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Research Article

Identification of hydrogen peroxide as a major cytotoxic component in Maillard reaction mixtures and coffee

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The cytotoxic activity of Maillard reaction products and coffee was studied using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and the neutral red uptake (NRU) assay. Equimolar mixtures of sugars and lysine were heated at 120°C and used to stimulate bovine aorta endothelial cells for 24 h. The cytotoxic activity increased with increase in educt concentration and heating time. Mixtures containing ribose were most active, followed by lactose and glucose. Hydrogen peroxide, which was present in the Maillard mixtures in concentrations between 7 and 87 μM, was identified as one of their major cytotoxic components. H_2O_2-concentrations increased further up to 130 μM under cell culture conditions. Filter coffee, espresso, and green coffee extract reduced cell viability significantly to 10, 19, and 83% of PBS-treated control. The effect was largely attenuated by the addition of catalase. Nil, 33, and 41 μM H_2O_2 was measured in green coffee extract, filter coffee, and espresso, respectively, increasing to 13, 369, and 333 μM during cell culture conditions. No additional H_2O_2 formation was detected when coffee was incubated for up to 5 h without further treatment. In conclusion, hydrogen peroxide is a major product in Maillard mixtures and coffee inducing cell death *in vitro*.

Keywords: Bovine aorta endothelial cells / Coffee / Cytotoxicity / Hydrogen peroxide / Maillard reaction

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1 Introduction

During coffee roasting, the green coffee beans are heated for 2–15 min at temperatures between 200 and 230°C [1]. During this process, sugars contained in the green coffee and reactive carbonyl compounds formed during roasting react with proteins, amino acids, and peptides leading to low molecular weight Maillard products and high molecular weight melanoidins. It was calculated that melanoidins amount to about 25% of the dry matter of the coffee beverage [2]. The structure of coffee melanoidins is barely elucidated, but they obviously contain mainly degradation products of not only sugars and amino acids or peptides, but also polysaccharides and polyphenols [3–6].

The physiological activity of primary coffee components such as caffeine, cafestol, and kahweol is well-established [7, 8]. On the other hand, relatively little is known about the health impact of the neoformed Maillard products and melanoidins in coffee. Coffee shows strong antioxidative activity, determined by its radical chain breaking activity, ferric reducing ability, or inhibition of hexanal degradation, which is at least partially related to Maillard products [3, 9–12]. These results are in good agreement with previous studies proving an antioxidative activity of heated reaction mixtures of sugars and amino acids, as well as defined Maillard products. For example, Maillard products were able to inhibit oxidation of human low-density lipoproteins, metal-induced oxidation of various substrates, or radical chain reactions [13–16]. More recently, it was shown that coffee melanoidins are able to inhibit the Angiotensin-I converting enzyme, which could imply an antihypertensive

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Abbreviations: FOX, ferrous oxidation in xylenol orange; **MEM,** minimum essential medium; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **NRU,** neutral red uptake; **PCA,** perchloric acid; **MRM,** Maillard reaction mixtures; **ROS,** reactive oxygen species

activity [17]. Furthermore, coffee extracts led to nuclear translocation of the transcription factor NF-κB in macrophages, which may be related to an immunomodulatory activity in the gut [18].

A cytotoxic effect of various heated lysine–sugar mixtures has been demonstrated for rat C6 glioma cells and human lymphocytes as well as -under certain conditions- for Caco-2 cells [19–21]. Furthermore, different proteins, which were incubated with glucose, glucose and fructose, or glyceraldehyde led to a considerable loss of cell viability [22, 23]. Hereby, the cytotoxic effect of glycated proteins depended on the sugar type and protein moiety, the modification rate, and the cell type [24, 25]. In another study, no cytotoxic effect was observed after incubation of COS-7 and HL-60 cells with differently glycated β-lactoglobulin [26]. Furthermore, coffee and melanoidins, isolated from coffee, increased or decreased metal-induced cytoxicity, depending on the metal and melanoidin concentration [27, 28].

The purpose of this study was to investigate the cytotoxic potential of Maillard products and coffee in detail and to identify active components in the mixture.

2 Materials and methods

2.1 Chemicals

Eagle's minimum essential medium (MEM, with Earle's salts), fetal bovine serum, penicillin/streptomycin (10 000 U/10 000 µg/mL), 75 cm^2 plastic flasks and 24-well tissue culture plates were purchased from Biochrom AG (Berlin, Germany). D-ribose (Rib), D-lactose monohydrate (Lac), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Fluka (Buchs, Switzerland). Neutral red (NR), L-lysine hydrochloride (Lys), and perchloric acid (PCA) were from Merck KGaA (Darmstadt, Germany); D-glucose (Glc) and glacial acetic acid from Riedel-de Haën (Hannover, Germany). Ammonium ferrous sulfate, catalase (EC 1.11.1.6), and xylenol orange were supplied by Sigma–Aldrich (Taufkirchen, Germany). Roasted and green coffee beans (filter coffee and espresso, both 100% *Coffea arabica*) as well as instant coffee (Nescafé gold) were obtained from a local retailer. Water was purified by a Millipore Synergy 185 device (Millipore, Schwalbach, Germany).

