

Caffeic acid phenethyl ester, an antioxidant from *propolis*,
protects peripheral blood mononuclear cells of competitive
cyclists against hyperthermal stress

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Abstract

Hyperthermal stress and resulting free radical generation is known to impair endurance capacity and immune cell redistribution during prolonged exercise. Caffeic acid phenethyl ester (CAPE), a phenolic compound purified from propolis, has many biological and pharmacological activities including antioxidation. To examine whether CAPE has protective effect against hyperthermal stress in athletes, we isolated peripheral blood mononuclear cells (MNC) from competitive cyclists and assessed their response to hyperthermia with or without CAPE pretreatment. We found that pretreatment of cyclists' MNC with CAPE (0, 1, 2, 4 $\mu\text{g/ml}$) reversed or reduced hyperthermia-induced survival inhibition, necrosis, superoxide production, GSH depletion, and intracellular superoxide burst in a dose-dependent manner. These results suggest that CAPE may enhance the hyperthermal tolerance in immune mononuclear cells of competitive cyclists.

Keywords: Propolis; Cyclist; Mononuclear cell; Hyperthermia; Caffeic acid phenethyl ester (CAPE)

1. Introduction

Heat stress is known to impair endurance capacity during moderate prolonged exercise. A hyperthermal environment adds to thermal constraints and increases the risk of cardiovascular collapse when the evaporation of sweat is augmented, especially during exercise. Blood velocity in the middle cerebral artery is reduced with hyperthermia during prolonged exercise (Nybo and Nielsen, 2001).

Hyperthermia is considered the main factor underlying the early fatigue and dehydration seen during prolonged exercise in the heat (Gonzalez-Alonso, et al., 1999). It has been reported that, to the extent that it causes sympathoadrenal activation, hyperthermia may mediate exercise-induced immune cell redistribution (Rhind, et al., 1999). Exercise causes heat generation and may induce hyperthermia (muscle temperatures of up to 45°C, core temperatures of up to 44°C) and generation of superoxide anion and hydrogen peroxide (Salo, et al., 1991).

Honeybee propolis has been widely used as a folk medicine. Caffeic acid phenethyl ester (CAPE), an active ingredient of propolis (Grunberger, et al., 1988), has a broad spectrum of biological activities including antioxidant (Bhimani, et al., 1993; Jaiswal, et al., 1997; Sud'ina, et al., 1993), anti-inflammatory (Frenkel, et al., 1993; Michaluart, et al., 1999; Mirzoeva and Calder, 1996), and anti-viral actions (Fesen, et al., 1994);

prevention of re-perfusion injury (Koltuksuz, et al.,1999); and anti-cancer effects (Chen, et al., 1996; Lee, et al., 2000; Chen, et al., 2001).

Since hyperthermia and free radical generation are related to exercise-induced physical damage, it is reasonable to test whether an antioxidant can prevent or reduce hyperthermia-induced free radical generation and damage. If it were effective in this way, it might not only promote athletic performance but also prevent injury secondary to endurance-exercise-induced hyperthermia.

We designed this study to test whether CAPE can prevent hyperthermia-induced damage in cyclists' MNC and therefore, enhance their tolerance to hyperthermal stress. We assessed the protective effect of caffeic acid phenethyl ester against hyperthermia-induced changes in cell viability, morphology, superoxide production, and intracellular superoxide content in peripheral blood mononuclear cells (MNC) of competitive cyclists.

2. Materials and methods

2.1. Subjects

Competitive cyclists (N = 5) were recruited. All the subjects were male. Each cyclist had been engaged in endurance training for 3-4 years before the study. No subjects participated in any competitions or intensive training (cycling more than 150 km per week) in the 4 months prior to the study. No clinical illnesses were noted, and no medical or surgical treatment was given during the 4 months prior to blood sampling. After subjects had rested quietly for 30 minutes, blood samples were taken.

2.2. Measurement of cardiopulmonary fitness

To verify the ability to endurance exercise in subjects, their cardiopulmonary fitness was evaluated. Maximal oxygen uptake (VO_{2max}) was measured using a graded maximal treadmill protocol similar to our previous study (Chiang, et al., 2000). In brief, oxygen uptake and ventilation were measured using a system for cardiopulmonary exercise testing (Q-plus IW/Corival 400, Seattle, WA) while exercising on a treadmill (Quinton-645). The subject warmed up by walking at 3.0 mph on a 10% grade initially and ran at increasing speeds with an increment of 0.5 mph per min. A polar pacer heart rate monitor was used to record the heart rate, and the maximal heart rate was noted.

2.3. Isolation of mononuclear cells

Mononuclear cells (MNCs) from the subjects' blood samples were separated by centrifugation on a density gradient (Ficoll-Hypaque, 1.077 gm/ml, Pharmacia Fine Chemicals), and a concentration of 1.5×10^6 cells/ml was incubated in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Hyclone, Logan, UT) and penicillin/streptomycin until treatment.

