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Research Article

Impact of apple polyphenols on *GSTT2* gene expression, subsequent protection of DNA and modulation of proliferation using LT97 human colon adenoma cells

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Apple extract (AE) enhances expression of glutathione *S*-transferases (*e.g.*, *GSTT2*) in human colon cells (LT97). Therefore, aim of the present study was to identify functional consequences of *GSTT2* induction by AE and to determine the relation of AE effects to isolated compounds. Polyphenol composition of AE was analyzed. LT97 cells were treated with AE or synthetic polyphenol mixture (SPM) under conditions that induced *GSTT2*, and challenged with GSTT2-2 substrate cumene hydroperoxide (CumOOH) to determine DNA damage using comet assay. Modulation of *GSTT2* expression (real-time PCR) was reassessed, and the influence on cell proliferation and pro-oxidative potential of AE and SPM were assessed to understand additional mechanisms. Induction of *GSTT2* by AE was accompanied by protection of LT97 cells from CumOOH-induced genotoxicity. Although SPM was unable to reflect AE-specific bioactivity related to *GSTT2* modulation and anti-genotoxicity, inhibition of LT97 cell proliferation by SPM was comparable. Storage of AE caused changes in phenolic composition along with loss of activity regarding *GSTT2* induction and amplified growth inhibition. At the applied concentrations, no H_2O_2 formation was detectable with any of the substances. AE can protect against oxidatively induced DNA damage. Nevertheless, chemopreventive effects of AE strongly depend on the specific composition, which is modified by storage.

Keywords: Apple polyphenols / Chemoprevention / Cumene hydroperoxide / GSTT2 / LT97

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1 Introduction

Even though the recent report of the World Cancer Research Fund appraised the protective effects of fruits on the risk for colon cancer as limitedly suggestive, it is widely believed that a diet rich in fruits and vegetables shows protective effects against colorectal cancer [1, 2]. Many studies demonstrated chemopreventive properties of phytochemicals, among them antioxidant activities, induction or inhibition of enzymes and anti-proliferative effects [3]. In particular the modulation of drug-metabolizing enzymes seems to be important in terms of chemoprevention since these enzymes can inactivate carcinogens [4].

Apples are widely consumed in the Western diet and they are a major source of flavonoids [5–7]. Recently, *in vivo* studies with rats revealed properties of apples related to anti-carcinogenic activities. Treatment of rats with a cloudy apple juice resulted in reduced DNA damage, pre-neoplastic lesions and number of aberrant crypt foci initiated by an injection of the colon carcinogens dimethylhydrazine or azoxymethane [8, 9]. Apple extract (AE) was found to inhibit protein kinase C activity in a cell-free system and was shown to induce apoptosis in HT29 colon carcinoma cells [10]. Studies with the procyanidin-enriched fraction of

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Abbreviations: AE, apple extract; **CumOOH**, cumene hydroperoxide; **DAPI**, 4′,6-Diamidino-2-phenylindol; **EC_{50}**, effective concentration that reduces number of cells by 50%; **FCS**, fetal calf serum; **FOX**, ferrous iron in xylenol orange; **GSH**, glutathione; **GST**, glutathione S-transferase; **SPM**, synthetic polyphenol mixture

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an AE have shown that this compound increases the protein expression of extracellular signal-regulated kinase 1 and 2, c-Jun N-terminal kinase and the activity of caspase-3, indicating induction of apoptosis resulting in cell death [11]. In the same study the authors also showed that apple procyanidins arrest cells in G2/M phase of the cell cycle and reduce activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase in a human colorectal carcinoma cell line (SW620) and thus, inhibit promotion of colon carcinogenesis.