2.2 Preparation of Maillard model reaction mixtures (MRM)

The model systems consisted of Lys and sugar (Glc, Lac, or Rib) in an equimolar ratio (0–110 mM each) and 15 mL PBS with the pH adjusted to 7.4 using 0.5 N NaOH. The solutions were prepared in capped 25 mL screw neck bottles and heated for up to 5 h at 120°C. After the incubation, the mixtures were cooled in an ice bath. The pH was subsequently readjusted to 7.4 with 2 N NaOH. The solutions were then transferred to sterile 15 mL polypropylene tubes using sterile syringe filters (∅ 33 mm, 0.2 µm, PVDF).

In order to model the development of H_2O_2 concentration during cell stimulation, the pH of the MRM was adjusted to 7.4. Aliquots of 50 µL of MRM were then diluted in 500 µL of MEM or water and incubated for 24 h under cell culture conditions (37°C, 5% CO_2). Since the concentration of the active components is not known, the given concentrations of the MRM reflect the original concentration of the sugar, respectively Lys, which was used to prepare the MRM. It reflects the final concentration as present in the cell culture experiments. For example, a 5 mM MRM is prepared by heating 55 mM sugar and 55 mM Lys, and is then further diluted for cell culture experiments.

2.3 Activity guided fractionation of the MRM

MRM were fractionated using a Jasco HPLC system (Jasco, Gross-Umstadt, Germany) equipped with two pumps (model Pu-2087Plus), an EP 30/16 Nucleosil 100-7 C18 guard column, a VP 250/21 Nucleosil 100-7 C18 column (both Macherey-Nagel, Dueren, Germany), a column oven (model CO-200), and a UV/Vis detector (model UV-2077Plus). The columns were operated at 30°C with a flow rate of 20 mL/min and a gradient composed of water (solvent A) and ACN (solvent B) as follows: (t (min):% B)(0:2)(5:2)(10:85)(12:85)(17:2)(20:2). The detection was simultaneously carried out at three wavelengths (λ = 205, 275, and 295 nm). In each run, 1 mL of MRM was applied. The fractions were collected at 0°C with an Advantec fraction collector (model CHF122SB, Jasco, Gross-Umstadt). Following chromatographic separation, the fractions were rotovaporized and freeze-dried. The remnants were dissolved in a volume of water equal to the amount of initially fractionated MRM and subsequently filter sterilized.

2.4 Preparation of coffee extracts

Using a standardized preparation, 3.75 g of ground coffee was extracted with 45 mL of boiling water (100°C) in a 100 mL beaker and allowed to stand for 10 min. The suspension was filtered into a 50 mL volumetric flask and cooled to 25°C in a water bath. The flask was filled with water to yield precisely 50.0 mL coffee extract after pH adjustment to 7.4 with 0.5 N NaOH, and subsequently filter sterilized. Green coffee beans were treated with liquid nitrogen prior to grinding. The extract was prepared equally. In some experiments, coffee was prepared in domestic style. For this purpose, quantities of 15.2 g of ground green coffee or 7.2 g of ground roasted coffee were extracted using a conventional drip brewer, whereas 23.1 g espresso powder was used in a coffee percolator, and 2.1 g of instant coffee was suspended in boiling water. All extractions were performed with 250 mL of tap water.

2.5 Cell culture

Bovine aorta endothelial cells (GM-7373, a gift from the Nikolaus Fiebiger Centre of Molecular Medicine, University of Erlangen-Nuremberg) were cultured in 75 cm^2 plastic flasks and maintained in an incubator (37°C) under a humidified atmosphere of 5% CO_2. MEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 μg/mL) was used as a basal growth medium. Cells were plated at 5.8×10^4 cells/mL (600 μL/well) in 24-well tissue culture plates and allowed to grow for 48 h. The cell culture medium was replaced by MEM (500 μL/well), adding MRM, their fractions, or coffee extracts, respectively (50 μL/well). In some experiments, cells were exposed to Maillard reaction products in MEM containing catalase (100 U/mL). After 24 h of treatment, cell viability was assessed by two different endpoints, namely MTT and neutral red uptake (NRU) assay. The viability was expressed as percent of control for which cells were treated with PBS in MEM as a reference (100% of control).

2.6 MTT assay

MTT assay was performed according to the method of Mosmann [29] with slight modifications. After exposure to the stimulants, the supernatant was removed and the cells were treated with MTT (1 mg/mL in MEM) for 3 h at 37°C. In order to dissolve the generated formazan crystals, isopropanol/1 N HCl (25:1) was added to the cells. The cells with the solvent were then kept on a rotary shaker for 10 min. The solutions were transferred to a 96-well plate and the absorbance was recorded at a wavelength of 595 nm by a microplate reader (Model 550, BioRad, Munich, Germany).

2.7 NRU assay

Cell viability was further determined by measuring the uptake of NR into viable cells [30]. After removal of the Maillard reaction products, the cells were incubated for 3 h at 37°C in MEM containing 50 μg NR/mL. Cells were briefly washed and fixed using an aqueous solution of $CaCl_2$ (10 mg/mL) and formaldehyde (35%). Subsequently, incorporated NR dye was extracted from the cells by adding 50% ethanol/1% acetic acid in water. After shaking the plates vigorously for 10 min on a rotary shaker, the extracted dye was transferred to a 96-well plate. Absorbance was measured at a wavelength of 550 nm with a microplate reader.

