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Protection against damaged DNA in the single cell by polyphenols

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The protective properties of seven polyphenols against hydrogen peroxide induced DNA damage in human peripheral blood lymphocytes (PBL) were studied using single cell micro-gel electrophoresis. Hydrogen peroxide causes a concentration-dependent increase in single cell DNA strand breakage in human PBL. Quercetin and 7,8-dihydroxy-4-methyl coumarin exhibited the strongest protection, significantly inhibiting 50 μM H_2O_2-induced DNA damage at a range of concentrations of 3.1–25 μM. Curcumin, resveratrol and vanillin protected against DNA damage induced by 50 μM H_2O_2 at a range of concentrations of 6.25–25 μM, but rutin and 7-hydroxy-4-methyl coumarin failed to provide any protection even at concentrations up to 50 μM. Quercetin, 7,8-dihydroxy-4-methyl coumarin, curcumin, resveratrol and vanillin are therefore effective in protection of human single cell DNA from oxidative attack.

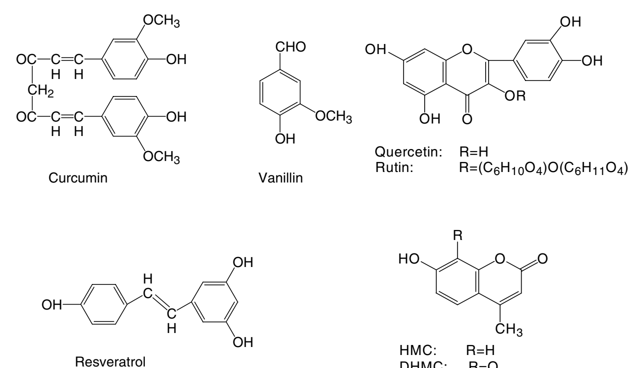
1. Introduction

Phenolic compounds and coumarins are widespread in the plant kingdom, including herbs used for centuries in China as traditional medicines. Some are used as yellow coloring and flavoring agents in food. They have been studied for many years, but inconsistent results have been obtained regarding their antioxidant and reactive oxygen species (ROS) scavenging properties [1, 2]. ROS may cause DNA damage [3] that is believed to be involved in at least two major human problems, aging and cancer.

Single cell micro gel electrophoresis (SCGE), or comet assay, is a rapid, simple and sensitive technique for measuring and analyzing DNA breakage in single mammalian cells [4]. In the present study, seven polyphenols: rutin, quercetin, curcumin, *O*-vanillin, resveratrol and two coumarins, 7-hydroxy-4-methyl coumarin (HMC) and 7,8-dihydroxy-4-methyl coumarin (DHMC) were examined for their protective activities against H_2O_2 induced DNA damage in normal human peripheral blood lymphocytes (PBL) using the comet assay.

2. Investigations, results and discussion

Separated PBL treated with various concentrations of polyphenols were subsequently exposed to 50 μM hydrogen peroxide and the DNA damage was examined by comet assay. Cell viability was tested using trypan blue dye exclusion. The cell viability of each treated group was more than 90%. DNA damaged cells appear as a comet after single cell electrophoresis, while cells with intact DNA retain a circular appearance (Fig. 1).



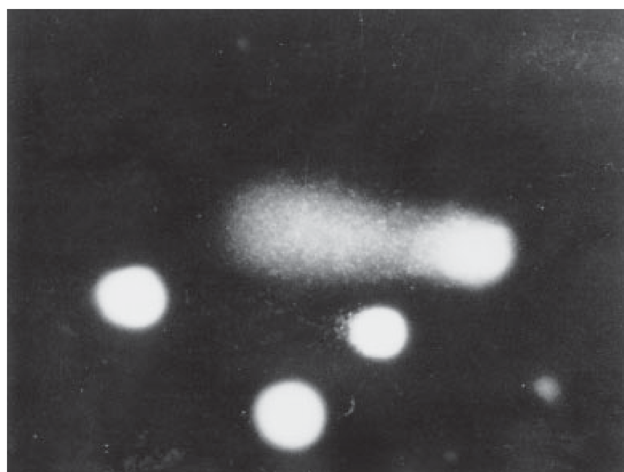


Fig. 1: Cells image after single cell micro gel electrophoresis. Cells with (a long tail) and without (circular) damaged DNA

