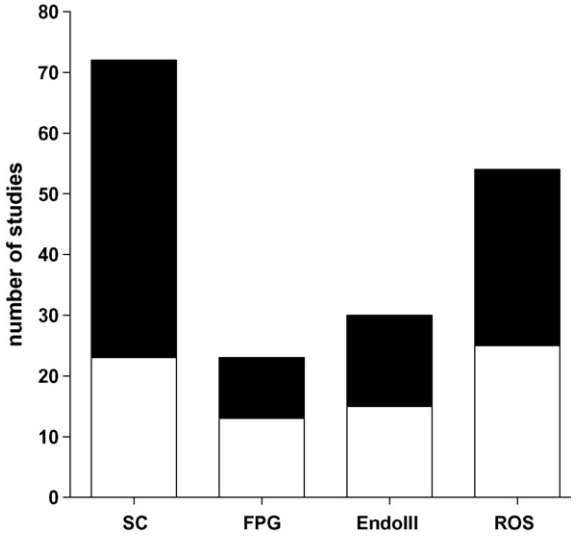

Fig. 1. Evaluation of results obtained in SCGE experiments in human dietary intervention trails. SC: standard conditions, ROS: damage induced by reactive oxygen species (H_2O_2 treatment or radiation); white bars: positive results, grey bars: negative results.

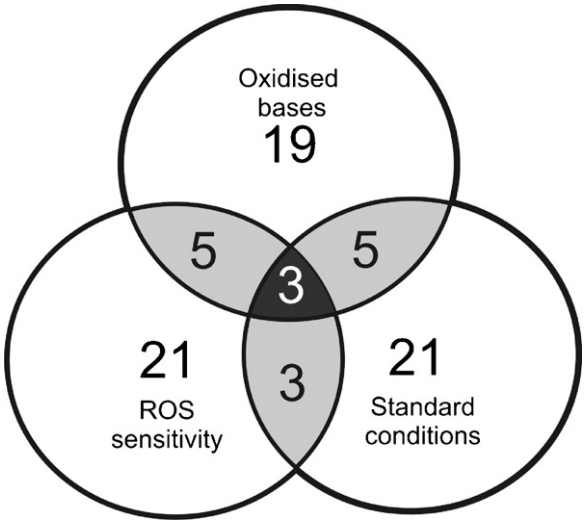

Fig. 2. Overlap of positive results obtained in different endpoints. In total, 32 studies were found in which DNA-migration was measured under standard conditions, after ROS challenge and also treatment of the nuclei with EndoIII, FPG or with both enzymes.

Table 1
Results of human intervention trials, which were published since 2005

Dietary factor	Design of the study	SCGE results[a]	Remarks	Ref.
Complex foods				
Almond consumption 84 g/p/d	Randomised, crossover $n = 60$ ♂ smokers, control $n = 30$ ♂ non-smokers 4 w intervention, 4 w wash-out period Sampling: weeks 0 and 4	%DNA ↓ SC (+)	Serum: α-tocopherol (+), ↑ SOD (++), ↑ GPX (+) (only in smokers)	[27]
Almond consumption 84 g/p/d or 168 g/p/d	Sequential $n = 20$ ♂ smokers, 10 in each group, control $n = 10$ smokers 4 w intervention Sampling: weeks 0 and 4	%DNA ↓ SC (++) in high consumption group only	Plasma: ↔ SOD, ↔ GPx, ↓ MDA (++) and (+++) in low and high consumption groups Urine: ↓ 8-OH-dG (+++) in both group	[40]
Apples 1000 g/p/d either organically or conventionally produced	Double-blind, randomised, cross-over $n = 6$ ♂ 3 d run-in, 1 d intervention, 1 w wash-out Sampling: 0 h, 1–6, 9, 12 and 24 h	%DNA ↔ SC, ↔ FPG ↓ EndoIII (+++) ↓ $FeCl_3$[b] (++) ↔ H_2O_2	Plasma: ↔ antioxidant capacity of low-density lipoproteins	[28]
Brussels sprouts 300 g/p/d	Sequential $n = 8$ ♂+♀, 5 d run-in, 6 d intervention Sampling: 0 d, 6 d	TL ↔ SC, ↓ H_2O_2 (++) ↓ FPG (++), ↓ EndoIII (++)	LY: ↓ SULT1A1 (++, which activates PhIP), ↔ GPx, Plasma: ↑ vitamin C (++)	[18]
Raw watercress 85 g/p/d	Single-blind, randomised, cross-over $n = 30$ ♂ + 30 ♀ 8 w intervention, 7 w wash-out Sampling: weeks 0, 8, 15 and 23	%DNA ↓ SC (+), ↓ FPG (+) ↔ H_2O_2	Beneficial changes were significantly higher in smokers than in nonsmokers; Plasma: ↑ lutein (+++) and ↑β-carotene (++)	[19]
Sumach (*Rhus coriaria* L) 3 g/p/d	Placebo- controlled, randomised $n = 16$ (8 sumach, 8 placebo) 3 d intervention Sampling: 0 d, 3 d	TL ↓ SC (+), ↓ FPG (++) ↓ EndoIII (++) ↓ H_2O_2 (++)	Plasma: ↑ SOD (+), ↓ GPX (+), ↑ GST (++) ↑ GST-pi (+)	[31]
Virgin olive oil (high phenol extra virgin) 50 g/p/d (equals 30 mg phenols/p/d)	Randomised cross-over (high phenol oil vs low phenol oil) $n = 10$ ♀, post-menopausal 8 w intervention, 8 w wash-out Sampling: 2 w (5 times first period, 5 times second period)	%DNA High phenol oil: ↓ SC (++) ↓ FPG (++) ↔ H_2O_2	High phenol oil: Plasma: ↔ antioxidant capacity ↑ (+++) Olive oil phenolics in urine (hydroxytyrosol, homovanillyl alcohol)	[32]
Wheat sprouts 70 g/p/d	Sequential $n = 13$ ♂ + ♀ 5 d run-in, 5 d intervention, 10 d wash out Sampling: 0 d, 5 d, 10 d	TL ↔ SC ↓ FPG (++)	In parallel CBMN assay in LY was carried out ↓ Apoptosis (+), ↔ MN, ↔ NPB ↔ Nbud, ↔ necrosis, ↔ NDI	[37]
Beverages				
Breast or cow's milk	Sequential $n = 35$ ♂+♀ breast milk fed, $n = 35$ ♂+♀ 6 m intervention Sampling: at age 10 m	Arbitrary units ↓ SC (++) in breast milk fed		[39]
Coffee 600 ml/p/d (150 ml metal filtered + 450 ml paper filtered)	Sequential $n = 9$ ♂ + ♀ 5 d run in, 5 d intervention Sampling: 0 d, 5 d	TL/TM ↔ SC ↓ FPG (++) ↓ EndoIII (++) ↓ H_2O_2 (+)	LY: ↑ SOD (++), ↔GPx	[12]
Coffee 600 ml/p/d (200 ml metal filtered + 400 ml paper filtered)	Sequential $n = 8$ ♂ + ♀ 5 d run-in, 5 d intervention Sampling: 0 d, 5 d	TL ↔ SC ↓ FPG (++) ↓ EndoIII (++) ↓ H_2O_2 (++)	LY: ↑ SOD (++), ↔ GPx	[13]
Coffee 1 l/p/d Metal filtered	Sequential $n = 7$ ♂+♀, 14 d run in, 6 d intervention Sampling: 0 d, 6 d	TL ↔ SC	Plasma and salvia: ↔ overall GST Plasma: ↑ (+++) GST-pi, ↔ plasma GST-α	[14]