2.8 Ferrous oxidation in xylenol orange (FOX) assay

H_2O_2 was determined by a modified FOX assay using PCA (PCA-FOX) as described previously [31]. A 50 μL of coffee or MRM, respectively, were diluted in 500 μL of water prior to analysis. Briefly, an aliquot of 60 μL of each diluted sample was vortexed with 20 μL of water and allowed to stand for 15 min at room temperature. A 20 μL of this solution was mixed with 180 μL of PCA-FOX reagent (0.45 mM xylenol orange and 0.45 mM $Fe(NH_4)_2(SO_4)_2$ in 0.11 N $HClO_4$) in a 96-well plate. After 30 min, the absorbance was recorded at 550 nm and corrected using 60 μL of water as a blank. The same procedure was executed with a sample aliquot in which the water was replaced by 20 μL of a catalase solution (500 U/mL water). The H_2O_2 concentration was calculated from the absorbance difference (± catalase) utilizing a freshly prepared standard curve. Thus, only compounds are recorded which produce the color reaction and which are degradable by catalase. H_2O_2 content of stock solutions was evaluated using the molar extinction coefficient of 0.0394 $cm^2/\mu mol$ at 240 nm [32].

2.9 Statistical analysis

Numeric data are expressed as means ± SD. The significance of differences of individual mean values was calculated employing the Student's *t*-test (two-tailed, unpaired) assuming unequal variances as indicated by the F-test (ANOVA, confidence interval at 95%). Different levels of confidence are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All statistical parameters were calculated using Microsoft Excel 2003.

3 Results

3.1 Cytotoxicity of Maillard reaction mixtures

MRM were prepared by heating various sugars with L-lysine. Bovine aorta endothelial cells (GM-7373) were treated with the reaction mixtures for 24 h and cytotoxicity was determined by the MTT and NRU assay.

The influence of educt concentration and heating time on the cytotoxicity of a Glc–Lys–MRM is exemplified in Fig. 1. The given concentrations indicate the original sugar concentration of the mixture before heating, taking into account the dilution prior to cell stimulation. With an increase in both concentration and heating time, cell viability significantly decreased compared to the PBS-treated control. Cell viability dropped to 4.0 and 3.4% of the PBS stimulated control as detected in the MTT and NRU assay, respectively ($p < 0.001$), when cells were exposed to 10 mM Glc–Lys–MRM heated for 5 h at 120°C.

Furthermore, the sugar component had a major impact on the cytotoxicity of the model solutions. The cytotoxic effect exerted by different MRM derived from glucose, lactose, or ribose and lysine is depicted in Fig. 2. Following incubation with MRM that were heated for 5 h at 120°C, the model solution containing ribose and lysine showed the most deleterious impact on GM-7373 cell viability followed by Lac–Lys- and Glc–Lys–MRM. However, a sig-

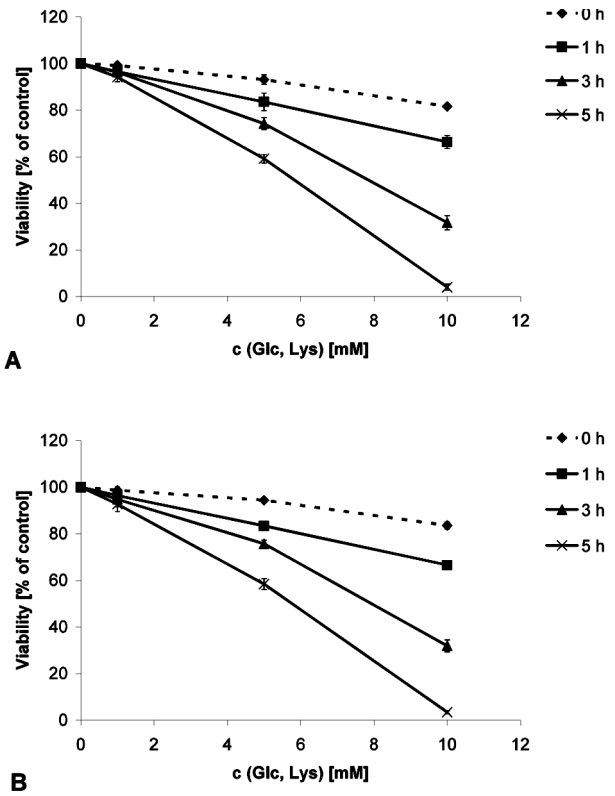

Figure 1. Viability of GM-7373 cells after incubation with different concentrations of Glc – Lys – MRM which were heated for 0 to 5 h at 120°C. Cell viability was assessed by (A) MTT and (B) NRU assay following 24 h of incubation with MRM at 37°C, 5% CO_2. Values represent means ± SD of three independent experiments, each in quadruplicate. The given concentrations indicate the original sugar concentration of the mixture before heating, taking into account the dilution prior to cell stimulation.