In a previous study we have shown that the exposure of LT97 colon adenoma cells to 128 μg/mL AE (7.5 μM phloridzin equivalents) for 24 h induced *GSTT2* mRNA (real-time PCR: 1.8-fold, $p \leq 0.001$) [12]. The gene products of *GSTT2* may biologically activate selected industrial chemicals, such as ethylene oxide, halogenated alkanes and epoxides, and thus enhance their toxicity [13, 14]. Alternatively, human GSTT2-2 has a peroxidase activity with *t*-butyl hydroperoxide and most pronounced cumene hydroperoxide (CumOOH) as substrates, whereas there was no detectable activity for hydrogen peroxide (H_2O_2) [15 – 17]. While the physiologically existing 15-hydroxyperoxide of arachidonic acid was identified as a substrate for a rat Theta class GST, *i.e.*, Yrs-Yrs, no natural substrate for human GSTT2-2 has yet been identified [15, 18, 19]. The identification of such a natural substrate is critical to define a specific function of this enzyme within the human defense system. So far, it can be only speculated that human GSTT2-2 may help to protect cells from oxidative stress induced by peroxides generated during peroxidation of lipids or arachidonic acid.

Although GSTT2-2 has pronounced substrate specificity for CumOOH, GSTA2-2 and GSTK1-1 as well as mu-class GST enzymes have also been reported to show affinity for this compound [15, 19, 20]. In addition, CumOOH is also a substrate for glutathione peroxidase [21].

Based on the *GSTT2* gene expression results, we assumed that a pre-treatment of human LT97 colon adenoma cells with AE would alter the genotoxic response to CumOOH. The AE batch (termed AE[A]) used for previous studies [12, 22, 23] ran out during experiments. Therefore, we had to order a new batch of AE (termed AE[B]) from the same charge, cultivar year and production line, but different storage conditions. To explore the effects of AE more in depth, a synthetic polyphenol mixture (SPM) of phenolic compounds as detected in the AE[A] was prepared and its effects were compared to results obtained with this AE. Furthermore, modulation of LT97 cell proliferation by AE batches and SPM was compared. Considering that polyphenols are not only known for their antioxidant effects but also for their pro-oxidative potential *in vitro* and *in vivo* [24 – 28], the ability of AE compounds to induce formation of H_2O_2 in cell culture media was investigated to clarify whether H_2O_2 has to be considered for AE-mediated modulation of *GSTT2* expression and proliferation.

Overall, the aim of the study was to identify functional consequences of *GSTT2* induction by AE and the relation of AE effects to isolated compounds.

2 Materials and methods

2.1 AE and SPM

Clear apple juice from the cultivar year 2002 was produced from a defined mixture of table apples as has been described before [22]. Polyphenols of 100 L of that juice were retained on a 5-L adsorber resin (XAD 16 HP, Rohm & Haas, Frankfurt, Germany) packed onto a Pharmacia glass column (BPG 100, 100 × 10 cm). After washing out water-soluble juice ingredients, polyphenols were eluted with three bed volumes of ethanol (96%). The ethanolic fraction was concentrated by evaporation, transferred into the water phase and freeze dried [29]. AE[A] was stored at 4°C until further use. An aliquot was used for analysis of polyphenol contents [23, 30], other aliquots were needed to treat the colon cells prior to determining biological activities. AE[A] was in use from 01/2003 to 01/2006. In contrast, AE[B] was stored approximately 4 years at room temperature until use starting in 03/2007.

To assess whether the interaction of compounds in AE are responsible for the effects of *GSTT2* gene expression modulation and the anti-genotoxic potential of the AE, an SPM was created. Commercially available polyphenols, corresponding to those found in the analysis of AE[A] (highlighted in Table 1), were dissolved at corresponding concentrations in ethanol or methanol, and were freshly mixed for each experiment to avoid any effects on the activity of SPM (chlorogenic acid, caffeic acid, phloretin-2′-*O*-glucoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-rutinosid and (−)-epicatechin were purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany), whereas quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside and the procyanidins B_1 and B_2 were obtained from Fluka (Deisenhofen, Germany). Because of the different solubility of the SPM constituents, two different alcoholic solvents were used to obtain the conditions required for cell culture experiments. The total polyphenol content of the SPM was equal to about 50% of the AE[A] (480.4 μg/mL in AE[A]; 264.2 μg/mL in SPM). To exclude any effects of the solvents on cell metabolism, the final concentrations did not exceed 0.2% for ethanol and 0.05% for methanol.

