

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. Ferik ^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, Rozvojová 135, Prague, Czech Republic

Received 24 October 2005; accepted 2 February 2007

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 (H₂O₂ 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 H₂O₂ induced DNA-damage was observed with coffee at low concentrations (≤ 25 μ l/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.

© 2007 Elsevier Ltd. All rights reserved.

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

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).

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).

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 Stavrlic (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 (Nishinagasaki, Japan); hydrogen peroxide (H₂O₂) 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 H₂O₂ 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 aspirated 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 $\geq 80\%$ 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 (Hözl, 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 ($p \leq 0.05$) 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 ≤ 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 ≥ 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 sub-toxic 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

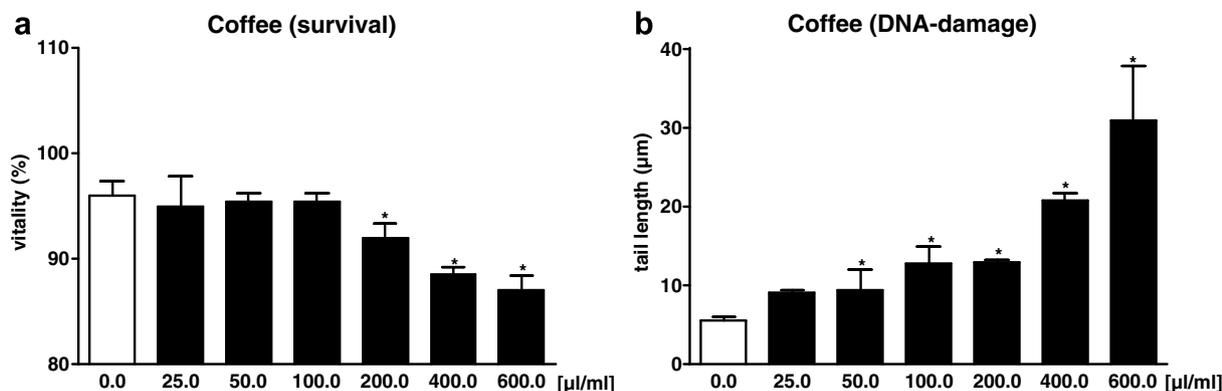


Fig. 1. Effect of coffee on the viability (a) and DNA-migration (b) of peripheral human lymphocytes. Values on the x-axis indicate the amount of coffee. The lymphocytes were exposed to the coffee for 30 min. Subsequently, the cell viability was determined with trypan blue and comet formation was monitored.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

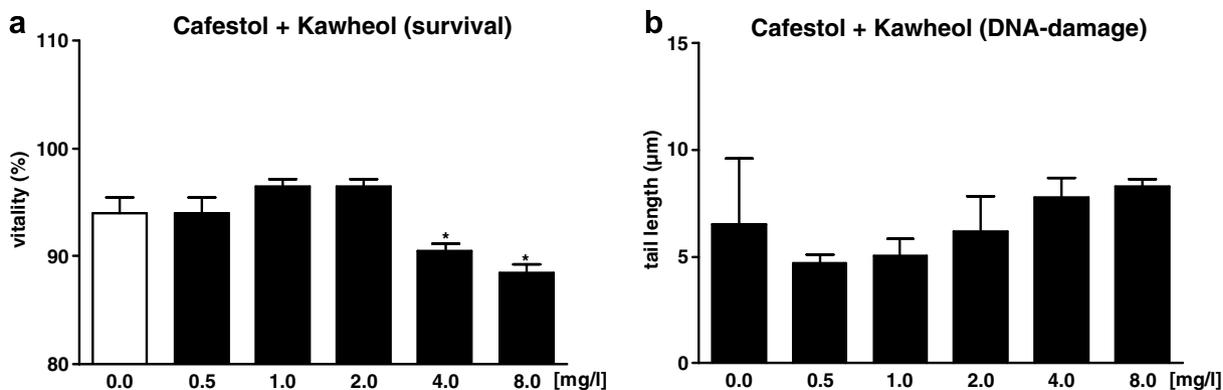


Fig. 2. Effect of the coffee specific diterpenoids cafestol and kahweol on the viability (a) and on DNA-migration (b) of human lymphocytes. The diterpenoids were dissolved in DMSO and different concentrations were added to the cell suspensions for 30 min. Controls were exposed to the solvent only. Subsequently,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