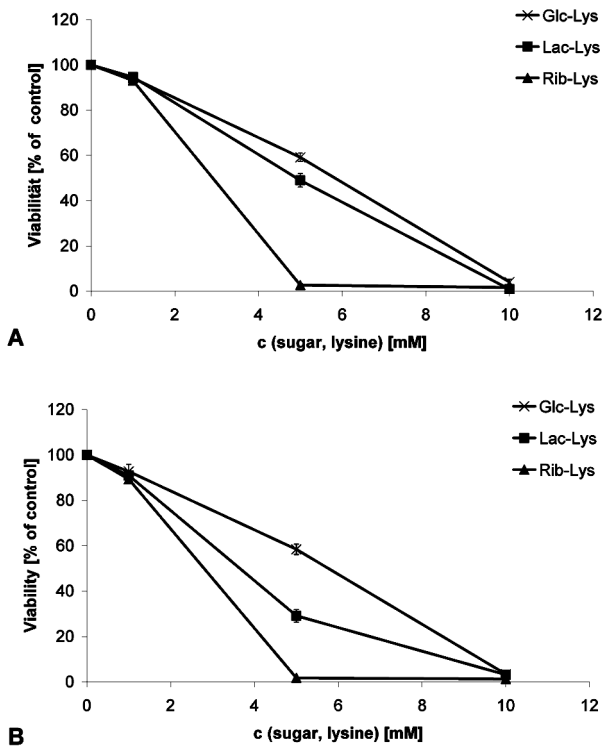

Figure 2. Comparison of the cytotoxic activity of MRM derived from different sugars. MRM consisting of Glc, Lac, or Rib and Lys were heated for 5 h at 120°C. Viability of GM-7373 cells was assessed by (A) MTT and (B) NRU assay after 24 h of incubation with MRM at 37°C, 5% CO_2. Data are expressed as means ± SD of three independent experiments, each in quadruplicate. The given concentrations indicate the original sugar concentration of the mixture before heating, taking into account the dilution prior to cell stimulation.

nificant difference in sugar-dependent cytotoxicity ($p < 0.01$) could only be observed after administration of MRM at a concentration of 5 mM. The unheated control did not show a significant influence on the cell viability (Fig. 3B). Since the results obtained by the MTT assay and NRU assay were very similar, only experiments using the MTT assay are shown hereafter.

3.2 Activity guided fractionation of a Rib – Lys – MRM

In order to gain a deeper insight into active components causing MRM-induced cytotoxicity, a Rib – Lys – MRM (55 mM, pH 7.4, 5 h at 120°C) was fractionated by semi-preparative RP HPLC (Fig. 3A). Since the original MRM is further diluted for cell culture experiments, the 55 mM MRM corresponds to the 5 mM MRM shown in Fig. 2 (for details see Section 2). The resulting six fractions were then tested with the MTT assay as illustrated in Fig. 3B.

Only fraction 1 exerted a significant cytotoxic effect compared to the PBS-treated control ($p < 0.01$) diminishing the viability of the endothelial cells to 77% of control (Fig. 3B). Since the recombined fractions 1 – 6 (Fc in Fig. 3B) showed full activity, a loss of cytotoxic components during fractionation was excluded. More likely, the results can be explained by synergistic effects of two or more components.

In order to further elucidate these effects and to identify active components, different combinations of the HPLC fractions were tested for their cytotoxic activity by MTT and NRU assay. First, mixtures of five HPLC fractions were incubated for 24 h with the endothelial cells prior to evaluation of cell viability by MTT assay.

As can be inferred from Table 1, only the mixtures containing fraction 1 displayed a cytotoxic effect, whereas the recombined fractions 2 – 6 did not significantly reduce cell viability compared to the PBS-treated control. However, all mixtures of five fractions showed enhanced activity compared to fraction 1 alone. Similar results were obtained, when 2, 3, or 4 fractions were combined. In all cases, cyto-

Table 1. Cytotoxic effect of recombined fractions obtained from preparative HPLC of a Rib–Lys–MRM (55 mM in PBS, incubated for 5 h at 120°C, pH 7.4, the fractions were diluted 1:10 for cell culture experiments)

Single fractions		Combination of two fractions		Combination of three fractions		Combination of four fractions		Combination of five fractions	
F (yield% of all fractions)	Viability (% oc)	F	Viability (% oc)	F	Viability (% oc)	F	Viability (% oc)	w/o F	Viability (% oc
1 (45)	77 ± 3	1, 2	64 ± 6	1, 2, 3	28 ± 4	1, 2, 3, 4	6 ± 3	1	96 ± 2
2 (31.1)	104 ± 5	1, 3	40 ± 5	1, 2, 4	57 ± 5	1, 2, 3, 5	9 ± 4	2	21 ± 3
3 (10.3)	106 ± 4	1, 4	69 ± 4	1, 2, 5	59 ± 5	1, 2, 3, 6	14 ± 5	3	38 ± 4
4 (5.6)	104 ± 3	1, 5	81 ± 3	1, 2, 6	66 ± 7	1, 2, 4, 5	44 ± 2	4	18 ± 7
5 (3.7)	102 ± 4	1, 6	76 ± 4	1, 3, 4	26 ± 10	1, 2, 4, 6	42 ± 4	5	19 ± 5
6 (4.2)	101 ± 4			1, 3, 5	37 ± 13	1, 2, 5, 6	40 ± 4	6	19 ± 6
				1, 3, 6	34 ± 4	1, 3, 4, 5	26 ± 4		
				1, 4, 5	62 ± 2	1, 3, 4, 6	20 ± 3		
				1, 4, 6	58 ± 4	1, 3, 5, 6	30 ± 3		
				1, 5, 6	72 ± 3	1, 4, 5, 6	68 ± 3		

The yield of dry matter of each fraction is given in parenthesis. Cell viability was assessed *via* MTT assay; (% oc):% of control, F: fraction(s). Data are expressed as mean ± SD of one representative experiment in quadruplicate (MTT).

toxic activity was only detected in the presence of fraction 1, but the addition of almost all other fractions synergistically enhanced the biological activity. The highest synergistic effect was detected for fraction 3, the lowest for fractions 5 and 6, but the effect was not strictly related to the yield of each fraction (Table 1).