Table 1 (*Continued*)

Dietary factor	Design of the study	SCGE results[a]	Remarks	Ref.
Juices				
Blueberry/apple juice 1000 ml/p/d	Sequential n = 168 (54 ♂+114 ♀) 5 d wash-out, 4 w intervention Sampling: 0 d and 28 d	TM ↓ H_2O_2 (+) ♂ Stronger effect than ♀	Plasma: ↑ quercetin (+++), ↑ (+) vitamin C and ↑ TEAC (+)	[29]
Blood orange juice 300 ml/p/d or drink supplemented with the same amount of vitamin C (150 mg), or sugar drink (control)	Sequential n = 7 ♀ received each drink on 3 different occasions 2 w apart Sampling: every h till 8 h and 24 h after the intake of each drink	LC/%DNA ↔ SC ↓ H_2O_2 (+)	Plasma: ↑ (++) vitamin C content after orange and supplemented drink till 8 h after consumption	[17]
Red mixed berry juice (rich in polyphenols TEAC 19:1) 700 ml/p/d	Placebo-controlled parallel n = 27 ♂ (treatment:18, placebo: 9) 3 w run-in, 4 w intervention or placebo, 3 w wash out Sampling: 0 d, after each week, from 1 to 9	LC/%DNA ↓ SC (++) ↓ FPG (++)	Plasma: ↔ malondialedehyde Blood: ↑ (+) total glutathione, ↑ (+) reduced glutathione, ↔ oxidised glutathione	[16]
Tomato-drink (rich in caroteniods) 250 ml/p/d	Double-blind, placebo-controlled cross-over n = 13 ♂+13 ♀ 26 d placebo, 26 d intervention, 26 d wash-out Sampling: 26 d per phase (placebo/wash out/intervention)	%DNA ↔ SC	Blood: ↑ (+) INF-γ after placebo intake, ↔ after tomato drink, ↓ (++) TNF-α	[33]
Individual food components				
Carotenoids: lutein, lycopene or ß-carotene 12 mg/p/d of each or 4 mg each in a mix/p/d	Blinded, placebo-controlled parallel n = 37 ♀, postmenopausal, 57 d intervention Sampling: 0 d, 15 d, 29 d, 43 d, 57 d	VIS ↓ SC (+) ↔ H_2O_2	Plasma: ↑ (++) caroteinoids	[38]
Coenzyme Q 3 mg/kg/p/d	Sequential n = 10 ♀ 28 d intervention Sampling: 0 d, 14 d, 28 d, 12 w	TM ↓ FPG (+)	Plasma: ↓ (+) redox-status, ↑ coenzyme Q (+++), Platelets: ↓ (+) redox-status, ↑ coenzyme Q (+), White blood cells: ↑ coenzyme Q (+)	[30]
Gallic acid (GA), 0.2 mg/kg/kg/p/d	Sequential, placebo-controlled, randomised 3 d intervention n = 16 ♂+♀ (8 GA, 8 placebo) Sampling: 0 d, 3 d	TL ↓ SC (+), ↓ (++) FPG ↓ (++) EndoIII ↓ (++) H_2O_2	Plasma: ↑ SOD (++), ↑ GPX (+), ↔ GST, ↑ GST-pi (+++)	[20]
Vitamins C and E 500 + 300 mg/p/d	Sequential n = 17 ♂+♀ exposed to anaesthetic gases, n = 19 ♂+♀ control 12 w intervention Sampling: 0 d, 12 w	VIS ↓ (++) SC	No other investigations	[34]
Vitamins C and E 9 pts – vit E 400 mg/p/d, 9 pts – vit E 200 mg/d, 9 pts – vit C 250 mg/d, 8 - placebo	Randomised placebo-controlled n = 27 ♂ and 9 ♀, pts with COPD 12 w intervention Sampling: 0 d, 12 w	LC/%DNA ↔ SC ↓ (++) H_2O_2	Plasma: ↔ TBARS	[35]
Vitasay, complex of 10 vitamins + 10 minerals	Double-blind randomised 4 w intervention n = 80 (40 ♂ + 40 ♀) Sampling: weeks 0 and 4	%DNA ↓ SC ↓ H_2O_2	No effect of age and gender	[36]

Abbreviations: d, days; COPD, chronic obstructive pulmonary disease; EndoIII, endonuclease III; FPG, formamidopyrimidine glycosylase; GPx, glutathione peroxidase; GST, glutathione-S-transferase; INF-γ, interferon-γ; LC, leucocytes; L, liter; m, months; Nbud, nuclear buds; NDI, nuclear division index; NPB, nucleoplasmatic bridges; p, person; pts, patients; SC, standard conditions of the comet assay (single strand breaks; double strand breaks; alkali-labile sites); SOD, superoxide dismutase; SULT –sulfotransferase; TEAC, trolox equivalent antioxidative capacity; TL, tail length; TM, tail moment; VIS, visual score of comets; TNF-α, tumor necrosis factor α; w, weeks; ↓ decrease; ↑ increase; ↔ no alteration; + either increase or decrease of 25%; ++ either increase or decrease less than 50%; +++ either increase or decrease less than 75% compared to control.

[a] Unless otherwise indicates, lymphocytes were used as indicator cells.

[b] $FeCl_3$ was used to induce oxidative damage of DNA.

We also evaluated the correlation between studies in which formation of oxidised purines and pyrimidines were evaluated in parallel. In total, 16 studies were identified, and in 50% of them, protection was seen in both endpoints.

