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Abstract

The antigenotoxic effects of umbelliferone (UMB), herniarin (HER) and 7-isopentenyloxy coumarin (7-IP), common natural dietary coumarins, were evaluated on the human lymphocyte DNA damage using single-cell gel electrophoresis. H₂O₂-induced DNA break was measured based on the percentage of DNA in tail, and the antigenotoxic effects of the tested compounds were compared with that of ascorbic acid (10, 25, 50, 100 and 200 μM), UMB, HER and 7-IP did not show any genotoxicity, as compared to phosphate-buffered saline. Treatment with UMB, HER and 7-IP led to a significant reduction in the percentage of DNA in tail induced by H₂O₂ ($p<0.001$) at all concentrations. The presence of prenyl moiety in the chemical structure of 7-IP may contribute to its better antigenotoxic property, compared to UMB. The results of this study showed that 7-IP possessed the best antigenotoxic activity among the tested compounds.

Keywords

Antioxidant, comet assay, DNA, herniarin, oxidative stress, umbelliferone

Introduction

Oxidative stress (OS) is the main reason of several human diseases and plays a key role in the etiology of cancer, cardiovascular diseases and chronic infections (Bonomin et al., 2008; Ishii, 2007; Laviano et al., 2007; Seven et al., 2008). Reactive oxygen species (ROS) are usually produced during regular cell metabolism in the body and lead to OS and DNA damage (Barzilai, 2004). Free radicals are capable of damaging proteins, lipids, DNA and other cell components (Hamilton et al., 2001).

Today, there is an upward trend to substitute synthetic antioxidants by natural ones, and a lot of studies on a wide range of foods, plant derivatives and medicinal herbs explain that these natural products can act as effective anti-inflammatory, -oxidant or -cancer agents (Aravindaram & Yen, 2008). Some natural products showed considerable protective effects against OS (Glei & Pool-Zobel, 2006; Plazar et al., 2008; Ruberto & Baratta, 2000). Therefore, identification and/or developing new antioxidant agents have been the major focus of many research studies (Hung & Yen, 2002; Kogure et al., 2004; Torres et al., 2006).

Coumarins constitute the main class of plant derivatives largely found in families Apiaceae and Rutaceae (Curini et al., 2006; Iranshahi et al., 2003). Plant-derived phenolic coumarins largely occurred in fruits and vegetables and play beneficial roles in the human body as dietary antioxidants. Simple coumarins, such as umbelliferone (UMB) and herniarin (HER), are widespread natural coumarins. They occur in many familiar plants of the family Apiaceae such as carrot, coriander and wild celery and showed promising biological properties (Bhattacharyya et al., 2009; Weber et al., 1998).

For example, Ramesh and Pugalendi have shown that UMB could reverse lipid peroxidation markers (e.g. malondialdehyde and exhaled ethane) to near normalcy and improves antioxidants’ status. Therefore, UMB could be considered as a potent antioxidant (Ramesh & Pugalendi, 2006). 7-Prenyloxy coumarins (another class of coumarins), including 7-isopentenyloxy coumarin (7-IP), auraptene and umbelliprenin, have been known as bioactive prenylated coumarins (Askari et al., 2009), which are produced by diverse plant genus, such as Ferula and Citrus (Iranshahi et al., 2007; Ju-Ichi, 2005). They showed promising biological activities, including anti-inflammatory and cancer chemopreventive effects (Epifano et al., 2009; Iranshahi et al., 2008; Soltani et al., 2009, 2010).

To find a logical correlation between the antigenotoxic effects of these compounds and their chemical structures, we evaluated the antigenotoxicity of UMB, HER and 7-IP on human peripheral lymphocytes. With respect to the occurrence of these natural coumarins in many foods and medicinal
plants, we aimed to study their antigenotoxic activity using comet assay. Antigenotoxic properties of these compounds have not yet been reported. As this method is performed on living cells, it gives us a better view of actual antioxidant activities of compounds in physiological conditions, comparing to antioxidant assays done in chemical environments.