In order to elucidate the cytotoxic component in fraction 1, several Maillard products, which are known to elute in fraction 1, were tested for their cytotoxic activity. Among those, H_2O_2, which coelutes with the major peak in fraction 1, showed a considerable cytotoxic effect on the cells. The concentration of H_2O_2 was determined in the different fractions by FOX assay as follows (means of a triplicate determination): F1, 19 μM; F2, 7 μM; F3, 7 μM; F4, 1 μM; F5, 1 μM, F6, 0 μM.

3.3 Involvement of H_2O_2 in MRM-induced cytotoxicity

To investigate the role of H_2O_2 in MRM-induced cytotoxicity, the concentration of H_2O_2 in different sugar–Lys–MRM was monitored depending on concentration and heating time of the model mixtures. Furthermore, the influence of an additional 24 h incubation of MRM in MEM under cell culture conditions, *i.e.*, conditions simulating MRM treatment of GM-7373 cells, on H_2O_2 formation was evaluated. The concentrations of H_2O_2 determined after heating of MRM for 5 h at 120°C as well as after further incubation for 24 h under cell culture conditions, but in the absence of cells, are listed in Table 2. Generation of H_2O_2 increased both with prolonged heating at 120°C (data not shown) and the concentration of the sugar–Lys–MRM. Following thermal treatment at 120°C, the highest concentrations of H_2O_2 were observed in mixtures of Rib and Lys, whereas solutions of Glc or Lac and Lys contained approximately equal amounts of H_2O_2. A subsequent incubation of heated MRM under cell culture conditions in the absence of cells resulted in a considerable rise of H_2O_2 content. The sugar-dependent production of H_2O_2 followed the order Rib–Lys > Glc–Lys > Lac–Lys. Thus, H_2O_2 detected in the fractions before may also partially be formed during or after the HPLC separation. For comparison, aqueous solutions of H_2O_2 were added in the highest concentration found in heated model mixtures (Rib–Lys–MRM, 5 h at 120°C) after 24 h incubation under cell culture conditions, which were 14 μM H_2O_2 corresponding to the 1 mM MRM, 59 μM H_2O_2 corresponding to 5 mM MRM, and 130 μM H_2O_2 corresponding to 10 mM MRM (Table 2). These H_2O_2 solutions exerted a dose-dependent cytotoxic activity (HOOH–CAT), but to a lesser extent than the corresponding model solutions (Rib–Lys – CAT, Fig. 4).

Furthermore, cytotoxicity was attenuated by the addition of catalase, an enzyme that readily catalyzes the decomposition of H_2O_2. Upon addition of catalase, the lethal impact of aqueous H_2O_2 solutions on GM-7373 cells was completely neutralized. However, Rib–Lys–MRM still exhibited a noticeable residual cytotoxic effect. When the endothelial cells were stimulated by the reaction mixtures, which had been used for fractionation (55 mM), or with the recombined fractions in the presence of catalase, cell viability was restored to 31 and 37% of control, respectively (data not shown).

3.4 Cytotoxicity of coffee samples

Since coffee contains high concentrations of Maillard products, it was tested if a similar mechanism as observed for the MRM also takes place in coffee.

Incubation of GM-7373 cells with coffee samples obtained from standardized preparation resulted in a high