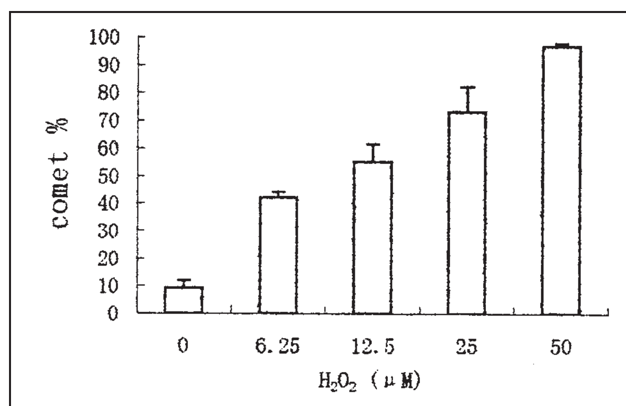


Fig. 2: DNA damage induced by H_2O_2 in human PBL

Hydrogen peroxide caused a concentration-dependent increase in DNA strand breakage in human PBL. Control cells, exposed to phosphate buffered saline (PBS) instead of H_2O_2, showed a comet-like tail in 9.4% of the cells, indicating basal DNA damage. Cells treated with 50 μM hydrogen peroxide for 10 min had a comet-like tail in 96.9% of the cells, indicating extensive DNA damage (Fig. 2). These cells were regarded as negative controls.

The inhibitive effects of the polyphenols are shown in Fig. 3. 50 μM H_2O_2-induced DNA damage was signifi-

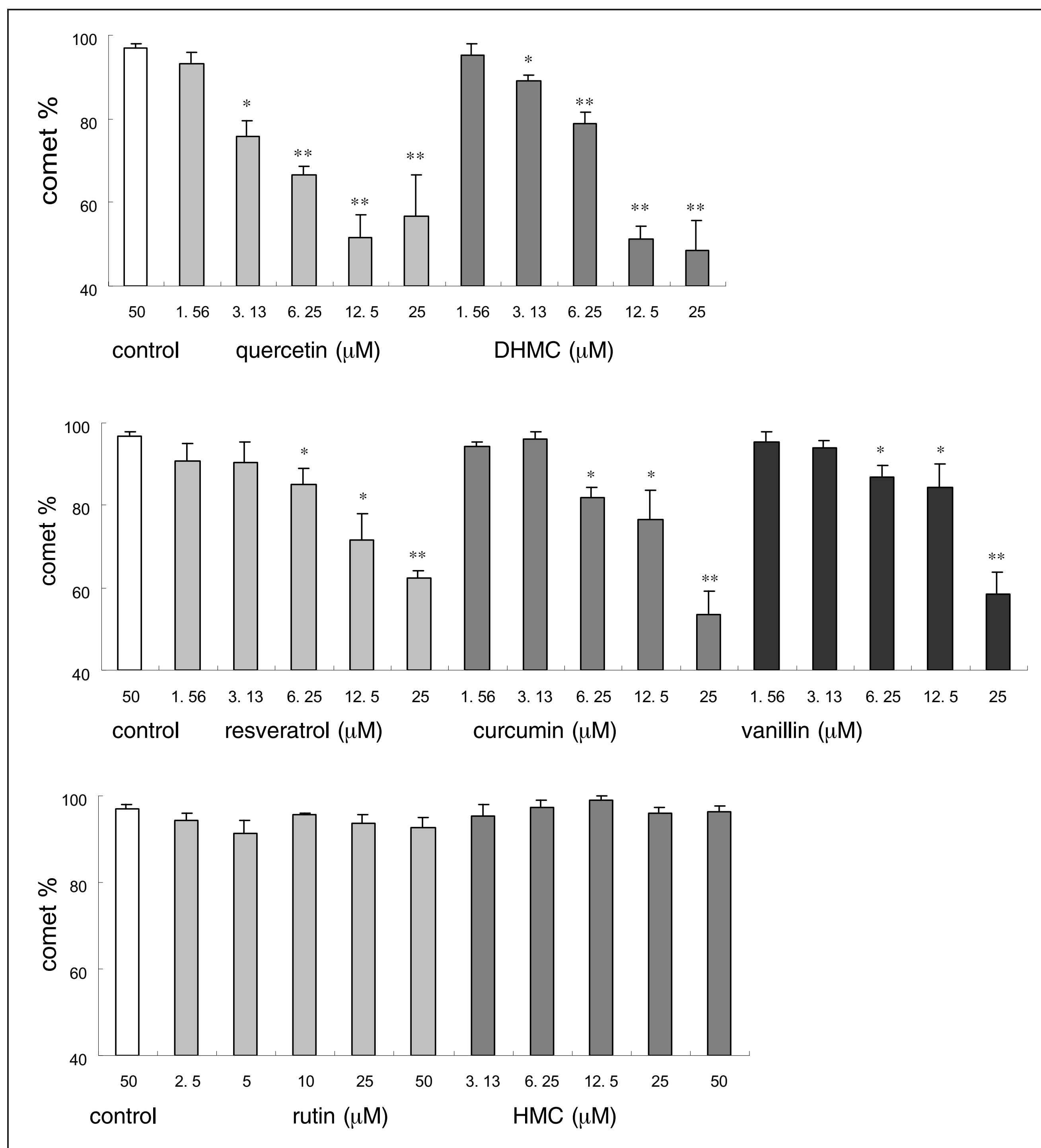


Fig. 3: Protection by various polyphenols against DNA damage induced by H_2O_2 50 μM. ** $P < 0.01$, * $P < 0.05$ vs. control, $x \pm s$, $n = 3 \times 50$ cells