2.2 Analysis of phenolic compounds

The polyphenol content of the AE[B] was measured in 2007 in the same way as for AE[A], which was analyzed in 2002 [23], to compare differences in composition between the batches. An aliquot of the AE[B] was diluted with the internal standard 3,4,5-trimethoxycinnamic acid and directly submitted to a HPLC-DAD system. Detailed infor-

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Table 1. Phenolic composition in mg/g and fold differences of AE[A] and AE[B] determined by HPLC-DAD analysis (details see Section 2). The polyphenols used for preparing SPM according to AE[A] are in italics.

Substance	AE[A]	AE[B]	Fold difference (AE[A]/AE[B])
Chlorogenic acid	181.5	149.5	1.2
Caffeic acid	4.8	2.7	1.8
p-Coumaric acid	–	1.7	–
4-Coumaroyl-quinic acid	77.3	19.3	4.0
Procyanidin B1	7.0	1.7	4.1
Procyanidin B2	15.1	6.1	2.5
(−)-Epicatechin	19.15	0.6	32.0
(+)-Catechin	–	0.6	–
Phloridzin	27.9	20.0	1.4
Phloretin	–	0.5	–
Phloretin-xyloglucoside	138.9	34.6	4.0
Quercetin-3-O-glucoside	1.4	–	–
Quercetin-3-O-galactoside	0.8	0.5	1.6
Quercetin-3-O-rutinoside	2.6	0.2	13.0
Quercetin-3-O-rhamnoside	4.1	0.6	6.8
Quercetin	–	1.1	–
Sum	480.4	215.9	

mation about HPLC conditions and evaluation has been published elsewhere [31, 32].

2.3 Cell lines and culture conditions

The human colorectal adenoma cell line LT97 represents an early stage of tumor development (kind gift from Professor B. Marian, Institute for Cancer Research, University of Vienna, Austria) [33, 34]. It was established from colon microadenoma of a patient with hereditary familiar polyposis, and has an almost normal karyotype [34]. LT97 cells were maintained in MCDB culture medium according to Klenow *et al*. [35].

2.4 Challenge with CumOOH or H_2O_2 using comet assay

To investigate the influence of AE and the SPM on protection against DNA damage, LT97 cells were incubated with 128 μg/mL AE or SPM (corresponding to AE[A]), respectively, for 24 h. The cells were harvested and challenged with different concentrations of CumOOH (120 – 300 μM; Sigma-Aldrich) or H_2O_2 (18.8 – 150 μM; 30% aqueous solution from Merck, Darmstadt, Germany) in vials prior to embedding them on glass slides. Neither incubation with AE or SPM nor the damage with genotoxic compounds affected cell viability. The working solutions were freshly prepared with PBS for each experiment. First, cells were incubated for 5 min at 4°C to avoid repair of the induced oxidative DNA damage and inhibit enzymatic processes, according to [36]. Second, the protocol for the challenge was modified to 37°C for 5 min to allow enzymatic activity, *i.e.*, GSTT2-2 activity. The comet assay was carried out under the conditions described by Tice *et al*. [37]. DNA damage was microscopically quantified using a ZEISS Axiovert 25 microscope (Carl Zeiss Jena GmbH, Jena, Germany) and the Comet 4.0 image analysis system (Kinetic Imaging Corp., Liverpool, UK). Per sample, 150 cells were analyzed and the percentage of fluorescence in the tail (TI, "tail intensity") was evaluated.

2.5 *GSTT2* expression using real-time PCR

The same treatment conditions as described for the comet assay were applied to LT97 cells before total RNA was isolated using Qiagen RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The quality of RNA samples were characterized by the RNA Integrity Number (RIN) ranging from 8.5 to 9.7 using an Agilent 2100 Bioanalyzer (Santa Clara, United States). First-strand cDNA synthesis from 2.5 μg total RNA was performed by Super Script™II Reverse Transcriptase (Invitrogen, GmbH, Karlsruhe, Germany) using poly (dT) primers. Furthermore, *GSTT2* gene expression in LT97 cells was evaluated by real-time PCR using SYBR Green© as DNA stain (iCycler IQ®, Bio-Rad GmbH, München, Germany) as described by Veeriah *et al*. [22]. The relative mRNA modulation of *GSTT2* is expressed as fold difference to the medium-treated controls normalized to the housekeeping gene *GAPDH*.