The results of 20 newer studies are summarised in Table 1. One of the interesting findings is the observation of a significant protective effect of coffee consumption [12–14] (600–1000 ml/day, 5–7 days), and it is notable that no protective effects were seen in earlier trials after consumption of 600 g of a mixed fruit/vegetable diet [15] or consumption of fruit/vegetable juice [16,17]. Newer studies with cruciferous vegetables [18,19] yielded evidence for strong protective properties; and gallic acid, which occurs in specific plant foods (e.g., mangos and rhubarb), was also extremely effective [20,21]. Comparisons with the results obtained in human studies with vitamin C under similar conditions showed that gallic acid is more active in regard to prevention of oxidative damage [20]. These results suggest that only certain plant derived foods and beverages demonstrate protective effects as indicated by the comet assay. It is notable that Giovannelli et al. [22] reported on the basis of a questionnaire-based study that coffee consumption is associated with increased DNA migration attributable to FPG lesions. However, in the same study also lycopene consumption was found to cause DNA damage due to formation of oxidised purines, and it is likely that the outcome of the study was biased by confounding factors as tomatoes as well as lycopene were found to be active in a number of earlier controlled intervention trials in which the SCGE technique was used [8]. Furthermore, the observation of protective effects of coffee consumption towards FPG-induced lesions could be confirmed in two large follow-up studies (forty individuals per study) with different types of coffee (Knasmüller et al., unpublished). However, it is also notable that coffee may also cause adverse effects, for example, an increase of DNA damage induced by the heterocyclic aromatic amine PhIP was seen in a small trial which may be due to its interaction with enzymes involved in the activation of this amine [21].

Other potential adverse effects of coffee may be increased risks for cardiovascular diseases and increased probability of spontaneous abortions or impaired fetal growth [23]. As described in a recent review [24], it may be possible to design coffees with increased beneficial health effects on the basis of the identification of the specific components which account for its antioxidant and DNA-protective effects which were not only found in human trials but also in a number of *in vitro* studies [25,26] (see Table 1 [12–14,16–20,27–40]).

3. Correlations with other markers of damage

In several SCGE experiments additional parameters that are indicative for antioxidant effects were measured. Overall, the findings are inconsistent: in a study by Bub et al. [41] with fruit juices, the thiobarbituric acid reactive substance (TBARS) level was significantly reduced in plasma whereas no changes were seen in ferrous oxidation of xylenol orange (version 2, FOX2) and ferric-reducing antioxidant power (FRAP) measurements. Weisel et al. [16] investigated the effect of an anthocyanin-rich juice and found protection in SCGE assays and increased glutathione levels but no impact on plasma malondialdehyde (MDA) concentrations or on urinary isoprostane excretion. In a trial with olive oil, DNA migration was reduced when the experiments were conducted under standard conditions and with FPG and the effects were paralleled by an increase of the total antioxidant capacity (TAC) of plasma [32].

In some of the studies, the impact of dietary factors on the activities of antioxidant enzymes was monitored. For example, the activity of superoxide dismutase (SOD) was found to be elevated after consumption of sumach [31], Brussels sprouts [42], coffee [13] and gallic acid [20,21], whereas no alterations of the glutathione peroxidase activity (GPx) were observed in any of these trials.

Another relevant marker of DNA oxidation that has been used in a number of human studies is the excretion of 8-oxoguanine (8-oxoGua) in urine. According to our knowledge, no results are available from intervention trials in which the urine levels of oxidised deoxyguanosine and lymphocyte comets were comparatively evaluated. However, Gedik et al. [43] compared in a small study the occurrence of 8-oxoGua in urine and in lymphocytes (by HPLC) and FPG-induced comet formation in lymphocytes. Overnight urinary 8-oxoGua concentrations correlated both, with the overall 8-oxoGua levels in lymphocytes measured by HPLC, and with FPG-sensitive sites in these cells, although individual values of DNA base oxidation from HPLC and the comet assay did not correlate with each other.

Dotan et al. [44] compared the results of numerous studies in which different endpoints of oxidative stress were measured in parallel. They concluded that different markers of lipid peroxidation correlate well among themselves (e.g., F2-isoprostanes, MDA and lipid hydroperoxides), but failed to find correlations between these parameters and DNA migration. A recent biomonitoring study [45] on 141 subjects (43 controls and 98 exposed) also demonstrated no correlation of MDA levels with DNA strand breaks or oxidative DNA damage although there was a positive correlation of DNA damage with cholesterol and triglycerides.

On the other hand, this study revealed a strong inverse correlation of oxidised purines and pyrimidines with glutathione S-transferase activity, that was significant in almost all groups and subgroups. Associations of oxidative DNA damage with plasma concentrations of antioxidant vitamins (α- and β-tocopherol, β-carotene and retinol) were also found.

In a recent comprehensive evaluation of techniques used to monitor antioxidant effects of food components, we suggested that methods which enable the detection of oxidative DNA damage (i.e., DNA migration and also other endpoints such as 8-OH-dG measurements) may be in particular highly relevant since DNA damage is considered to be a key mechanism which is associated with the aetiology of a number of diseases. Other potentially valuable endpoints are probably the measurement of oxidised LDL and of isoprostanes levels which are also considered to be related to risks of cardiovascular disorders (for review see Knasmüller et al., 2008 [5]).

4. Use of SCGE trials to detect protection against DNA-reactive carcinogens

Humans are exposed to a broad variety of genotoxic carcinogens either via environmental contamination or by consumption of foods which contain various groups of DNA-reactive carcinogens [46]. Over the last few years, we attempted to develop protocols for SCGE experiments that can be used to detect alterations of the sensitivity of lymphocytes towards specific genotoxic carcinogens.

DNA-reactive carcinogens which can be used in experiments with human lymphocytes are for example heterocyclic aromatic amines (PhIP; 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; IQ; 2-amino-3-methylimidazo[4,5-*f*]-quinoline, MeIQx; 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; AαC—2-amino-9H-pyrido[2,3-*b*]-indole; Trp-P-2; 3-amino-1-methyl-5H-pyrido-[4,3-*b*]indole), thermal degradation products (acrylamide, furan, glycidamide), metals (V^{5+}, Cd^{3+}, Cr^{6+}), methylating agents (methyl methanesulfonate, *N*-methyl-*N*-nitrosourea, *N*-methyl-*N*-nitroso-dimethylamine) and also other compounds such as the mycotoxin ochratoxin A or the cytostatic drug cyclophosphamide (for a complete list see Nersesyan et al. [21]).

Table 2
Results of human intervention trials in which alterations of the sensitivity of peripheral lymphocytes towards DNA-damage induced by different carcinogens were investigated

Model carcinogen/treatment	Intervention trial	Result	Ref.
BPDE (0.40 μM, 30 min)	Coffee (unfiltered) 1.0 l/d, 5 d *n* = 7	↓ (45%) TL ↔ Plasma and salvia overall GST ↑ (206%) plasma GST-pi ↔ Plasma GST-α	[14]
	Sumach 3.0 g/d, 3 d *n* = 4 ♂ + 4 ♀	↓ (59%) TL ↑ (85%) plasma GST-α ↑ (39%) total GST ↑ (48%) GST-pi	[31]
Trp-P-2 (0.20 mM, 30 min)	Brussels sprouts 300 g/d, 6 d *n* = 4 ♂ + 4 ♀	↔ TL ↓ (23%) SULT1A1 (which activates PhIP) ↔ SOD, ↔ GPx ↑ (37%) plasma vitamin C	[18]
	Coffee (metal filtered) 600 ml/d, 5 d, *n* = 4 ♂ + 4 ♀	↓ (35%) TL ↑ (38%) SOD	[13]
PhIP (0.70 mM, 30 min)	Brussels sprouts 300 g/d, 6 d *n* = 4 ♂ + 4 ♀	↓ (97%) TL ↓ (23%) SULT1A1 (which activates PhIP) ↔ SOD, ↔ GPx ↑ (37%) plasma vitamin C	[18]
	Coffee (metal filtered, French press) 600 ml/d, 5 d, *n* = 4 ♂ + 4 ♀	↑ (22%) TL ↑ (38%) SOD	[12]
MNU (0.55 mM, 30 min)	Coffee (metal filtered, French press) 800 ml/d, 5 d *n* = 5 ♂ + 10 ♀	↓ (19%) TL	[21]
DMNA (0.40 mM, 30 min)	Coffee (metal filtered, French press) 800 ml/d, 5 d *n* = 5 ♂ + 10 ♀	↓ (23%) TL	[21]