2.4. Treatments

Freshly isolated MNC from each subject were divided into 5 groups of 5×10^5 /ml each: A, incubation at 37°C for 1 hour (control group); B, incubation at 43°C for 1 hour; C, incubation with 1 µg/ml CAPE for 30 min at 37°C then at 43°C for 1 hour; D, incubation with 2 µg/ml CAPE for 30 min at 37°C then at 43°C for 1 hour; E, incubation with 4 µg/ml CAPE for 30 min at 37°C then at 43°C for 1 hour. Following the allotted treatment times, the cells were incubated at 37°C until analysis. CAPE (structure shown in Fig. 1) was purchased from Sigma (Sigma, St. Louis, MO) and dissolved in absolute ethanol so that the final ethanol concentration was 0.1% v/v regardless of the concentration of CAPE. In our previous studies, this concentration of

ethanol had no significant effect on the viability of MNC.

2.5. Cell viability and surviving fraction

The numbers of cultured viable MNC were counted at 24 hours after treatment using the trypan blue dye exclusion test. Adherent cells were collected by gently rubbing the dishes with a rubber policeman (Bellco Glass, Vineland, NJ) before counting. The surviving fraction of MNC was calculated as follows:

$$\frac{\text{Number of viable cells in each group after 24 hours}}{\text{Number of viable cells in control group after 24 hours}}$$

2.6. Cell morphology

After 24 hours of induction, the MNC were harvested and cytocentrifuged onto a microscope slide using a Cytospin^{2R} (Shandon Southern Instrument Inc., German) and then stained with Wright's stain. Morphological examination was performed by observing 200-400 stained cells under a microscope at a magnification of 1000X. The percentages of necrotic monocytes and lymphocytes were calculated separately.

2.7. Assay for superoxide production

The production of superoxide by monocytes was detected by the nitroblue tetrazolium (NBT, Sigma) reduction test (Johnston, et al., 1975). Cells collected from cultures were suspended in RPMI 1640 medium at a concentration of 1×10^6 /ml and incubated for 30 min at 37°C with an equal volume of NBT test stock solution (containing 2 mg of NBT and 1 μ M of phorbol myristate acetate per ml of PBS). Cytospin preparations were counter-stained with 0.5% safranin O. The percentage of formazan-containing monocytes was assessed microscopically out of 200 cells.

2.8. Fluorocytometric analysis of intracellular glutathione and superoxide anion levels

Intracellular glutathione (GSH) and superoxide anion levels were assessed by staining with 5-chloromethylfluorescein diacetate (CMF-DA) (Molecular Probes, Eugene, OR) and dihydroethidine (HE) (Sigma), respectively. Briefly, PBS-washed MNC were incubated with 1 μ M CMF-DA or 10 μ M HE for 15 min at 37°C before the fluorocytometric analysis. The GSH level was measured with the FL-1 channel (green fluorescence with excitation and emission settings of 488 and 530 nm) and the superoxide level was assessed with the FL-2 channel (red fluorescence with excitation

and emission settings of 488 and 585 nm). The CMF-DA was used to measure the amount of intracellular non-protein thiol. Coates et al have reported that, using this method, the GSH content is proportional to the amount of non-protein thiol (Coates and Tripp, 1995).

3. Statistics

Data were expressed as mean \pm standard error of the mean (SEM). The changes in cell numbers, intracellular GSH and superoxide levels between cells of various groups were evaluated by analysis of variance.

4. Results

4.1. Resting heart rate, maximal heart rate and maximal oxygen uptake (VO_{2max}) of subjects

The resting and maximal heart rate of the cyclists were 51.4 ± 3.0 and 183.3 ± 2.9 beats/min, respectively. The mean VO_{2max} was 75.7 ± 3.2 ml/kg/min.

4.2. Surviving fraction of MNC

The surviving fraction of MNC after 24-hour incubation in the groups B (hyperthermia alone) and E (hyperthermia following 4 μ g/ml CAPE administration) was $78.5 \pm 3.2\%$ and $99.5 \pm 2.7\%$ ($p < 0.05$), respectively. The protective effect of CAPE on hyperthermia-induced inhibition of surviving fraction shows a dose-dependent pattern (Fig. 2).

4.3. Morphological changes

After 24-hour incubation, the percentages of necrotic monocytes and lymphocytes in control group A (37°C) were $6.4 \pm 1.4\%$ and $18.5 \pm 3.8\%$, respectively. These ratios of necrotic cells were markedly higher in group B but not in the CAPE-pretreated groups (Fig. 3).

4.4. Superoxide production

After 24 hours of incubation, $18.1 \pm 1.3\%$ of monocytes in group A, $30.3 \pm 2.1\%$ in group B and $3.3 \pm 1.0\%$ in group E exhibited a positive NBT reduction reaction.

CAPE inhibited the overproduction of superoxide caused by hyperthermia in a dose-dependent manner (Fig. 4).

4.5. Intracellular GSH levels

After hyperthermia treatment, the intracellular GSH levels of monocytes and lymphocytes were only $82.3 \pm 6.6\%$ and $89.5 \pm 3.4\%$ of the levels of group A (control 37°C) cells. Pretreatment of MNC with CAPE reversed this decrease of GSH levels in lymphocytes but not in monocytes (Fig. 5).