2.6 Determination of LT97 cell number

Cell proliferation in the presence of AE or SPM was determined in 96-well microtiter plates using the 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) assay. LT97 cells were grown to a confluence of approximately 40%, exposed

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to 0 – 510 μg/mL AE or corresponding concentrations of SPM and maintained in culture for 24 h. Cell number analysis was carried out as previously described [23]. For each sample and control, the fluorescence of DAPI-stained DNA, which reflects the cell number, was measured (excitation 360 nm/emission 465 nm, TECAN Spectrafluor GmbH, Grailsheim, Germany).

2.7 Determination of H_2O_2 formation

Peroxide production or rather H_2O_2 formation in the cell-free MCDB culture medium in the presence of AE or SPM was analyzed using the ferric-xylenol orange assay (FOX) method [38, 39]. Different concentrations of the test substances (0 – 510 μg/mL, in culture medium) were incubated for 24 h at 37°C in a humidified incubator to allow H_2O_2 formation. Then 40 μL of the test concentrations were mixed with 360 μL of the FOX solution and incubated for 30 min at room temperature. H_2O_2 oxidizes ferrous iron to ferric iron, which can be detected by the formation of a colored complex with xylenol orange. Serial concentrations of H_2O_2 (0 – 300 μM) were processed in parallel for calibration. After centrifugation (10 000 × *g*, 10 min), 2 × 100 μL of the supernatant were transferred to a 96-well plate and absorption was recorded at 550 nm (TECAN Spectrafluor). Concentrations of H_2O_2 were determined from the standard curve.

2.8 Statistical analysis

Statistical analysis was performed using the GraphPad® Prism software Version 4.0 (GraphPad® Software Inc., San Diego, USA). All experiments performed *in vitro* were repeated at least three times independently unless otherwise indicated. Repeated measures (one-way) ANOVA, including Bonferroni's multiple comparison test were used to compare the significance of differences between PBS-treated controls and CumOOH- or H_2O_2-treated cells. Comparisons of medium-treated controls with AE treatments were calculated using repeated measures (mixed model) ANOVA with matched subjects, including paired-wise Bonferroni's multiple comparison test. Differences with p values = 0.05 were considered significant. Formation of significant amounts of H_2O_2 was tested using one-way ANOVA, including Bonferroni's multiple comparison test.

3 Results

3.1 AE batches differed in phenolic composition

The analysis of AE[B] revealed that the overall amount of polyphenols was reduced compared to the previous analysis of AE[A] in 2002 (Table 1). In particular the flavan-3-ols like procyanidins and (–)-epicatechin, and the flavonols, *i.e.*, quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside and quercetin-3-*O*-rutinoside, were present in much higher concentration in AE[A]. Also some deglycosylated metabolites, like phloretin and quercetin were detected in AE[B], indicating degradation processes during storage. To mimic the mixture of AE[A] composition, SPM was prepared to assess whether these particular compounds could be responsible for the effects of AE[A] batch.