Abbreviations: BPDE, benzo(a)pyrene-7,8-diol-9,10-epoxide; DNMA, *N*,*N*-dimethyl-*N*-nitrosoamine; MNU, *N*-methyl-*N*-nitrosourea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; TL, tail length; Trp-P-2, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole; SULT, sulfotransferase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GST, glutathione S-transferase.

The activities of phase I enzymes which are required for the activation of procarcinogens are quite low in peripheral lymphocytes [47], therefore negative results are obtained with compounds such as benzo(a)pyrene and also with the mycotoxin aflatoxin B_1. In order to detect protective effects towards these carcinogens, it is possible to use their DNA-reactive metabolites, for example benzo(a)pyrene-7,8-diol-9,10-epoxide (BPDE) [48]. In the case of aflatoxin B_1, its DNA-reactive metabolite (the *exo* isomer of AFB_1 8,9-epoxide) is highly unstable [49], but it is possible to activate the mycotoxin by preincubation with exogenous liver homogenate (S9 mix) [50]. Also heterocyclic aromatic amines (HAs) can be activated with this procedure [51], but we, as well as others [52,53] showed that positive effects can be obtained in lymphocytes also with high concentrations of the parent compounds.

Table 2 summarises the results of intervention studies with selected genotoxic carcinogens which we conducted in recent years. It can be seen, that in some cases protective effects were observed which could be explained by alterations of the activities of activating and/or detoxifying enzymes. For example, the attenuation of DNA damage by PhIP after consumption of Brussels sprouts could be attributed to inhibition of sulfotransferase (SULT1A1) that is required for the activation of this heterocyclic aromatic amine [18]. Interestingly, no protective effect was observed in the same study towards Trp-P-2, which is structurally related but does not require activation by SULT [18]. On the contrary, a significant increase of PhIP-induced DNA migration was observed in a recent coffee study (consumption of 600 ml/d during 5 days) [12]. The reason for this adverse effect may be the induction of enzymes such as CYP1A1 and CYP1A2 which was found in earlier experiments with rats by Huber et al. [54]. In the same intervention study, changes of the sensitivity of the cells towards the alkylating agent methyl methanesulfonate were also found, whereas no clear effect was seen with dimethylnitrosamine. It was shown recently in animal experiments that the coffee diterpenoids kahweol and kafestol induce the activity of the enzyme O^6-methylguanine DNA methyltransferase which removes methyl groups from guanosine [54], and it is possible that the induction of this repair enzyme may account for the protective effect towards methyl methane sulfonate [12].

5. Effects of dietary interventions on DNA repair

In 2001, Collins et al. [55] published a protocol for an SCGE-based *in vitro* assay for repair of oxidative DNA damage, which can be applied to human lymphocytes collected in dietary intervention studies. Cell free extracts of lymphocytes are incubated with agarose-embedded nucleoids from cells previously treated with photosensitiser plus light to induce elevated levels of 8-oxoGua. Accumulation of breaks resulting from incision at oxidised bases by the repair enzyme 8-oxoguanine DNA glycosylase (OGG1) leads to increased comet formation. In two human intervention studies, one with coenzyme Q10 [56], and one with kiwifruit [57], an increase of the DNA repair capacity was seen. Recently we found a similar effect in the Oslo Antioxidant Study, in which volunteers took a diet high in antioxidant-rich foods for several weeks [58]. In the kiwifruit study, mRNA levels were measured for OGG1 and also for the enzyme that follows OGG1, AP endonuclease 1 (APE1); there was no sign of a change in gene expression being responsible for the increased enzyme activity. Also several other studies with dietary components failed to find an increase of OGG1 at the

transcriptional level, but in general it seems to be the case that differences in OGG1 enzyme activity are poorly reflected in gene-expression levels [59].

More recently we developed an analogous assay for nucleotide excision (NER) repair, in which UV-irradiated cells provide the substrate DNA and are currently applying this protocol in human nutritional studies [60]. The further validation of the different repair protocols is currently under progress.

It is theoretically possible to monitor cellular repair more directly, by treating cells with DNA-damaging agents and incubating them in medium at 37 °C, measuring the residual damage at intervals. In an early experiment [61], we gave volunteers 1 g of vitamin C and, 2 h later, isolated lymphocytes; and incubated them upto 24 h. Rejoining of strand breaks in lymphocytes is slow, taking several hours (compared with minutes in standard cultured cell lines such as HeLa). Eight volunteers varied in their response to vitamin C. Two, who showed the largest increase in plasma concentration, also showed an apparent acceleration of repair, whereas the others did not. We also performed similar trials with β-carotene (waiting 24 h after supplementation, since it is slowly absorbed compared to vitamin C) [62]. Again, the responses varied, but cells from some volunteers appeared to rejoin breaks more quickly if isolated after the β-carotene supplementation.

However, we later concluded [63] that this apparent stimulation of repair probably has another explanation. Immediately after isolation, the blood cells show a transient increase in strand breaks (in the absence of H_2O_2) that might reflect the effect of sudden exposure to atmospheric oxygen. In the case of cells treated with H_2O_2, a continuing input of oxidative damage, working against the removal of the H_2O_2-induced breaks, would give an impression of slow repair; and carotenoids or vitamin C, by protecting against atmospheric oxygen, would appear to speed up repair. Lymphocytes show much faster rejoining if incubated for 24 h before challenging with H_2O_2 [64]; but we have not pursued this approach to the nutritional modulation of repair, since the experimental results are hard to interpret and the *in vitro* approach seems more reliable.

6. Indicator cells and media

The majority of investigations (≥90%) were carried out with peripheral lymphocytes whereas only in a few studies leucocytes were used.

When cells are treated with ROS-generating chemicals or radiation they are in most cases kept in artificial media. However, it is also possible to conduct experiments in which cells are maintained in the plasma of the donors. The latter protocol detects extracellular ROS-scavenging effects caused by a dietary factors, whereas the first reflects changes of intercellular ROS defence system and both approaches should be used in human studies in order to obtain a more complete picture [21].