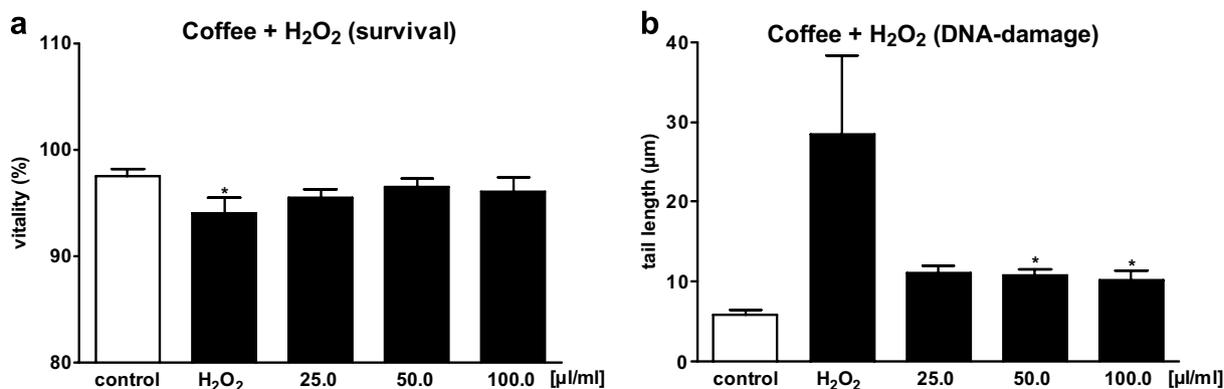


Fig. 3. Effect of coffee on the acute toxicity (a) and on DNA-damage (b) caused by H₂O₂ in peripheral human lymphocytes. The experiment was carried out as described in the legend of Fig. 1, but after exposure of the cells to subtoxic concentrations coffee, H₂O₂ (50 µM) was added to the cells for 5 min on ice, controls were exposed to the solvent only. Per experimental point three, cultures were prepared in parallel. *Indicates statistical significance (p -value ≤ 0.05 , analysis of variance ANOVA). Bars indicate means \pm SD results obtained with three slides (per slide 50 cells were evaluated).

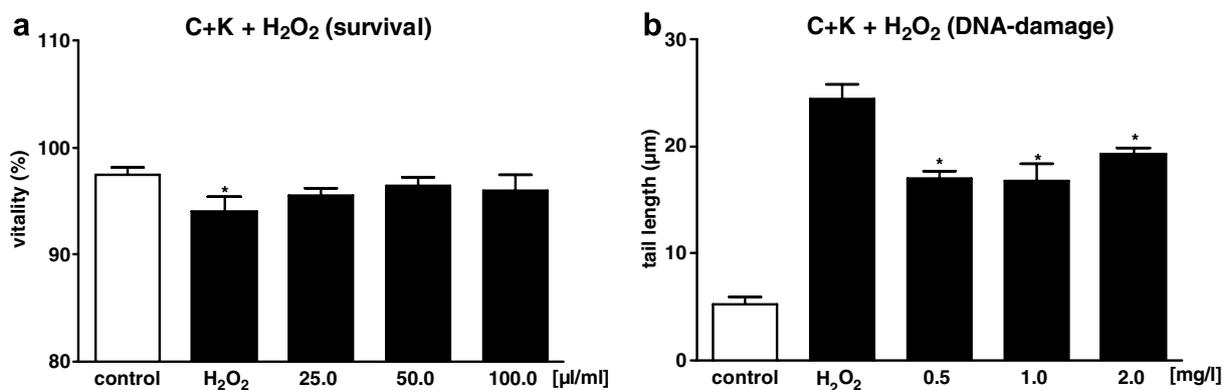


Fig. 4. Effect of cafestol and kahweol on the acute toxicity (a) and on DNA-damage (b) caused by H₂O₂ in peripheral human lymphocytes. The experiment was carried out as described in the legend of Fig. 2, but after treatment with the diterpenoids, H₂O₂ (50 µM) was added to the cells for 5 min. Controls were exposed to the solvent only. Per experimental point three, cultures were prepared in parallel. *Indicates statistical significance (p -value ≤ 0.05 , analysis of variance ANOVA). Bars indicate means \pm 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