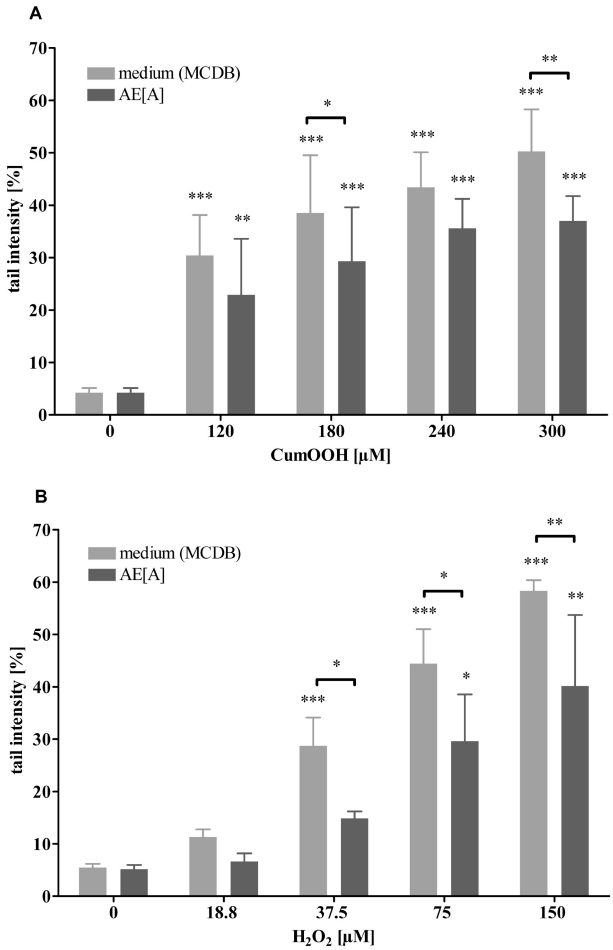

Figure 1. Levels of DNA damage in LT97 cells induced by: (A) 120 – 300 μM CumOOH (5 min at 4°C, n=3) or (B) 18.8 – 150 μM H_2O_2 (5 min at 4°C, n=3) after pre-incubation with AE[A] (128 μg/mL, dark gray bars) or medium control (light gray bars) measured with the standard version of the alkaline comet assay (mean + SD). The significant differences to the untreated control were calculated by one-way ANOVA including Bonferroni's multiple comparison test. The effect of the AE pre-incubation was calculated using two-way ANOVA (repeated measures) including Bonferroni's multiple comparison test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

3.2 Genotoxicity of CumOOH and H_2O_2 was modulated by AE[A]

CumOOH and H_2O_2 were significantly genotoxic in LT97 cells (light gray bars in Figs. 1A and B). In the first set of

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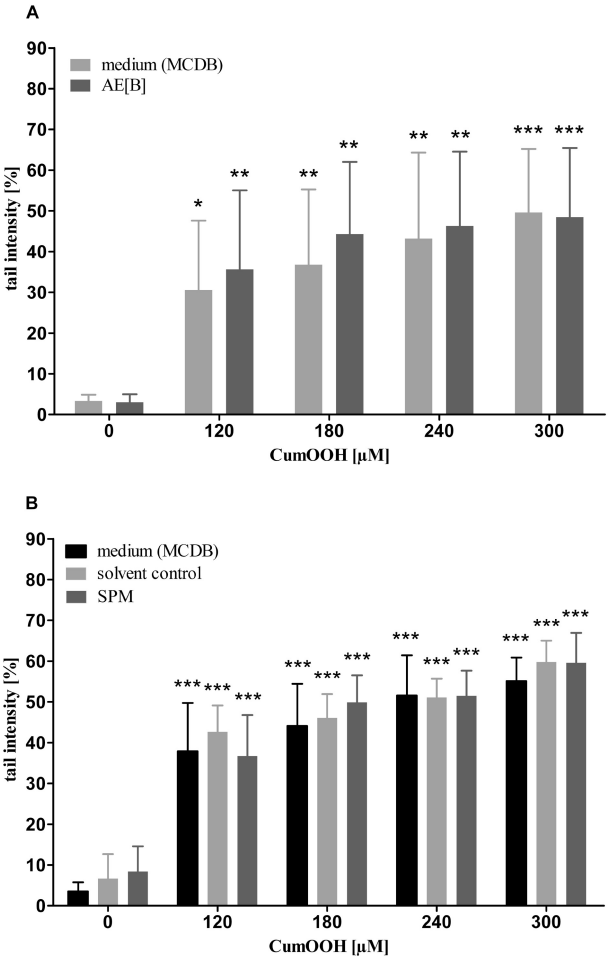

Figure 2. Levels of DNA damage in LT97 cells induced by 120 – 300 µM CumOOH (5 min at 37°C, *n*=3) after pre-incubation with: (A) AE[B] (128 µg/mL, dark gray bars) or (B) SPM (corresponding to 128 µg/mL of AE[A], dark gray bars) measured with the standard version of the alkaline comet assay (mean + SD). The significant differences to the untreated control were calculated by one-way ANOVA including Bonferroni's multiple comparison test. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