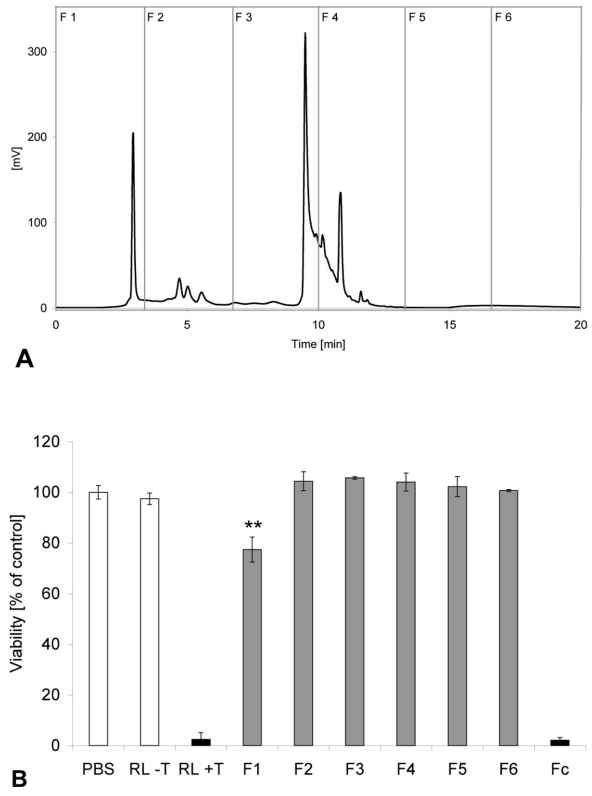

Figure 3. (A) Chromatogram of a Rib – Lys – MRM (55 mM in PBS, incubated for 5 h at 120°C, pH 7.4) separated by preparative HPLC and recorded at $\lambda = 275$ nm. The windows indicate the collected fractions. (B) Viability of GM-7373 cells after 24 h of incubation (37°C, 5% CO_2) with HPLC fractions as determined by MTT assay. Viability of endothelial cells was assessed by the MTT assay after 24 h of incubation at 37°C, 5% CO_2. PBS: control, RL-T: unheated Rib – Lys – MRM (5 mM in PBS, pH 7.4), RL + T: Rib – Lys – MRM (5 mM in PBS, incubated for 5 h at 120°C, pH 7.4), F1: fraction 1 *etc.*, Fc: recombined mixture of all fractions. Values represent means ± SD of two representative experiments, each in quadruplicate. ** $p < 0.01$ in comparison with control (PBS).

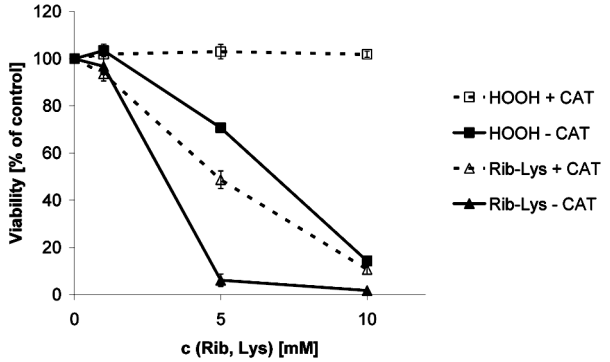

Figure 4. Influence of catalase (CAT) on the cytotoxicity of Rib-Lys-MRM (5 h at 120°C, pH 7.4) and aqueous solutions of H_2O_2. The concentrations of the latter resemble H_2O_2 contents in the depicted Rib – Lys – MRM after 24 h incubation as determined by PCA-FOX assay (*cf.* Table 2). Data are expressed as means ± SD of three independent experiments, each in duplicate. The given concentrations indicate the original sugar concentration of the mixture before heating, taking into account the dilution prior to cell stimulation.

Table 2. Concentrations of H_2O_2 in different MRM (incubated for 5 h at 120°C, pH 7.4) as determined *via* the PCA-FOX method

MRM (5 h at 120°C)	Concentrations (MRM)	Concentrations (H_2O_2) (μM)	
		Water	24 h MEM
Glc – Lys	1 mM	7 ± 0	12 ± 1
	5 mM	14 ± 0	34 ± 1
	10 mM	23 ± 1	53 ± 0
Lac – Lys	1 mM	6 ± 4	9 ± 1
	5 mM	11 ± 0	15 ± 3
	10 mM	21 ± 1	29 ± 3
Rib – Lys	1 mM	10 ± 1	14 ± 2
	5 mM	31 ± 1	59 ± 3
	10 mM	87 ± 1	130 ± 6

H_2O_2 was measured after 1:10 dilution of heated MRM in water as well as MEM and following incubation for 24 h under cell culture conditions (37°C, 5% CO_2). The pH of MRM was readjusted to 7.4 prior to dilution. Data are expressed as mean ± SD (three independent experiments, each in triplicate).

diminution of cell viability (Fig. 5). Filter coffee and espresso significantly reduced the cell viability compared to the PBS-treated control (10 and 19% of control, $p < 0.001$ and $p < 0.01$, respectively) while green coffee exerted only a weak cytotoxic effect (83% of control, $p < 0.01$). Cytotoxicity was mostly attenuated by administration of catalase together with the coffee samples. However, the addition of catalase did not completely neutralize the deleterious effects induced by all coffee samples so that a significant cytotoxic activity remained ($p < 0.001$) compared to the PBS-treated control.

Additionally, the H_2O_2 content of coffee samples obtained from the standardized protocol was monitored directly after preparation as well as after additional 24 h incubation under cell culture conditions. Freshly brewed filter coffee and espresso samples were shown to produce H_2O_2 after dilution in water as displayed in Fig. 6 A. No H_2O_2 was detected in hot water extracts of green coffee. Incubation of coffee samples under cell culture conditions yielded an up to 11-fold increase in H_2O_2 contents of filter coffee and espresso samples. Little H_2O_2 (13 μM) was generated by brewed green coffee. Figure 6B shows the time course of H_2O_2 production of coffee prepared in domestic style which was allowed to stand at room temperature for up to 5 h. The samples were diluted in water prior to measurement. The initially determined content of H_2O_2 in freshly prepared coffee samples did not increase with time.

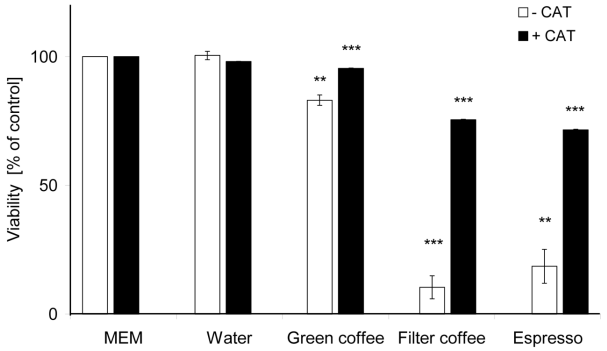

Figure 5. Influence of catalase (CAT) on the cytotoxicity of coffee samples obtained from standardized preparation. Viability of GM-7373 cells was determined *via* MTT assay after 24 h of incubation at 37°C, 5% CO_2. Data are expressed as means ± SD of three different experiments, each in duplicate. ** $p < 0.01$, *** $p < 0.001$ in comparison with control (MEM ± -CAT).