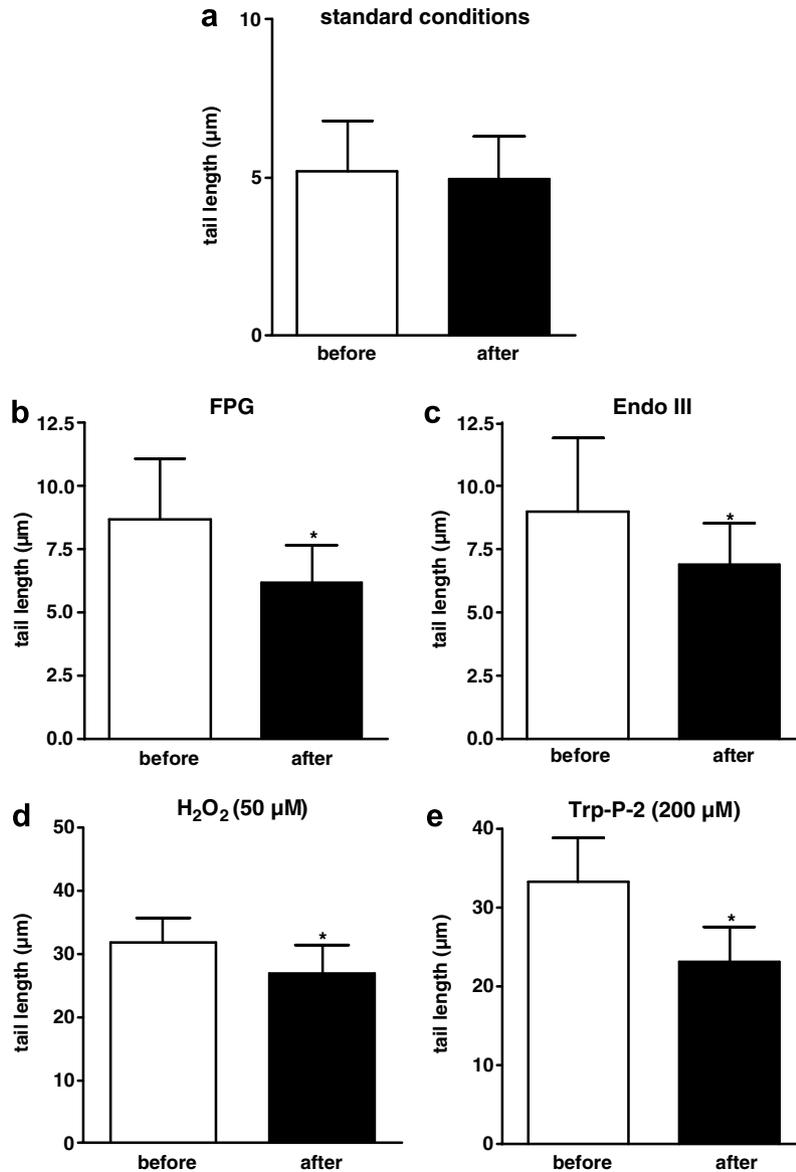


Fig. 5. Effect of coffee consumption on endogenous and chemically induced DNA-damage. Eight individuals participated in the intervention trial. Each of them consumed 600 ml coffee consecutively over a period of five days. Before and after the intervention lymphocytes were isolated from blood and analysed for DNA-migration in SCGE experiments. (a) Shows the results obtained with standard electrophoresis conditions. In experiments with H₂O₂ (d) and Trp-P-2 (e), three cultures per participant were prepared in PBS and the cells were treated on ice with H₂O₂ (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 ≤ 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 H₂O₂ (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