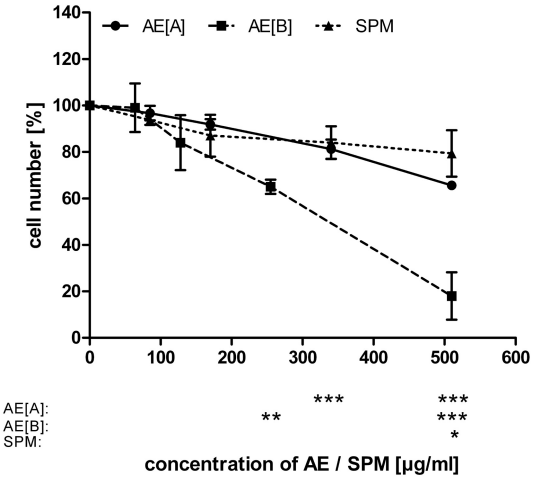

Figure 3. Modulation of cell number by both tested AE batches (0 – 510 µg/mL) and SPM (corresponding to AE[A]). LT97 cells were grown to a maximum confluence of 40% and incubated in a humidified incubator (37°C, 5% CO_2, 95% humidity) for 24 h ($n = 3$). Significant differences to the untreated medium control (cell number = 100%) were calculated by one-way ANOVA, including Bonferroni's multiple comparison test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). The anti-proliferative activity of the AE[A] was already published previously [23].

experiments, LT97 cells were pre-incubated with AE or SPM, respectively, for 24 h and challenged with CumOOH at 4°C for 5 min. AE[A] significantly reduced the CumOOH-induced DNA damage at 180 µM by 23% ($p \leq 0.05$) and at 300 µM by 26% ($p \leq 0.01$) compared to the medium control (Fig. 1A, dark gray bars). For pre-treatment with AE[B] and SPM, respectively, no reduction of CumOOH genotoxicity was detected (data not shown).

Comparable to CumOOH, pre-treatment with AE[A] for 24 h resulted in a significant reduction of H_2O_2-induced genotoxicity of up to 48% compared to medium control (Fig. 1B, dark gray bars).

To simulate physiological conditions, the second set of experiments with AE[B] and the SPM were performed at 37°C for 5 min. Again, AE[B] and SPM had no impact on CumOOH genotoxicity (Figs. 2A and B). Because of depletion of the sample during the experimental work, this experiment could not be performed with AE[A].

3.3 AE[B] and SPM did not increase *GSTT2* mRNA expression

To understand the inactivity of AE[B] and SPM in the comet assay, their impact on *GSTT2* expression was investigated. Veeriah *et al*. [12] summarized the induction of gene expression obtained with AE[A] in LT97 cells with different platforms (Superarray: 3.0-fold, Custom array: 1.5-fold and real-time PCR: 1.8-fold). Repetition of *GSTT2* gene expression analyses with AE[B] and SPM using real-time PCR revealed that neither AE[B] nor SPM were as effective as AE[A] (fold change of 1.0 for AE[B] and 0.7 for SPM).

3.4 Number of cells was reduced more by AE[B] compared to AE[A] and SPM

The anti-proliferative activity of the AE[A] was shown in our previous publication [23]. In the present study, the potential of AE[B] and SPM was compared to AE[A] (Fig. 3). Treatment of LT97 cells with AE[B] with the same concentration range (0 – 510 µg/mL) and SPM (corresponding to AE[A]) caused a concentration-dependent decrease of cell number. After 24 h, the anti-proliferative activities of the SPM was comparable to AE[A]. Only SPM equivalent to 510 µg/mL AE[A] caused a significantly different

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result ($p \leq 0.05$). However, there was a vast difference in response to AE[B]. The reduction of number of cells was much more pronounced. The EC_{50} value of this batch was 333 ± 18 μg/mL, whereas the EC_{50} value was not determinable for the other two test mixtures (AE[A] and SPM).

3.5 Minor concentrations of H_2O_2 formed by AE and SPM

To identify mechanisms behind the detected effects of AE on transcriptional level and the differences of both AE batches and the SPM, their pro-oxidative potential was investigated using the FOX assay. After incubation of the AE (0–510 μg/mL) in LT97 cell culture medium for 24 h, H_2O_2 was detectable but in very low concentrations. The formation of H_2O_2 induced by AE[A] and that by AE[B] were not significantly different. Only for 510 μg/mL AE[A] significant amounts of H_2O_2 (7 μM) were detectable compared to medium control. After addition of SPM to cell culture medium, no H_2O_2 generation was detectable for any tested concentration or for the solvent control. The concentration used for gene expression and comet assay, thus resulted in <1 μM H_2O_2 in LT97 cultures (128 μg/mL AE).