In a few intervention trials, buccal cells have been used in SCGE experiments. Szeto et al. [65] described recently the development of an improved protocol in which agarose embedded cells of epithelial origin from the mouth were digested with trypsin and proteinase K. In the same study, a small intervention trial with carotene rich berry juice was included and a significant reduction of comet formation was observed. According to these authors [65] it is not possible to use an earlier protocol developed by Rojas et al. [66] as it leads to extremely high background levels.

Another approach was followed by Osswald et al. [67] who isolated lymphocytes from suspensions of cells collected from the mouth and developed a technique for SCGE analyses which was used successfully in an intervention trial with supplemented bread by Glei et al. [68]. More recently, it was shown by Kleinsasser and co-workers [69,70] that it is possible to establish mini organ cultures with oral mucosal cells and use them to study the DNA-damaging effects of dietary genotoxins in SCGE trials. This approach may be also useful to monitor protective effects of dietary compounds.

One of the advantages of the use of buccal cells is that they can be collected with non-invasive methods. Since more than 90% of human cancers arise from epithelial cells it has been postulated that experiments with these cells may have particular relevance for the detection of cancer preventive effects [71]. In this context it is also notable that micronucleus assays with exfoliated oral cells have been successfully used to predict health risks due to occupational exposure, life style factors and also to identify protective factors in the human diet [72–74].

Table 3
Design of human intervention trials[a]

Design	Sequential (%)	Crossover (%)
Simple	28 (33.3)	4 (4.9)
Placebo	27 (32.1)	2 (2.5)
Run-in period	5 (6.0)	–
Wash-out period	6 (7.1)	7 (8.4)
Placebo + run-in	1 (1.1)	–
Placebo + wash-out	1 (1.1)	1 (1.1)
Run-in + wash-out	1 (1.1)	–
Placebo, run-in + wash-out	1 (1.1)	–
Total	70 (83.3)	14 (16.7)

[a] In total 84 studies have been published.

7. Experimental design of human studies

One of the most problematic issues of human SCGE trials concerns the adequate design of the studies. Table 3 shows that a broad variety of different protocols has been used in earlier investigations.

The experimental design of intervention trials has been critically discussed by Møller and Loft [6], who attempted to define criteria for the quality of such studies. They stressed that the experiments should have a placebo-controlled, parallel design. However, in studies with fruits and vegetables, it is problematic to design adequate placebos. A crossover design, in which the subjects are randomly split into two (or more) groups, receiving supplement(s) or baseline diet in different orders, allows for the possibility of 'seasonal changes' affecting results, as the groups (control diet and supplemented) are compared at the same time. Sequential and crossover trials have the advantage that the subjects can be used as their own controls, which reduces the interindividual variations of the study.

In studies with a crossover design, it is important to include wash-out periods between the different phases. Møller and Loft [6] emphasise that positive results of some poorly controlled earlier studies may have been biased by seasonal variations or by changes of the lifestyle of the subjects over time. An evaluation of currently available reports shows that approximately 70% of all investigations published so far lasted less than 4 weeks. Therefore, it is unlikely that such changes would have had a substantial impact on the results of these trials. However, in longer studies such effects may be relevant; for example, Verschaeve et al. [75] found higher basal values of DNA damage (strand breaks) in summer than in other seasons in a Belgian study, whereas Smolkova et al. [76] failed to detect such effects in Slovakia.

The time factor can be excluded in 'ecological' or questionnaire-based studies in which the aim is to seek correlations between general nutritional habits and DNA damage. Krajcovicova-

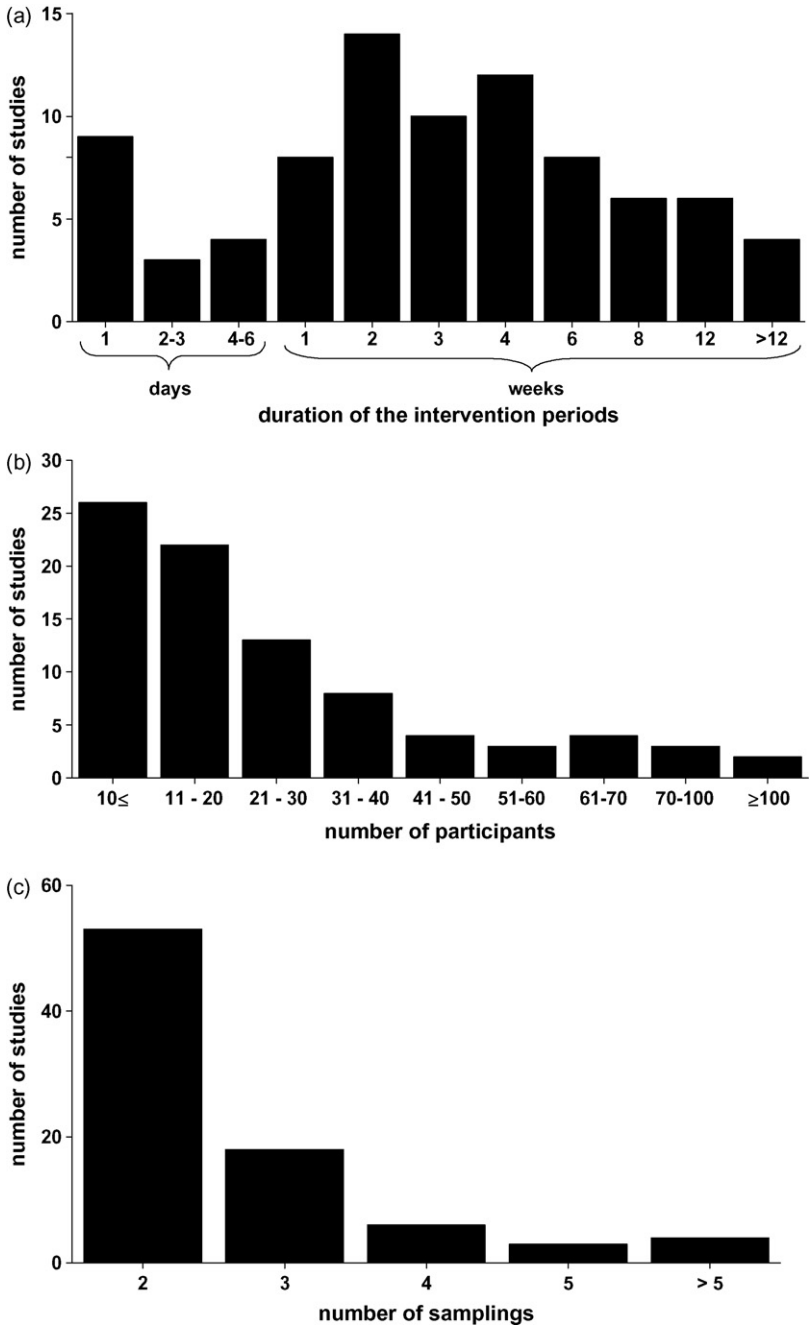

Fig. 3. (a–c) Experimental design of intervention trials. The bars are based on the evaluation of results published in peer-reviewed journals between 1996 and 2008. (a) Number of studies different intervention periods; (b) number of participants in intervention trials; (c) number of blood samplings.