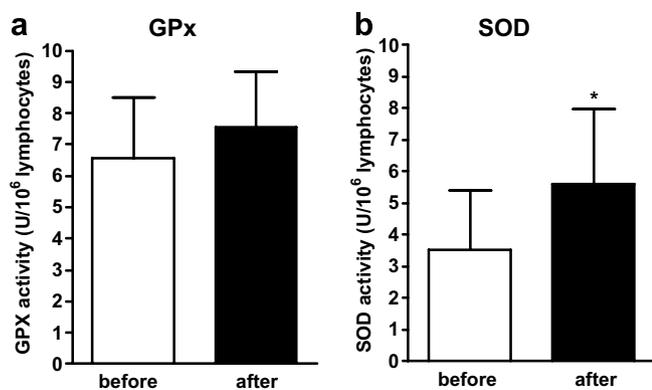


Fig. 6. Effect of coffee consumption on the activity of antioxidant enzymes. The measurements were carried out with cytosolic fractions of lymphocytes of the participants of the intervention trial. The intervention was carried out as described in the legend of Fig. 5. GPx (a) was measured according to 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 \pm 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 \leq 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 H₂O₂ 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 H₂O₂. 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 H₂O₂, 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 splenectomized 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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.

Acknowledgements

The study was supported by a Grant from the Institute for Scientific Information on Coffee (ISIC), La Tour de Peilz, Switzerland.

References

- Aeschbacher, H.U., Meier, H., Ruch, E., Wurzner, H.P., 1984. Investigation of coffee in sister chromatid exchange and micronucleus tests *in vivo*. Food Chem. Toxicol. 22, 803–807.
- Azam, S., Hadi, N., Khan, N.U., Hadi, S.M., 2003. Antioxidant and prooxidant properties of caffeine, theobromine and xanthine. Med. Sci. Monit. 9, BR325–BR330.
- Cavin, C., Holzhauser, D., Constable, A., Huggett, A.C., Schilter, B., 1998. The coffee-specific diterpenes cafestol and kahweol protect against aflatoxin B1-induced genotoxicity through a dual mechanism. Carcinogenesis 19, 1369–1375.
- Chen, A.T., Reidy, J.A., Annest, J.L., Welty, T.K., Zhou, H.G., 1989. Increased chromosome fragility as a consequence of blood folate levels, smoking status, and coffee consumption. Environ. Mol. Mutagen 13, 319–324.
- Clarke, R.J., Vitzthum, O.G., 2001. Coffee – Recent Developments. Blackwell Science, Berlin.