4 Discussion

Phenolic compounds isolated from apples are implicated in preventing diseases related to oxidative stress, like cancer development [40]. For AE[A], prepared from apple juice, an induction of *GSTT2* mRNA has been shown in human colon cells *in vitro* [22]. Since little is known about *GSTT2*, it was of interest to determine whether the modulation of *GSTT2* could result in functional consequences and reduce DNA damage induced by the GSTT2-2 substrate CumOOH. Since the original AE[A] batch was depleted during experimental work, another batch from the same cultivar year and production line (AE[B]) was used for further investigations. Storage conditions of the two AE batches differed, thus the phenolic composition of AE[B] was analyzed to compare this batch to AE[A]. Vast differences in phenolic composition and concentration were detected. It is already known that phenolic compounds undergo various changes during storage caused by oxygen [41], pH [42], UV light [43] and temperature [41, 43, 44]. The resulting degradation products most probably have different biological activities. Storage of the AE resulted in decrease of polyphenols, especially of procyanidins, (-)-epicatechin and quercetin glycosides, whereas aglycons like quercetin and the apple-specific phloretin appeared. These results confirm the findings of Spanos *et al.* [45] that storage of apple juice concentrates for nine month at 25°C causes a decline of cinnamic acids, quercetin glycosides and total loss of procyanidins. Also, experiments with a polyphenol extract of *Myrtus communis* L. demonstrated the instability of flavonoids during 1 year of storage. The unaltered polyphenol composition was only preserved for the initial 3 months and the antioxidant activity of this extract could be provided over this time period [46]. For SPM, polyphenol concentrations equivalent to those of AE[A] were mixed, but the limited commercial availability of the single compounds prohibited the complete reproduction of AE. Substances in SPM were found in both AE batches, but in different amounts. Nevertheless, it should be pointed out that the total composition of the AE has not yet been completely elucidated. It has been estimated that only 50% of all polyphenols in the AE have so far been identified. Recently, Huemmer *et al.* [31] published a method that enabled the detection and quantification of oligomeric and polymeric procyanidins; using this method the elucidation of nearly all the phenolic AE composition is possible.

In a first set of experiments using AE[A] aimed at determining whether the already detected up-regulation of *GSTT2* resulted in reduced DNA damage generated by the GSTT2-2 substrate CumOOH, a distinct protection was seen against CumOOH, as well as against H_2O_2, in LT97 adenoma cells. These data were obtained under conditions that make distinct enzymatic activity unexpected. Thus, there are only non-enzymatic processes that are responsible for detected protective effects. Excluding direct interaction between polyphenols and the genotoxic compounds CumOOH/H_2O_2, modulation of redox state by polyphenols *via* increasing intracellular glutathione (GSH) level is possible. GSH represents one of the most important endogenous antioxidant defense systems, and GSH synthesis is known to be induced in response to polyphenols [47, 48]. The tripeptide GSH can directly detoxify H_2O_2, other peroxides and free radicals; hence, increased GSH levels implicate an enhanced cell protection against exogenously induced oxidative stress [49, 50]. Therefore, GSH should be considered in further investigations using a freshly extracted polyphenol mixture from apple juice.

We have been able to show that pre-incubation of HT29 cells with chlorogenic acid reduced the DNA-damaging effects of H_2O_2 [51]. Since chlorogenic acid is the main polyphenolic compound in both AE batches and SPM, we conclude that the protective action against H_2O_2 could be achieved by this substance. As AE[B] and SPM contain equal amounts of chlorogenic acid, this hydroxycinnamate may be irrelevant for protection against CumOOH.

To investigate possible effects of enzymatic detoxification, the protocol was modified. As the supply of AE[A] was used up, these experiments were not possible with AE[A]. Neither AE[B] nor SPM induced *GSTT2* or protected against CumOOH-induced DNA damage. Thus, we could not confirm a causal correlation between *GSTT2* up-regulation and protection against CumOOH found by others in genetically modified cells [52].