cantly inhibited by quercetin and DHMC at concentrations in the range 3.1–25 μM, while curcumin, resveratrol and vanillin showed inhibitation at concentrations of 6.25–25 μM. Therefore quercetin and DHMC exhibited the strongest protective abilities. Curcumin, resveratrol and vanillin were less active than the of former two compounds. However, rutin and HMC failed to afford any protection even at concentrations of up to 50 μM.

DHMC and HMC have different antioxidant actions in the peroxidation of human low-density lipoprotein [5]. In our study, DHMC exhibited strong inhibition of DNA damage induced by H_2O_2. Therefore, the inhibition by DHMC may be contributed by the 8′-hydroxy group of the coumarin which for DHMC has a hydroxyl at the 8 site of benzene while HMC has none. Vanillin, a naturally occurring food flavoring agent, is also antioxidative [6] and non-genotoxic [7]. Resveratrol, a phenolic compound of the stilbene family present in wines and grapes, has been reported as having stronger activity in scavenging $OH^•$ than vanillin [8]. Our results are different, suggesting that under our experimental conditions the protective effects on DNA damage are not due to scavenging of $OH^•$. Rutin and quercetin belong to the flavonoids, and rutin has stronger activity than quercetin both in scavenging superoxide anions and inhibiting lipid peroxidation [9]. In the present study, quercetin had the strongest antioxidation ac-

tivity of all these compounds but rutin was not effective, suggesting that as the most abundant flavonoid in human diet, quercetin may play an important role in protecting human DNA from oxidative attack.

3. Experimental

3.1. Reagents

Rutin, quercetin, resveratrol, low and normal melting point agarose (LMA, NMA), Triton-x-100, and ethidium bromide were purchased from Sigma Chemical Company. Sodium lauroyl sarcosine was produced by Amresco. Curcumin and *O*-vanillin were produced by Shanghai First Reagent Company. Two synthetic coumarins were provided by courtesy of Liu Z. Q. et al. [5]. Lymphocyte separation medium was produced by Shanghai Second Reagent company. All other reagents were analytical purity grade.

3.2. Method

PBLs were separated from whole blood (obtained from a healthy adult female donor) using lymphocyte separation medium, washed and resuspended in PBS at a concentration of 500,000 cells/ml. The PBLs were then incubated with various concentrations of polyphenols in PBS for 20 min at 37 °C, followed by 10 min incubation at 4 °C in the presence of 50 μM H_2O_2. Cells were washed twice with PBS and viability was tested using trypan blue dye exclusion.
The SCGE was performed as described by Singh [10] with minor modifications. 25 μl cell suspension were mixed with 50 μl of 1% LMA, and then placed on a microscope slide (precoated with 0.5% agarose) at 37 °C and covered with a cover glass. The slides were kept at 4 °C for 5 min for solidification of the agarose. After removing the cover glass, the slide was immersed in lysing solution (1% sodium lauroyl sarcosine, 2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10, and 1% Triton-x-100, 10% DMSO added freshly) for 70 min. This is to lyse the cells and permit DNA unfolding. The slide was then placed in electrophoresis buffer (1 mM Na_2-EDTA and 300 mM NaOH) for 20 min for DNA unwinding, followed by 20 min electrophoresis at 34 V, 310 mA. These electrophoresis conditions had been established in prior experiments for finding the suitable range of DNA damage in PBL.
All the steps described above were conducted in subdued light to prevent DNA damage from UV light. Afterwards, the slides were washed twice with ice cold neutralizing buffer (0.4 M Tris, pH 7.5), dried gently and stained with 25 μl of 20 μg/ml ethidium bromide per slide, and then covered with a cover glass.
Cells were viewed at × 200 magnification with a fluorescent microscope (Olympus BH-2, excited by green light and barrier filter of 590 um) within 24 h. Photomicrographs were taken using Lucky black and white film ASA 400. For each sample three slides were scored, with at least 50 cells counted randomly on each slide. DNA with strand breaks migrates towards the anode during electrophoresis, and the cell appears as a comet. Undamaged cells retain a circular appearance. Cells were graded by eye into 2 categories corresponding to the length of the tail. Comets were defined as having a tail greater than half the length of the head according to Everet [11]. So the percentage of comets (comets%) reflected the levels of DNA damage in cells.

3.3. Statistics

Cells were counted by one investigator (LGA), and tested for significance using the T-test. Two-tailed *p* values were calculated, a value of less 0.05 was taken to imply statistical significance.

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