- Collins, A., Dusinska, M., Franklin, M., Somorovska, M., Petrovska, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslova, K., Vaughan, N., 1997. Comet assay in human biomonitoring studies: reliability, validation, and applications. *Environ. Mol. Mutat.* 30, 139–146.
- Collins, A.R., Dusinska, M., 2002. Oxidative stress biomarkers and antioxidant protocols. In: Collins, A.R., Dusinska, M. (Eds.), *Oxidation of Cellular DNA Measured with the Comet Assay*. Humana Press Inc., Totowa, NJ, p. 336.
- Collins, A.R., Duthie, S.J., Dobson, V.L., 1993. Direct enzymic detection of endogenous oxidative base damage in human lymphocyte DNA. *Carcinogenesis* 14, 1733–1735.
- Corrao, G., Zambon, A., Bagnardi, V., D'Amicis, A., Klatsky, A., 2001. Coffee, caffeine, and the risk of liver cirrhosis. *Ann. Epidemiol.* 11, 458–465.
- Daglia, M., Papetti, A., Gregotti, C., Berte, F., Gazzani, G., 2000. *In vitro* antioxidant and *ex vivo* protective activities of green and roasted coffee. *J. Agric. Food Chem.* 48, 1449–1454.
- Daglia, M., Racchi, M., Papetti, A., Lanni, C., Govoni, S., Gazzani, G., 2004. *In vitro* and *ex vivo* antihydroxyl radical activity of green and roasted coffee. *J. Agric. Food Chem.* 52, 1700–1704.
- Dashwood, R.H., 2002. Modulation of heterocyclic amine-induced mutagenicity and carcinogenicity: an 'A-to-Z' guide to chemopreventive agents, promoters, and transgenic models. *Mutat. Res.* 511, 89–112.
- Devasagayam, T.P., Kamat, J.P., Mohan, H., Kesavan, P.C., 1996. Caffeine as an antioxidant: inhibition of lipid peroxidation induced by reactive oxygen species. *Biochim. Biophys. Acta.* 1282, 63–70.
- Duthie, S.J., Ma, A., Ross, M.A., Collins, A.R., 1996. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Res.* 56, 1291–1295.
- ESCODD, 2000. Comparison of different methods of measuring 8-oxoguanine as a marker of oxidative DNA damage. ESCODD (European Standards Committee on Oxidative DNA Damage). *Free Radic. Res.* 32, 333–341.
- Esposito, F., Morisco, F., Verde, V., Ritieni, A., Alezio, A., Caporaso, N., Fogliano, V., 2003. Moderate coffee consumption increases plasma glutathione but not homocysteine in healthy subjects. *Aliment Pharmacol. Ther.* 17, 595–601.
- Fujita, Y., Wakabayashi, K., Nagao, M., Sugimura, T., 1985. Characteristics of major mutagenicity of instant coffee. *Mutat. Res.* 142, 145–148.
- Gallus, S., Bertuzzi, M., Tavani, A., Bosetti, C., Negri, E., La Vecchia, C., Lagiou, P., Trichopoulos, D., 2002a. Does coffee protect against hepatocellular carcinoma? *Br. J. Cancer* 87, 956–959.
- Gallus, S., Tavani, A., Negri, E., La Vecchia, C., 2002b. Does coffee protect against liver cirrhosis? *Ann. Epidemiol.* 12, 202–205.
- Gebhardt, R., 2002. Oxidative stress, plant-derived antioxidants and liver fibrosis. *Planta Med.* 68, 289–296.
- Gelatti, U., Covolo, L., Franceschini, M., Piralì, F., Tagger, A., Ribero, M.L., Trevisi, P., Martelli, C., Nardi, G., Donato, F., 2005. Coffee consumption reduces the risk of hepatocellular carcinoma independently of its aetiology: a case-control study. *J. Hepatol.* 42, 528–534.
- Giovannelli, L., Saieva, C., Masala, G., Testa, G., Salvini, S., Pitozzi, V., Riboli, E., Dolara, P., Palli, D., 2002. Nutritional and lifestyle determinants of DNA oxidative damage: a study in a Mediterranean population. *Carcinogenesis* 23, 1483–1489.
- Giovannucci, E., 1998. Meta-analysis of coffee consumption and risk of colorectal cancer. *Am. J. Epidemiol.* 147, 1043–1052.
- Gunzler, W.A., Kremers, H., Flohe, L., 1974. An improved coupled test procedure for glutathione peroxidase (EC 1-11-1-9-) in blood. *Z. Klin. Chem. Klin. Biochem.* 12, 444–448.
- Harman, D., 1981. The aging process. *Proc. Natl. Acad. Sci. USA* 78, 7124–7128.
- Helma, C., Uhl, M., 2000. A public domain image-analysis program for the single-cell gel-electrophoresis (comet) assay. *Mutat. Res.* 466, 9–15.
- Hoelzl, C., Bichler, J., Ferk, F., Simic, T., Nersesyan, A., Elbling, L., Ehrlich, V.A., 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, 49–64.