Kudlackova et al. [77] published recently an interesting paper in which they found more oxidative DNA damage in older women than in young ones and lower levels of DNA damage in older vegetarians compared to non-vegetarians. The same group reported also a clear protective effect of consumption of fruits, vegetables and cereals towards formation of oxidised purines [78]. Giovannelli et al. [22] found correlations between both coffee consumption and tomato intake and the formation of oxidised purines—contrasting with intervention studies in which inverse associations were seen [13,21].

In a number of experiments [13,16,18,37,79,80] run-in-phases were included in which the participants consumed a controlled diet and did not consume the items under study. We strongly recommend the use of such phases in which the intake of dietary factors that are known to cause protective effects is controlled and in which the participants are asked to avoid physical exercise that may lead to comet formation [81,82]. Also wash-out periods will increase the quality of intervention studies, but the definition of their duration is problematic. In this context it is notable that Bub et al. [41] showed that the reversal of protective effects of a polyphenolics enriched diet takes several weeks. Also the duration of the intervention period itself is problematic and Fig. 3 shows that the periods used in previous studies vary over a broad range.

It can be expected that direct acting antioxidants cause effects already after few hours whereas compounds that act via induction of protective enzymes require longer periods to elicit protective effects. In the case of phenolics it was shown that the decrease in oxidative DNA damage occurs with some delay [83]. Boyle et al.

[84] studying the effects of a meal of fried onions, found a peak of plasma flavonoids at 4 h, whereas DNA oxidation reached the lowest level 4 h later. Multiple sampling which was used in a few of the studies [8,16,38,85,86] may help to solve the problem of defining the ideal intervention time, but usually financial limitations as well as the capacity of the laboratories are limiting factors. The number of participants that should be included in intervention trials is a further crucial question. Fig. 3 shows the numbers of participants enrolled in previous intervention trials. An adequate strategy to determine the ideal number of subjects is the calculation of the statistical power. Møller and Loft [8] compared the number of participants required to detect a 50% reduction of DNA migration on basis of earlier studies and found extremely strong variations, i.e., between 14 and 49 individuals.

The quality of intervention trials can be increased when factors are taken into consideration which may affect their outcome such as age [8,87,88], gender [87,89–91], body weight [92], seasonal effects [75,93], the lifestyles of the participants (nutritional habits, alcohol and tobacco consumption) [22,94–96] and physical activity [97], and also the latitude [89] and ethnicity may affect the results of SCGE trials [98]. According to a recent analysis by Møller [89] the most important factor is the age.

Most dietary studies were carried out with healthy individuals. However, some of the newer investigations were conducted with participants who suffered from diseases associated with oxidative stress (such as diabetes and renal failure) or had increased oxidative DNA damage due to physical exercise [99–103]. The results of some of these studies indicate that protective effects of specific dietary factors are more pronounced in individuals under oxidative stress; for example, Glei et al. [68] found protective effect with supplemented bread in smokers but not in non-smokers.

8. Unsolved problems and future perspectives

As described above, one of the problems associated with the use of SCGE assays in human intervention trials aimed to detect dietary protective factors concerns their experimental design. No efforts have been made so far to establish an international consortium which develops and validates appropriate strategies. The activities of the European Standards Committee on Oxidative DNA Damage (ESCODD) concerned primarily the standardisation of 8-oxoGua measurements, whereas groups concerned with the development of standardised SCGE guidelines [104–106] have addressed the problems associated with the adequate design of animal and *in vitro* studies, rather than human dietary intervention studies.

Recommendations on how to carry out human studies using the comet assay to monitor DNA damage and repair have recently been published by Dušinska and Collins [107]. The major critical steps, summarised below, have been addressed. As was already mentioned, the careful design of the study and the use of 'power calculations' are crucial to obtain statistically meaningful results. Inclusion and exclusion criteria have to be clearly defined and confounding factors (age, sex, physiological and clinical state, alcohol consumption, smoking, etc.), which may influence the background level and may bias the study, should be taken into consideration. A control group, either taking placebo or not (according to the type of study), carefully matched to the investigated groups, should be included and the control subjects should be sampled over the same period. The study must be ethically approved. To avoid subjectivity as much as possible, samples have to be coded, and analyses should be randomised and blinded wherever possible. Time and conditions of sampling, seasonal and geographical details, and other operational aspects including transport, storage and retrieval should be recorded as all these might increase the variability. Effects of individual differences in lifestyle and diet should be recorded (a detailed questionnaire is strongly recommended) together with individual and family history, and possible environmental or occupation exposure. Sampling of subjects should be performed at the same time of day, and in the same way throughout the study (if possible by taking fasted blood), sampling of subjects and controls should be done simultaneously (e.g., include at least a few subjects from the control group and if possible from each investigated group on the same day), rather than in consecutive phases. Even if it is possible to carry out the comet assay on the day the samples are collected, it is advisable also to freeze and store aliquots of lymphocytes at −80 °C or in liquid nitrogen. It is also necessary to consider whether to select frozen samples for the comet assay analysis completely randomly, or in batches (e.g., all samples from one subject together in the same comet assay experiment).

For each measured endpoint (strand breaks, FPG-sensitive sites, etc.), at least two parallels from each sample are recommended to be analysed. To control the standard experimental conditions, the use of 'reference standards'—the same cells (e.g., untreated lymphocytes from a single source, frozen in aliquots) in each comet assay experiment, is recommended. Inclusion of positive control cells (e.g., lymphocytes treated with H_2O_2) is also advisable.

As there are multiple factors that may increase the variation and modify final results, it is essential to employ the principles of Good Laboratory Practice and to follow the same procedure and the same protocol during the entire study. Furthermore, it is suggested to archive slides for possible future reanalysis. Several aspects of the planning and execution of trials and the analysis of data have been thoroughly discussed in a paper by Lovell and Omori [108].

Recently Møller [89] published a meta-analysis in which he calculated the baseline values of endpoints on the basis of earlier human studies (i.e., experiments conducted under standard conditions and after FPG and EndoIII treatment). The data he collected provide useful information if results obtained in future investigations lie in the normal range, but more information is required concerning the time and design of run-in and intervention phases, the duration of wash-out periods, the calibration of enzymes and other important factors which may influence the outcome of the experiments. One of the striking results obtained in the aforementioned analysis is, that neither alkali treatment nor the duration of the electrophoresis, had a significant impact on the levels of DNA damage.

