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

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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 (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 vitro experiments, inhibition of H₂O₂ induced DNA-damage was observed with coffee at low concentrations (≤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.

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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.

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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 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; Gelatt 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; Studler 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; Svilas 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 (H\textsubscript{2}O\textsubscript{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 \mu M H\textsubscript{2}O\textsubscript{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, cabbages, 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 saf) 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 ≥ 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$_2$O$_2$ (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 superoxido 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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 ± SD results obtained with three slides (per slide 50 cells were evaluated).](image-url)
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
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
DNA-migration (Fig. 1). This observation was not unex-pected. Specific diterpenoids (C + K), causes induction of the cells to high concentrations of coffee, but not to earlier investigations indicate that generation of H₂O₂ obtained (IARC, 1991; Stadler et al., 1994). A number of 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 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.

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4. Discussion

Aim of the present study was the investigation of poten-tial 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 (Iwashashi et al., 1990; Tsuji et al., 1991; Yamanaka et al., 1997), also caffeine was shown to possess prooxidant properties under specific con-ditions (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.

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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 the present study with data from earlier trials show that protective effects were detected. Comparisons of 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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