- Hözl, C., 2004. Schutzeffekte von Kohlsprossen vor freien Sauerstoffradikalen und heterozyklischen Aminen. Diplomarbeit, Universität Wien.
- Huber, W.W., McDaniel, L.P., Kaderlik, K.R., Teitel, C.H., Lang, N.P., Kadlubar, F.F., 1997. Chemoprotection against the formation of colon DNA adducts from the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the rat. *Mutat. Res.* 376, 115–122.
- Huber, W.W., Prustomersky, S., Delbanco, E., Uhl, M., Scharf, G., Turesky, R.J., Thier, R., Schulte-Hermann, R., 2002. Enhancement of the chemoprotective enzymes glucuronosyl transferase and glutathione transferase in specific organs of the rat by the coffee components kahweol and cafestol. *Arch. Toxicol.* 76, 209–217.
- IARC, 1991. IARC Monographs on the evaluation of the carcinogenic risks to humans: coffee, tea, mate, methylxanthines and methylglyoxal. In: IARC (Ed.), *Coffee*. International Agency for Research on Cancer. World Health Organisation, Lyon, pp. 41–206.
- IARC, 1993. IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans: some natural occurring substances: food items and constituents, heterocyclic aromatic amines and mykotoxines. Heterocyclic aromatic amines. In: IARC (Ed.), *International Agency for Research on Cancer*. World Health Organisation, Lyon, pp. 165–229.
- Ichiba, M., Maeta, Y., Mukoyama, T., Saeki, T., Yasui, S., Kanbe, T., Okano, J., Tanabe, Y., Hirooka, Y., Yamada, S., Kurimasa, A., Murawaki, Y., Shiota, G., 2003. Expression of 8-hydroxy-2'-deoxyguanosine in chronic liver disease and hepatocellular carcinoma. *Liver Int.* 23, 338–345.
- Inoue, M., Yoshimi, I., Sobue, T., Tsugane, S., 2005. Influence of coffee drinking on subsequent risk of hepatocellular carcinoma: a prospective study in Japan. *J. Natl. Cancer. Inst.* 97, 293–300.
- Iwahashi, H., Ishii, T., Sugata, R., Kido, R., 1990. The effects of caffeic acid and its related catechols on hydroxyl radical formation by 3-hydroxyanthranilic acid, ferric chloride, and hydrogen peroxide. *Arch. Biochem. Biophys.* 276, 242–247.
- Iwai, K., Kishimoto, N., Kakino, Y., Mochida, K., Fujita, T., 2004. *In vitro* antioxidative effects and tyrosinase inhibitory activities of seven hydroxycinnamoyl derivatives in green coffee beans. *J. Agric. Food Chem.* 52, 4893–4898.
- Khan, N.S., Ahmad, A., Hadi, S.M., 2000. Anti-oxidant, pro-oxidant properties of tannic acid and its binding to DNA. *Chem. Biol. Interact.* 125, 177–189.
- Kono, Y., Kobayashi, K., Tagawa, S., Adachi, K., Ueda, A., Sawa, Y., Shibata, H., 1997. Antioxidant activity of polyphenolics in diets. Rate constants of reactions of chlorogenic acid and caffeic acid with reactive species of oxygen and nitrogen. *Biochim. Biophys. Acta* 1335, 335–342.
- Kurozawa, Y., Ogimoto, I., Shibata, A., Nose, T., Yoshimura, T., Suzuki, H., Sakata, R., Fujita, Y., Ichikawa, S., Iwai, N., Fukuda, K., Tamakoshi, A., 2004. Dietary habits and risk of death due to hepatocellular carcinoma in a large scale cohort study in Japan. Univariate analysis of JACC study data. *Kurume Med. J.* 51, 141–149.
- Lindl, T., Bauer, J., 1994. *Zell- und Gewebekultur*. Stuttgart, Jena, New York.
- Majer, B.J., Hofer, E., Cavin, C., Lhoste, E., Uhl, M., Glatt, H., Meinel, W., Knasmüller, S., 2005. Coffee diterpenes prevent the genotoxic effects of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and *N*-nitrosodimethylamine in a human derived liver cell line (HepG2). *Food Chem. Toxicol.*
- Moller, P., Loft, S., 2002. Oxidative DNA damage in human white blood cells in dietary antioxidant intervention studies. *Am. J. Clin. Nutr.* 76, 303–310.
- Moller, P., Loft, S., 2004. Interventions with antioxidants and nutrients in relation to oxidative DNA damage and repair. *Mutat. Res.* 551, 79–89.
- Moller, P., Vogel, U., Pedersen, A., Dragsted, L.O., Sandstrom, B., Loft, S., 2003. No effect of 600 grams fruit and vegetables per day on