4 Discussion

The goal of this study was to investigate the cytotoxic activity of Maillard reaction mixtures (MRM) and coffee in detail and to identify active components. The results show that heated mixtures of sugars and lysine in the tested concentrations exert a strong cytotoxic effect on endothelial cells, which was dependent on the concentration of the educts, the heating time, and the type of sugar. The concentration of Maillard products used for cell culture experiments results from heating a 5–10 mM sugar and lysine solution. It is difficult to judge how well these concentrations represent those in food products. Raw coffee, for example, contains up to 9% mono- and disaccharides and up to 12% amino acids and proteins. The quantity of Maillard products used for cell culture experiments may overestimate the quantity of Maillard products which are taken up by nutrition. However, the concentrations were chosen so that the identification of active components and mechanisms is possible. Later it was verified that similar mechanisms take place when 1:10 diluted coffee was investigated: a high loss of cell viability was observed, when different coffee preparations were used for cell stimulation. These findings are in good agreement with previous studies, which showed cytotoxic activity for coffee [27], coffee melanoidins [28], glycated proteins [22–25], and glycated lysine [19–21].

Under the conditions applied here, H_2O_2 was clearly a major cause for the observed cytotoxic effect, because (i) H_2O_2 directly applied to the cells in similar concentrations as found in the MRM showed a strong cytotoxic effect, which was. however, lower than that of the corresponding MRM and (ii) the cytotoxic effect of the MRM could be attenuated by the addition of catalase. The latter effect was even more pronounced for coffee. Itagaki *et al*. [27] had

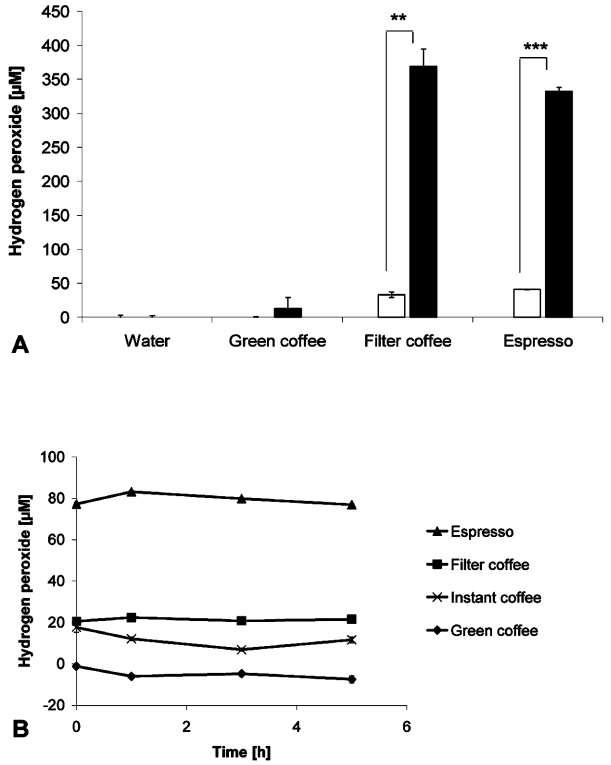

Figure 6. (A) Concentration of H_2O_2 in different coffees obtained from standardized preparation. H_2O_2 was determined after dilution of freshly prepared samples in water (white columns) and after additional 24 h incubation in MEM under cell culture conditions (37°C, 5% CO_2, black columns). (B) Time course of H_2O_2 concentrations in coffees prepared in domestic style. Coffee samples were stored at room temperature and diluted in water prior to determination of H_2O_2. All data are expressed as means ± SD of three independent experiments, each in triplicate. ** $p < 0.01$, *** $p < 0.001$.

shown before that the cytotoxicity of coffee can be inhibited by peroxidase, which degrades H_2O_2 and other peroxides. There is growing evidence that reactive oxygen species (ROS) are a functionally important class of compounds formed during the Maillard reaction. It was shown before that ROS formed during the Maillard reaction modified proteins to a similar extent as direct glycation reactions [33]. Furthermore, H_2O_2, which is formed during the Maillard reaction or during coffee roasting, causes NF-κB activation in several cell lines [18], fragmentation of Cu, Zn-superoxide dismutase [34], and collagen crosslinking [35]. Moreover, the cytotoxic effect of proteins which have been glycated with different agents (advanced glycation end-products) could be partially or fully reversed by the addition of antioxidants [22, 23, 36, 37]. It was hypothesized that advanced glycation end-products trigger signal transduction pathways, for example by their interaction with the receptor for advanced glycation end-products (RAGE), which include the intracellular formation of ROS [38]. The