One of the shortcomings associated with the interpretation of SCGE results in human trials is that we do not know whether a decrease in the level of DNA damage is translated into a beneficial effect on health. In the case of micronucleus assays, chromosomal aberration analysis and sister chromatid exchange tests, the efforts of Bonassi et al. [109,110] and Norppa et al. [111] showed that the first two endpoints, but not the last, correlate with the incidence of cancer in human populations indicating that dietary factors which reduce the number of micronuclei and/or chromosomal aberrations in humans lower the cancer risks. In the case of SCGE assays no results of long-term studies and meta-analyses to show whether comet formation correlates with cancer risks are available and it is questionable if the results of short-term interventions (which last only few days) indeed reflect long-term effects. It is, however of interest that increased comet formation was observed in patients with diabetes type 2 [112–114], hepatitis B and C [115,116], chronic inflammations such as rheumatoid arthritis [117,118], hyperlipidemic patients [118], obstructive pulmonary disease [119], renal dialysis patients [120], in Down syndrome [121] and in patients with psoriasis [121,122]. Most of these disorders are associated with increased cancer risks and prevention of DNA migration (seen in SCGE trials) may be taken tentatively as indicative for potential protective effects against

these diseases and reduction of the cancer risks associated with them. The latter assumption is also supported by a publication of Dusinska et al. [123] who found in an occupational study with fiber-exposed individuals that the MN-frequencies correlate well with levels of FPG- and EndoIII-sensitive sites in DNA.

However, it should be kept in mind, that the mechanisms of breaks leading to comet formation are not fully understood at present and that substantial further experimental evidence is required to draw firm conclusions between disease prevention and the reduction of comets by dietary factors.

The combination of SCGE experiments with omics-techniques which have been developed in the last decade and also with conventional biochemical methods may help to provide mechanistic explanation for DNA-protective effects [5,21]. A typical example is the explanation of the protective effects seen after Brussels sprouts consumption towards PhIP-induced DNA damage which is due to inhibition of sulfotransferases involved in the activation of this amine [42].

In a recent study, Hoelzl et al. found a significant increase of SOD in lymphocytes of individuals after consumption of Brussels sprouts in a proteomic study [42] which may explain the antioxidant effects (prevention of formation of oxidised DNA bases) which they found in a preceding SCGE trial with these vegetables in the same target cells [18].

One of the advantages of the SCGE technique is that it also can be used to monitor prevention of DNA damage in a variety of different tissues in laboratory rodents. This offers the possibility to investigate if protective effects seen in peripheral lymphocytes in humans are paralleled by a reduction of DNA damage in inner organs under identical experimental conditions. Furthermore, animal models can also provide valuable information concerning prevention of specific diseases associated with DNA damage. We have shown recently that gallic acid which is highly effective as antioxidant in peripheral lymphocytes of humans also protects against oxidative damage in inner organs of rats including brain, liver and lungs [21]. Subsequently, we could also demonstrate that the ROS-protective effect in the liver leads to reduction of radiation-induced preneoplastic hepatic lesions [21].

Despite all the different problems described above, the SCGE technique is probably one of the most effective methods for the detection of DNA-protective and antioxidant effects of dietary compounds in humans and it can be expected that the numbers of SCGE studies will increase in the near future.

Conflicts of Interest Statement

None.

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CURRICULUM VITAE

Name	Christine Johanna Hölzl
Date of Birth	13th December 1976, Schärding
Nationality	Austrian
Address	Seegasse 10/6, 1090 Vienna

EDUCATION

since Sept. 2005	Doctorate in Nutritional Sciences, Faculty of Life Sciences, University of Vienna "Effect of coffee consumption on the redox status in humans"
since Sept. 2007	Postgraduate course in Toxicology, Institute of Cancer Research, Medical University of Vienna
Sept. 1995- May 2004	Study of Nutritional Sciences (emphasis on marketing and nutrition ecology), Faculty of Life Sciences, University of Vienna, *Magistra rerum naturalium* (Mag.rer.nat.)
Sept. 1995- Sept. 1997	Study of international business administration, University of Vienna (no graduation)

SCHOLARSHIPS/ RESEARCH ACTIVITIES ABROAD

Feb. 2008	University of Belgrade, Faculty of Medicine, Institute of Biochemistry, Serbia, Training: ELISA techniques
Aug. 2005	Federal Environmental Agency, Bad Elster Branch, Germany; Application of the Ames Assay
Jul. 2004	German Institute of Nutrition (DIfE), Department of nutritional toxicology, Potsdam-Rehbrücke, Germany, Enzyme level and activity measurements
Sep.- Jan. 2002	Universidad Autónoma Madrid, Spain (ERASMUS)

ATTENDANCE AT WORKSHOPS

Genetic monitoring of human populations: planning, design and analysis of molecular epidemiology studies. Universitat Autónoma Barcelona, 11-16 June, 2006

Hands on workshop on plant-genotoxicity assays for environmental carcinogen screening, Medical University of Vienna, 28-30 June, 2006

AWARDS

Travel Award: 9th International Conference on Environmental Mutagens & 36th Annual Meeting of the Environmental Mutagen Society (EMS). September 3rd-8th, 2005, San Francisco, USA, *Poster Presentation*

Scholar In Award: 6th Annual AACR International Conference: Frontiers in Cancer Prevention Research. December 5th-8th, 2007, Philadelphia, USA, *Poster Presentation*

Astox-Award (Austrian Society of Toxikologie): Joint Meeting APHAR and ASTOX, November 22th-24th, Vienna, Austria, Co-author, *Best short lecture*

ACTIVE CONTRIBUTIONS AT CONFERENCES (SELECTED)

ORAL PRESENTATIONS

Feb. 2009 Short lecture. „DNA protective effects of Brussels sprouts: Results of human intervention studies". 24. Tagung der Gesellschaft für Umweltmutations-forschung; *Lifestyle and DNA-Stabilität*, Vienna, Austria

Oct. 2008 Oral presentation. „Gemüse gegen Krebs: die medizinische Wirkung der Kohlgemüse"; Schönbrunner Seminare, *Brokkoli, Pak Choi & Co*, Vienna, Austria

Mar. 2007 Oral presentation. „Antimutagenic and anticarcinogenic properties of coffee". *Synthetic and natural compounds in cancer therapy and prevention*; Bratislava, Slowakia

Sep. 2006 Oral presentation „DNA-schädigende Wirkung von Kanzerogenen in Lebensmitteln- humane Risiken und Präventionsstrategien"; Austrian conference for analytical food chemistry, *Schadstoffe in Lebensmitteln und Futtermitteln*, Vienna, Austria

Jan. 2007 Short lecture "Staining procedures have a strong impact on the outcome of human biomonitoring studies concerning formation of micronuclei (MN) in exfoliated epithelial cells"; National Meeting of the Environmental Mutagen Society India (EMSI), Coimbatore, Tamil Nadu, India

POSTER PRESENTATIONS

COST-meeting, 11-14 October, 2006, Vienna, *Molecular and physiological effects of bioactive food compounds*. "Use of single cell gel electrophoresis assays for the investigation of DNA-protective effects of phytochemicals."