- oxidative DNA damage and repair in healthy nonsmokers. *Cancer Epidemiol. Biomarkers Prev.* 12, 1016–1022.
- Nagao, M., Fujita, Y., Wakabayashi, K., Nukaya, H., Kosuge, T., Sugimura, T., 1986. Mutagens in coffee and other beverages. *Environ. Health Perspect.* 67, 89–91.
- Nardini, M., D'Aquino, M., Tomassi, G., Gentili, V., Di Felice, M., Scaccini, C., 1995. Inhibition of human low-density lipoprotein oxidation by caffeic acid and other hydroxycinnamic acid derivatives. *Free Radic. Biol. Med.* 19, 541–552.
- Nardini, M., Natella, F., Gentili, V., Di Felice, M., Scaccini, C., 1997. Effect of caffeic acid dietary supplementation on the antioxidant defense system in rat: an *in vivo* study. *Arch. Biochem. Biophys.* 342, 157–160.
- Nardini, M., Pisu, P., Gentili, V., Natella, F., Di Felice, M., Piccolella, E., Scaccini, C., 1998. Effect of caffeic acid on *tert*-butyl hydroperoxide-induced oxidative stress in U937. *Free Radic. Biol. Med.* 25, 1098–1105.
- Natella, F., Nardini, M., Giannetti, I., Dattilo, C., Scaccini, C., 2002. Coffee drinking influences plasma antioxidant capacity in humans. *J. Agric. Food Chem.* 50, 6211–6216.
- Pellegrini, N., Serafini, M., Colombi, B., Del Rio, D., Salvatore, S., Bianchi, M., Brighenti, F., 2003. Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different *in vitro* assays. *J. Nutr.* 133, 2812–2819.
- Reidy, J.A., Annett, J.L., Chen, A.T., Welty, T.K., 1988. Increased sister chromatid exchange associated with smoking and coffee consumption. *Environ. Mol. Mutagen.* 12, 311–318.
- Rinkus, S.J., Taylor, R.T., 1990. Analysis of hydrogen peroxide in freshly prepared coffees. *Food Chem. Toxicol.* 28, 323–331.
- Schwab, C.E., Huber, W.W., Parzefall, W., Hietsch, G., Kassie, F., Schulte-Hermann, R., Knasmüller, S., 2000. Search for compounds that inhibit the genotoxic and carcinogenic effects of heterocyclic aromatic amines. *Crit. Rev. Toxicol.* 30, 1–69.
- Shimazu, T., Tsubono, Y., Kuriyama, S., Ohmori, K., Koizumi, Y., Nishino, Y., Shibuya, D., Tsuji, I., 2005. Coffee consumption and the risk of primary liver cancer: pooled analysis of two prospective studies in Japan. *Int. J. Cancer* 116, 150–154.
- Shimizu, M., Yano, E., 1987. Mutagenicity of instant coffee and its interaction with dimethylnitrosamine in the micronucleus test. *Mutat. Res.* 189, 307–311.
- Smith, D.F., MacGregor, J.T., Hiatt, R.A., Hooper, N.K., Wehr, C.M., Peters, B., Goldman, L.R., Yuan, L.A., Smith, P.A., Becker, C.E., 1990. Micronucleated erythrocytes as an index of cytogenetic damage in humans: demographic and dietary factors associated with micronucleated erythrocytes in splenectomized subjects. *Canc. Res.* 50, 5049–5054.
- Somoza, V., Lindenmeier, M., Wenzel, E., Frank, O., Erbersdobler, H.F., Hofmann, T., 2003. Activity-guided identification of a chemopreventive compound in coffee beverage using *in vitro* and *in vivo* techniques. *J. Agric. Food Chem.* 51, 6861–6869.
- Squier, T.C., 2001. Oxidative stress and protein aggregation during biological aging. *Exp. Gerontol.* 36, 1539–1550.
- Stadler, R.H., Markovic, J., Turesky, R.J., 1995. *In vitro* anti- and pro-oxidative effects of natural polyphenols. *Biol. Trace. Elem. Res.* 47, 299–305.