present study supports an alternative hypothesis. During the Maillard reaction, ROS are formed directly, which then induce apoptosis or necrosis. Superoxide anion, H_2O_2, and hydroxy radical are formed, for example, by the autoxidation of carbohydrates [39]. Since the unheated mixtures of sugars and lysine were practically inactive in our experiments, however, it is more likely that H_2O_2 is generated by reaction products such as osones or the Amadori products [40]. It has also been shown that H_2O_2 is present in coffee. The measured H_2O_2 concentration seems largely dependent on the analytical method, particularly the pH value of the solution in which the coffee is diluted prior to analysis [41, 42]. In the present study, 21 mM H_2O_2 was measured in filter coffee, 18 μM in instant coffee, and 77 μM in espresso, which were prepared under domestic conditions and diluted in distilled water prior to the FOX assay. On the other hand, 33 μM H_2O_2 was found, if the coffee was prepared according to a standard protocol which included pH adjustment to 7.4 after extraction and subsequent dilution with distilled water. When the same standardized coffee preparation was diluted in MEM, 99 mM H_2O_2 was detected. Similar or higher results were obtained in other studies, when the H_2O_2 content in coffee was measured by the FOX assay [18, 42 – 44], whereas lower concentrations were found when chromogenic methods were applied after RP chromatography [41]. However, all studies agree that high amounts of H_2O_2 are produced from coffee during incubation at neutral pH. But in contrast to a previous study, an increase in the H_2O_2 content was not observed, when coffee prepared in a domestic drip brewer was allowed to stand for 5 h at its natural pH at room temperature. H_2O_2 formation is probably favored at neutral pH [41].

Whereas in earlier studies, the H_2O_2 formation was related to phenolic compounds, the present study rather suggests that the H_2O_2 in coffee is mainly formed by roasting products. In green coffee, which contains high levels of polyphenols, H_2O_2 was not detected and only low concentrations were formed during incubation for 24 h under cell culture conditions, which is in accordance to an earlier study [18]. The formation of H_2O_2 during incubation at cell culture conditions was also observed for MRM under the conditions applied in this study [18]. These results indicate that the observed H_2O_2 dependent cytotoxic effect of the MRM is not only caused by the H_2O_2 formed during the heating of the sugars but also with lysine. Moreover, the Maillard mixtures obviously contain compounds which are able to generate H_2O_2 at 37°C and neutral pH. Similar processes seem to be effective in coffee. The mechanism how H_2O_2 is generated from Maillard products and coffee components was not elucidated. In the literature, several mechanisms have been discussed: superoxide anion, which may be formed by reduction of oxygen with alkoxy radicals, may be further reduced to give H_2O_2. Alkoxy radicals can be produced by autoxidation of sugar degradation products or phenols [42, 45, 46]. Alternatively, metal catalyzed reduction of oxygen may occur *via* formation of a complex between oxygen, the metal ion, and an aminoreductone or enediol. Aminoreductone and enediol substructures can be part of advanced Maillard products or they are formed transiently, for example by tautomerization of the Amadori product [14, 47].

Further studies are also required to determine the dependence of H_2O_2 formation in MRM and coffee on the reaction conditions. It has been shown that not only the pH value may have a major influence, but also other factors such as temperature or the availability of oxygen must be considered [41]. Furthermore, other food components, such as phenols, may enhance or inhibit the process and again, these promoting and inhibitory effects are probably dependent on the reaction conditions. Thus, these factors must be elucidated in order to evaluate the relevance of H_2O_2 formation from Maillard products in food and *in vivo*.

The cytotoxic effect of H_2O_2 is well established, involving necrosis and/or apoptosis, depending on its concentration [48 – 50]. It was suggested that extracellular H_2O_2 penetrates the cell, where it triggers different signaling pathways, which eventually induces c-Jun N-terminal kinases. Their activation can then lead to apoptosis or necrosis [51]. Furthermore, H_2O_2 can induce cell death by increasing intracellular O_2^- levels through nitric oxide synthase and NADPH oxidase [52]. The physiological role of H_2O_2 is not clear yet. There is growing evidence that H_2O_2 is not only a toxic component, which must be eliminated by diverse detoxification systems, mainly catalase or glutathione peroxidase, but that it may also act as an inter- and intracellular signaling molecule [50]. Thus, it must be further investigated, if H_2O_2 that is taken up with food containing Maillard products is fully detoxified or if it exerts any physiological activity, which may be beneficial or harmful. Furthermore, ingested Maillard products that are able to generate H_2O_2 could be active systemically or locally. In two studies, elevated urinary H_2O_2 concentrations were measured after coffee consumption in healthy volunteers. These results indicate *de novo* formation of H_2O_2 from coffee components *in vivo* or partial resorption of H_2O_2 from the beverage [44, 53] and excretion.

The fractionation experiments in the present study show further that the cytotoxic effect of the MRM cannot be fully explained by the action of H_2O_2. Additionally, other factors may be active. Interestingly, these factors may not be cytotoxic by themselves, but enhance the cytotoxic activity of H_2O_2. Although this synergistic effect is more pronounced for some fractions, it does not seem to be specific. Therefore, the nature of this factor could not be revealed during this study.

In summary, this study provides evidence that the cytotoxic activity of MRM is largely related to H_2O_2 formed during the Maillard reaction at high temperatures. Furthermore, Maillard products are able to form H_2O_2 at 37°C and neutral pH. Roasting products in coffee behave in a similar

way. Further studies are now necessary to investigate if there are physiological consequences of these processes.

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