11th Scientific Symposium of the Austrian Pharmacological Society, 24-25 November, 2005, Vienna "Consumption of Brussels sprouts protects peripheral human lymphocytes against oxidative DNA-damage: results of a human intervention trial."

The 5th International conference on environmental mutagens in human populations. May 20-24, 2007. Antalya, Turkey. "Consumption of Brussels sprouts protects peripheral human lymphocytes against 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) and oxidative DNA-damage: results of a controlled human intervention trial."

2nd International Workshop on Analytical, Chemical and Biological Relevance of Heterocyclic Aromatic Amines, 10-12 Mai 2003. Poster presentation. "Investigation on the sensitivity of peripheral human lymphocytes towards genotoxic effects by heterocyclic aromatic amines."

PUBLICATION LIST

Peer reviewed as first or co-author

Knasmüller S., Mersch-Sundermann V., Kevekordes S., Darroudi F., Huber W.W., Hoelzl C., Bichler J., Majer B.J. **(2004)** Use of human-derived liver cell lines for the detection of environmental and dietary genotoxicants; current state of knowledge.Toxicology. 198(1-3):315-28. Review.

Hoelzl C., Bichler J., Ferk F., Simic T., Nersesyan A., Elbling L., Ehrlich V., Chakraborty A., Knasmüller S. **(2005)** Methods for the detection of antioxidants which prevent age related diseases: a critical review with particular emphasis on human intervention studies. J Physiol Pharmacol., 56 Suppl 2:49-64.

Steinkellner H., **Hoelzl C.**, Uhl M., Cavin C., Haidinger G., Gsur A., Schmid R., Kundi M., Bichler J., Knasmüller S. **(2005)** Coffee consumption induces GSTP in plasma and protects lymphocytes against (+/-)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide induced DNA-damage: results of controlled human intervention trials. Mutat Res, 11;591(1-2):264-75.

Bichler J., Cavin C., Simic T., Chakraborty A., Ferk F., **Hoelzl C.,** Schulte-Hermann R., Kundi M., Haidinger G., Angelis K., Knasmüller S. **(2006)** Coffee consumption protects human lymphocytes against oxidative and 3-amino-1-methyl-5H-pyrido[4,3-b]indole acetate (Trp-P-2) induced DNA-damage: results of an experimental study with human volunteers. Food Chem Toxicol. 45(8):1428-36

Ferk F., Mišík M., **Hoelzl C.**, Uhl M., Fuerhacker M., Grillitsch B., Parzefall W., Nersesyan A., Mičieta K., Grummt T., Knasmüller S. **(2007)** Benzalkonium chloride (BAC) and dimethyldioctadecyl-ammonium bromide (DDAB) – two common quaternary ammonium compounds, cause genotoxic effects in mammalian and plant cells at environmentally relevant concentrations. Mutagenesis 22(6): 363-70

Hoelzl C., Glatt H.R., Sontag G., Haidinger G., Kundi M., Simic T., Chakraborty A., Julia Bichler J:, Ferk F., Angelis K. and Knasmüller S. **(2007)** Consumption of Brussels sprouts protects peripheral human lymphocytes against 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP) and oxidative DNA-damage: results of a controlled human intervention trial. Molecular Nutrition and Food Research, 52, 330-341

Hoelzl C., Lorenz O., Haudek V., Gundacker N., Knasmüller S., Gerner C. **(2008)** Proteome alterations induced in human white blood cells by consumption of Brussels sprouts: Results of a pilot intervention study. Proteomics Clin. Appl., 2, 109-117

Knasmüller S., Nersesyan A., Mišík M., Gerner C., Mikulits W., Ehrlich V., **Hoelzl C.**, Szakmary A., Wagner K.H. **(2008)** Use of conventional and –omics based methods for health claims of dietary antioxidadants: a critical review. British Journal of Nutrition, E-Suppl. 1, ES3-ES52

Hoelzl C., Knasmüller S., Mišík M., Collins A., Dušinska M., Nersesyan A. **(2009)** Use of single cell gel electrophoresis assays for detection of DNA-protective effects of dietary factores in humans: recent results and trends. Mutation Research. 2009 Jan-Feb;681(1):68-79.

Chakraborty A., Ferk F., Simić T., Brantner A., Dušinská M., Kundi M., **Hoelzl C.**, Nersesyan A., Knasmüller S. **(2009)** DNA-protective effects of sumach (*Rhus coriaria* L.), a common spice: results of human and animal studies. Mutation Research 10;661(1-2):10-7

Ehrlich V., Nersesyan A., **Hoelzl C.**, Ferk F., Bichler J., Valic E., Schaffer A., Schulte-Hermann R., Fenech M., Wagner K.H., Knasmüller S. **(2008)** Inhalative Exposure to Vanadium Pentoxide Causes DNA Damage in Workers: Results of a Multiple End Point Study. Environmental Health Perspectives 116(12):1689-93

Reichhold S., Neubauer O., **Hoelzl C.**, Stadlmayr B., Valentini J., Ferk F., Kundi M., Knasmüller S., Wagner K.H. **(2009)** DNA damage in response to an Ironman triathlon. Free radical research, accepted

Book chapters and proceedings

Knasmüller S, **Hoelzl C**., Bichler J., Nersesyan A., Ehrlich V. **(2006)** Dietary compounds which protect against heterocyclic amines, In: Acrylamide and other hazardous compounds in heat treated foods. Skog K, Alexander J. Cambridge, England, Woodhead Publishing; pp. 536.

Ehrlich V., Nersesyan A., **Hoelzl C.**, Ferk F., Bichler J., Knasmueller S. **(2006)** Carcinogenic and genotoxic plant constituents, In: Handbuch der Lebensmitteltoxikologie. Dunkelberg H, Gebel T, Hartwig A. Weinheim, Germany, Wiley-VCH: 1915-1963.

Knasmüller S., Uhl M., Gottmann E., **Hoelzl C.**, Majer B.J. The Tradescantia Micronucleus Bioassay. **(2003)** In: Bioassays in Plant Cells for Improvement of Ecosystem and Human Health. Wydawnictwo Uniwersytetu Slaskiego, Katowice.

Hoelzl C., Bichler J., Ferk F., Ehrlich V., Cavin C., Simic T., Edelbauer L., Grasl-Kraupp B., Knasmüller S. **(2007)** Antimutagenic and Anticarcinogenic Properties of Coffee. In: The activity of natural compounds in diseases prevention and therapy. Ďuračková Z. and Knasmüller S. (eds): Bratislava, Slovakia, pp 127-149, *Conference proceedings*

Hoelzl C., Bichler J.; Ferk F., Ehrlich V., Cavin C., Simic T., Kundi M., Nersesyan A., Grasl-Kraupp B., Schaubschläger S., Knasmüller S **(2006)**. Mechanistic aspects of DNA- and cancer-protective effects of coffee. 21st colloquium Association for science and information on coffee*, Conference proceedings*

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