- Stadler, R.H., Richoz, J., Turesky, R.J., Welti, D.H., Fay, L.B., 1996a. Oxidation of caffeine and related methylxanthines in ascorbate and polyphenol-driven Fenton-type oxidations. *Free Radic. Res.* 24, 210–225.
- Stadler, R.H., Turesky, R.J., Muller, O., Markovic, J., Leong-Morgenthaler, P.M., 1994. The inhibitory effects of coffee on radical-mediated oxidation and mutagenicity. *Mutat. Res.* 308, 177–190.
- Stadler, R.H., Welti, D.H., Stämpfli, A.A., Fay, L.B., 1996b. Thermal decomposition of caffeic acid in model systems: identification of novel tetraoxygenated phenylindan isomers and their stability in aqueous solutions. *J. Agric. Food Chem.* 44, 898–905.
- Stavric, B., 1992. An update on research with coffee/caffeine (1989–1990). *Food Chem. Toxicol.* 30, 533–555.
- 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.* 591, 264–275.
- Sugimura, T., Nagao, M., Kawachi, T., Honda, M., Yahagi, T., Seino, Y., Sato, S., Matsukura, N., Matsushima, T., Shirai, A., Sawamura, M., Matsumoto, H., 1977. Origins of human cancer. In: Sugimura, T., Nagao, M., Kawachi, T., Honda, M., Yahagi, T., Seino, Y., Sato, S., Matsukura, N., Matsushima, T., Shirai, A., Sawamura, M., Matsumoto, H. (Eds.), *Mutagen-Carcinogens in Foods with Special Reference to Highly Mutagenic Pyrolytic Products in Broiled Foods*. Cold Spring Harbor, New York, pp. 1561–1577.
- Svilaas, A., Sakhi, A.K., Andersen, L.F., Svilaas, T., Strom, E.C., Jacobs Jr., D.R., Ose, L., Blomhoff, R., 2004. Intakes of antioxidants in coffee, wine, and vegetables are correlated with plasma carotenoids in humans. *J. Nutr.* 134, 562–567.
- Szuster-Ciesielska, A., Daniluk, J., Kandefers-Szerszen, M., 2002. Oxidative stress in the blood of patients with alcohol-related liver cirrhosis. *Med. Sci. Monit.* 8, CR419–CR424.
- Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.* 35, 206–221.
- Tsuji, S., Shibata, T., Ohara, K., Ito, Y., 1991. Studies on the factors affecting the formation of hydrogen peroxide in coffee. *J. Food Hyg. Soc. Japan* 32, 504–512.
- van den Berg, R., van Vliet, T., Broekmans, W.M., Cnubben, N.H., Vaes, W.H., Roza, L., Haenen, G.R., Bast, A., van den Berg, H., 2001. A vegetable/fruit concentrate with high antioxidant capacity has no effect on biomarkers of antioxidant status in male smokers. *J. Nutr.* 131, 1714–1722.
- van Zeeland, A.A., de Groot, A.J., Hall, J., Donato, F., 1999. 8-Hydroxydeoxyguanosine in DNA from leukocytes of healthy adults: relationship with cigarette smoking, environmental tobacco smoke, alcohol and coffee consumption. *Mutat. Res.* 439, 249–257.
- Wakabayashi, K., Shioya, M., Nagao, M., Sugimura, T., 1989. Medical, Biochemical and Chemical Aspects of Free Radicals. In: Wakabayashi, K., Shioya, M., Nagao, M., Sugimura, T. (Eds.), *Proceedings of the fourth Biennial General Meeting of the Society for Free Radical Research*, Kyoto, Japan, 9–13 April, 1988. Formation of 8-hydroxydeoxyguanosine residue in DNA by environmental mutagens/carcinogens. Elsevier, Amsterdam, pp. 1507–1510.
- Yamanaka, N., Oda, O., Nagao, S., 1997. Prooxidant activity of caffeic acid, dietary non-flavonoid phenolic acid, on Cu²⁺ induced low density lipoprotein oxidation. *FEBS Lett.* 405, 186–190.