**Methods**

**Preparation of UMB, HER and 7-IP**

7-IP was synthesized in 45% yield by a reaction between 7-hydroxycoumarin (1 M) and isopentenyl bromide (1.5 M) in acetone at room temperature. The reaction was carried out in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 2 M). After 24 hours, the mixture was concentrated under vacuum. 7-IP was purified by column chromatography (petroleum ether/ethyl acetate 9:1, v/v) as white crystals (CAS no.: 10387-50-5; purity, 95%; mp, 74.3–75.2°C). The products identity was confirmed by nuclear magnetic resonance (NMR) experiments, including 1H- and 13C-NMR (Askari et al., 2009). The chemical structures of these compounds are shown in Figure 1. UMB was purchased from Merck (CAS no.: 93-35-6; purity, 98%; mp, 120.0–121.0°C) and used as solvent for DMSO (DMSO; pH 10.0) and incubated at 4°C for 3 hours. Slides were then washed with cold PBS and placed in an electrophoresis tank. DNA was allowed to unwind for 30 minutes in fresh electrophoresis buffer (1 mM of Na2EDTA and 0.3 N of NaOH; pH 13.0). Electrophoresis was carried out at 1 V/Cm (300 mA) and 4°C for 15 minutes. A mixture of H2O2 (25 μM) with each coumarin (UMB, 7-IP and HER) at all concentrations or Vit C (positive control, 10, 25, 50, 100 and 200 μM) was also mixed with 50 μL of cell suspension as in the above-described conditions.

**Isolation of human lymphocytes**

Working with volunteers was in accordance with the guidelines of the declaration of Helsinki and Tokyo for humans and was approved by the ethics committee of Mashhad University of Medical Sciences (Mashhad, Iran). Whole blood was obtained from 10 healthy volunteers (25–30 years of age). Ethylenediamine tetraacetic acid (EDTA; 10%) was collected into a centrifugation tube as an anticoagulant agent and 5 mL of fasting blood was diluted with phosphate-buffered saline (PBS; 1:1). An aliquot of 5 mL of lymphocyte separation medium (Ficoll) was collected in a tube, and the blood sample was carefully layered on it. All stages were performed on ice. Samples were centrifuged for 20 minutes at 2000 rpm, and supernatant was discarded quickly. Gradient-separated lymphocytes were diluted with PBS (1:1) and centrifuged at 1500 rpm for 10 minutes. The cell pellet was resuspended in 0.5 mL of PBS and counted in a Neubauer chamber. Cell concentration was adjusted to 5000 cells/μL in preparation for comet assay. Lymphocyte viability was performed by the trypan blue dye exclusion technique.

**Determination of DNA damage (comet assay)**

The alkaline comet assay was conducted based on the method expressed by Singh et al. (1988). Stock solutions of compounds were prepared and stored at 4°C. Nine hundred and fifty microliters of H2O2 (25, 50, 100 and 200 μM) as a known genotoxic agent, UMB, 7-IP and HER (10, 25, 50, 100 and 200 μM) were mixed with 50 μL of cell suspension simultaneously and incubated at 4°C for 15 minutes. A mixture of H2O2 (25 μM) with each coumarin (UMB, 7-IP and HER) at all concentrations or Vit C (positive control, 10, 25, 50, 100 and 200 μM) was also mixed with 50 μL of cell suspension as in the above-described conditions.

**Statistical analysis**

Differences between groups were evaluated by means of Student’s t-test. Values were expressed as mean ± standard error of the mean (SEM). Statistical significance was accepted at p<0.05. To compare the potency of the compounds, the half-maximal effective concentration value of each compound and Vit C were separately calculated using SPSS software (version 11; SPSS, Inc., Chicago, IL).

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**Figure 1. Chemical structures of UMB (1), 7-IP (2) and HER (3).**
Results

According to trypan blue exclusion assay performed after lymphocytes separation, the viability of cells was approximately 96%. Isolated human lymphocytes were treated with different concentrations of H$_2$O$_2$, UMB, 7-IP and HER. H$_2$O$_2$ (25 μM) induced 45% DNA in tail and was chosen as a suitable oxidant (genotoxic) compound for comet assay pilot study (Figure 2). The cell suspension was incubated with different concentrations of compounds (UMB, HER and 7-IP) with or without H$_2$O$_2$ to evaluate the possible antigenotoxic and genotoxic effects, respectively (Figures 2 and 3). At various concentrations of UMB, 7-IP and HER alone (without H$_2$O$_2$), there was a very slight DNA damage with the percentage of DNA in tail ranging from 0.0 to 5.0%, and the difference with negative control was not significant (p > 0.05; Figures 2 and 4). In contrast, all compounds significantly inhibited H$_2$O$_2$-induced DNA damages (p < 0.001), as compared to positive control. All compounds exhibited a significant inhibitory effect on H$_2$O$_2$-induced DNA damage at the concentrations ranging from 10 to 200 μM (p < 0.001 for all concentrations). The best results were obtained by 7-IP, which reduced the percentage of DNA in tail to below 10% at all concentrations (p < 0.01, compared to UMB and HER). As shown in Figure 3, UMB, HER and 7-IP reduced the percentage of DNA in tail to 3.05–22.4%, 0.63–16.5% and 0.88–8% at the concentrations ranging from 10 to 200 μM, respectively.