The procyanidins with high molecular weight (400 mg/g AE[A] [31]) might play an important role for the detected

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chemopreventive effects of AE, since they are a predominant polyphenolic fraction in apples and though to have beneficial health properties [11, 53, 54]. Procyanidins have a pronounced instability during storage [45] and they are not commercially available. Thus, they were not present in either AE[B] or SPM, which may explain their inactivity regarding *GSTT2* up-regulation and protection against genotoxicity of CumOOH. Despite this inactivity, SPM showed almost the same effects on LT97 cell number as AE[A], indicating that modulation of *GSTT2* and inhibition of proliferation are mediated *via* different mechanisms. AE[A] and SPM were also equally anti-proliferative in HT29 cells after treatment for 24 h [22]. However, the impact of AE on cell number was more pronounced in LT97 cells compared to HT29 cells [23], and AE[B] showed an even larger effect on LT97 cell proliferation than AE[A]. This result was surprising since this batch contained far fewer polyphenols than the AE[A]. But AE[B] contained more aglycons as a consequence of glycoside degradation. A former analysis of the impact of quercetin glycosides on HT29 cell proliferation demonstrated that it did not reduce cell number even after treatment for 72 h, whereas reduction of HT29 cells was pronounced with quercetin [22]. It has also been demonstrated that reduction of LT97 cell number by quercetin results from apoptosis [55]. Therefore, the more active aglycons, which are only present in AE[B], could be responsible for the observed differences in growth inhibitory properties compared to AE[A] and SPM. The effective concentrations of single compounds in HT29 and LT97 cells by far exceed concentrations detected in AE[B]. For instance, the concentration of AE[B] that reduced LT97 cell number by 80% contained only 1.9 μM quercetin while Kaindl *et al.* [55] found an EC_{50} for quercetin of 48 μM. Furthermore, Srivastava *et al.* [44] found that storage of phenolic extracts reduces growth inhibitory potential of extracts rather than accelerating it.

The pro-oxidative capacity of phenolic substances in aqueous phases may be involved in modulation of cell growth [56]. Fridrich *et al.* [54] found that formation of H_2O_2 induced by apple polyphenols in cell culture medium modulates cellular response even though the mechanism seems to be complex. For LT97 cell culture medium, no or only marginal formation of H_2O_2 with AE or SPM was observed. Nonetheless, we assume that oxidation of apple phenolic compounds still occurs since we found evidence for the oxidation in the brownish color change as described by Kader *et al.* [57]. H_2O_2 formed by this oxidation processes is not detectable due to reduced stability in this particular medium [39]. In addition, results with AE[A] in HT29 cell culture medium (DMEM with 10% FCS), revealed an extensive formation of H_2O_2 (240 ± 8 μM H_2O_2 with 510 μg/mL AE after 24 h, data not shown). Both media differ in their concentration of transition metals. Higher amounts of transition metals in MCDB cell culture medium may result in H_2O_2 instability due to Fenton reaction, explaining low detectable H_2O_2 concentrations. Nevertheless, this would also mean that the cells are exposed to even more reactive oxygen species. Whether the resulting radicals are able to induce intracellular response is doubtful due to their high reactivity.

However, *in vitro* results generated with an isolated mixture of different polyphenols should be interpreted carefully, because recently published data about bioavailability and bacterial degradation of polyphenols demonstrate the actual and physiological situation in the human gut. Kahle *et al.* [58] have shown that after apple juice intake only a limited amount of ingested polyphenols reach the colon unmetabolized in healthy ileostomy patients, *e.g.*, recovery of chlorogenic acid and 3-*p*-coumaroylquinic acid in ileostomy bags was 10.2% and 33.1%, respectively. Therefore, regarding single compounds, 128 μg/mL AE seems to be quite high. But this concentration corresponds to a polyphenol content of approximately 0.5 – 1 L apple juice. Overall, it will be important to repeat the investigations with degradation products of polyphenols reflecting physiological conditions in the human gut more closely.

In conclusion, AE can reduce DNA damage induced by oxidative stress in adenoma cells *in vitro.* Nevertheless, our results indicate that storage conditions caused changes in stability, and thus in the particular composition of the polyphenolic extracts, accompanied by modifications in biological effectiveness of AE. Some possible active compounds of the AE are not part of the SPM as they are either not commercially available or have not been so far identified in the AE.

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