Discussion

OS causes damage to proteins, DNA, membrane lipids and cellular organelles and has a direct effect on the progress of early aging, cancers, cardiovascular, neurological and degenerative diseases (Pittella et al., 2009). OS arises during unregulated or prolonged production of ROS, resulting in DNA break. Mutation and increased cell proliferation are the main causes of carcinogenesis induced by DNA damage (Klaunig & Kamendulis, 2004; Wiseman et al., 1995). The antigenotoxic activity of several natural compounds has been previously reported by Mosaffa et al. (Mosaffa et al., 2006; Noroozi et al., 2009). In this study, we investigated the antigenotoxic activity of UMB, HER and 7-IP, using comet assay, which is a sensitive and feasible genotoxic assay (Valverde et al., 1999). These compounds occur widely in daily foods, including Citrus spp, carrot, coriander, golden apple and many other edible plants. Coumarins, a phenolic plant derivative class, are possible scavengers of reactive oxygen radicals (Romero-Jimenez et al., 2005).

To our knowledge, although there are numerous investigations regarding antitumor and other biological activities of these compounds, this is the first report on the antigenotoxic effects of UMB, HER and 7-IP. In our study, 7-IP, UMB and HER significantly inhibited the genotoxicity induced by H$_2$O$_2$ (p < 0.001). Vit C was used as a known antioxidant compound (Deutsch, 1998). 7-IP contains prenyl moiety in its structure and revealed inhibitory activity on phospholipid metabolism caused by tumor promoters in a recent study (Baba et al., 2002). The presence of prenyl and phenol groups may be responsible for this activity. In our study, 7-IP reduced the antigenotoxicity induced by H$_2$O$_2$ similar to Vit C. The protection trend against DNA damage was similar to that of Vit C. Both compounds (Vit C and 7-IP) showed slight increases in DNA damage at the concentration of 100 μM. UMB, which is a known metabolite of coumarins, also showed an inhibitory effect on DNA damage induced by H$_2$O$_2$. Another study also explained an inhibitory activity of UMB on cell proliferation of some carcinoma cell lines (Weber et al., 1998). Among the tested compounds, the best protective effects belonged to 7-IP and HER, followed by UMB. 7-IP contains prenyl moiety in its molecular structure, suggesting that the prenyl group may increase the antigenotoxic effects. Several findings revealed that the presence of prenyl moieties in the terpenoid coumarins plays an important role in their antitumor-promoting activity, as previously reported for xanthones, coumarins, flavonoids and phenylpropanoids (Iranshahi et al., 2008). These findings are confirmed by findings from auraptene and umbelliprenin in previous studies (Epifano et al., 2009; Soltani et al., 2009).

UMB, a hydrophilic compound with a hydroxyl group, showed the least protection on H$_2$O$_2$-induced DNA damage. According to the result of a study by Cao et al., free hydroxyls-containing substances may possess antioxidant activity, but it appears that the presence of prenyl groups may have a role in inhibiting DNA damage (Cao et al., 1997).
Another mechanism for the antigenotoxicity of coumarins is thought to be the interaction between the amino group in mutagens and the carbonyl group of coumarins (Marques & Lin, 2004). Inhibition of MEK1 might be the mechanism through which, some natural or synthetic coumarins show their anticancer and -inflammatory activities. The effect of inhibitors of the ERK/MAPK pathway was evaluated using an in vitro coupling assay for the MAP3Ks-MEK1-ERK2 kinase cascade. Many coumarin derivatives inhibited the activation of the inactivated human MEK1 by upstream MAP3Ks potentially. As it was studied in a human leukemia cell line, coumarins inhibit the production of tumor necrosis factor TNF alpha, which was induced by lipopolysaccharide LPS, and they also inhibit ERK phosphorylation. It was assumed that coumarins act by binding an allosteric site in the inactive conformation of MEK1. Therefore, anti-cancer and -inflammatory properties of coumarins may be the result of their potential to inhibit MEK1 (Han et al., 2005).

**Conclusion**

In conclusion, our results demonstrated that 7-IP, HER and UMB could be considered as antigenotoxic agents in vitro. Further studies at lower concentrations seem to be necessary and are recommended for understanding the exact mechanisms of their inhibitory effects against oxidative DNA damage.

**Declaration of interest**

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**References**