4.6. Intracellular superoxide levels

As demonstrated in Fig. 6, the intracellular superoxide level of group B cells was $147.9 \pm 5.0\%$ and $120.6 \pm 3.2\%$ of the level of group A control monocytes and lymphocytes, respectively. Pretreatment of MNC with CAPE reversed this increase of intracellular superoxide levels in a dose-dependent manner. In the same experiment, we used TPA 10^{-7}M as a positive control, resulting in large amounts of superoxide anion (data not shown).

5. Discussion

Our findings indicate that CAPE is a potent protector against hyperthermia-induced oxidative stress and injury in cyclists' MNC. This protective effect may result from antioxidative activity of CAPE.

Aerobic and endurance exercise, such as bicycling, causes an increase in core temperature (Brown, et al., 1993) as well as massive production of reactive oxygen species (Sjodin, et al., 1990), including superoxide anion, hydrogen peroxide and hydroxyl radical, etc. In this study, we found that hyperthermia not only increased the production of superoxide in cyclists' MNC but also decreased their intracellular GSH content. However, this phenomenon of an imbalanced redox state was returned nearly to normal by CAPE pretreatment. Most importantly, CAPE rescued MNC from hyperthermia-induced cell death. This protective effect against hyperthermia implies that CAPE might play a role in preventing immunosuppression during an acute endurance exercise such as a bicycling competition. In a previous study, we reported that CAPE depletes intracellular GSH content and scavenges intracellular hydrogen peroxide but not superoxide in human leukemic HL-60 cells (Chen, et al., 2001). The differential effect of CAPE on GSH and superoxide levels of normal MNC and leukemic cells remains to be elucidated.

Some investigators have reported that strenuous exercise increases heat shock protein (HSP) expression in leukocytes and skeletal muscle immediately after the exertion. This suggests HSP may have a protective role in maintaining function of athletes' cells after heavy exercise (Febbraio and Koukoulas, 2000; Fehrenbach, et al., 2000). We have done a preliminary study in this area, suggesting that CAPE increases expression of mRNA of HSP 70kD 3.1-fold in MNC as assessed by a cDNA microarray assay (data not shown). Therefore, the protective effect of CAPE against hyperthermia may be partly mediated by overexpression of HSP in MNC.

The protective effect of CAPE against hyperthermal stress in the other normal cells and tissues, such as skeletal muscle and myocardium needs to be further investigated.

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Fig. 1. Chemical structure of caffeic acid phenethyl ester (CAPE).

Fig. 2. Effect of CAPE on the surviving fraction of cyclists' MNC in groups B through E. MNC were treated with CAPE (0 – 4 µg/ml) for 30 min in 37°C followed by 43°C for 1 h. The number of viable cells were counted after 24 h and the surviving fraction was calculated by comparison with control group. Data from 5 separate experiments are expressed as mean ± SEM. * $p < 0.05$; significant difference compared with group B.

Fig. 3. Effect of CAPE on the necrotic ratio of cyclists' MNC. A, control; B, 43°C for 1 h; C, CAPE 1 µg/ml for 30 min then 43°C for 1 h; D, CAPE 2 µg/ml for 30 min then 43°C for 1 h; E, CAPE 4 µg/ml for 30 min then 43°C for 1 h. Cells were collected, cytopinned and stained after 24 h and the necrotic monocytes or lymphocytes were counted out of 400 cells. Data from 5 separate experiments are expressed as mean ± SEM. $p < 0.05$; significant difference in monocytes (*) or lymphocytes (#) compared with group B.

Fig. 4. Effect of CAPE on superoxide production in cyclists' monocytes. A, control; B,

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Fig. 5. Effect of CAPE on intracellular GSH content in cyclists' MNC. MNC were treated with CAPE (0 – 4 µg/ml) for 30 min in 37°C followed by 43°C for 1 h. After 24 h MNC were collected, stained with CMF-DA and analyzed by a flow cytometer. * $p < 0.05$; significant difference compared with group B.

Fig. 6. Effect of CAPE on intracellular superoxide anion level in cyclists' MNC. MNC were treated with CAPE (0 – 4 µg/ml) for 30 min in 37°C followed by 43°C for 1 h. After 24 h MNC were collected, stained with HE and analyzed by a flow cytometer. $p < 0.05$; significant difference in monocytes (*) or lymphocytes (#) compared with group B.

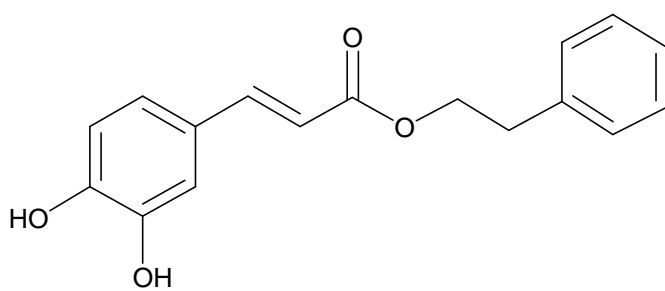


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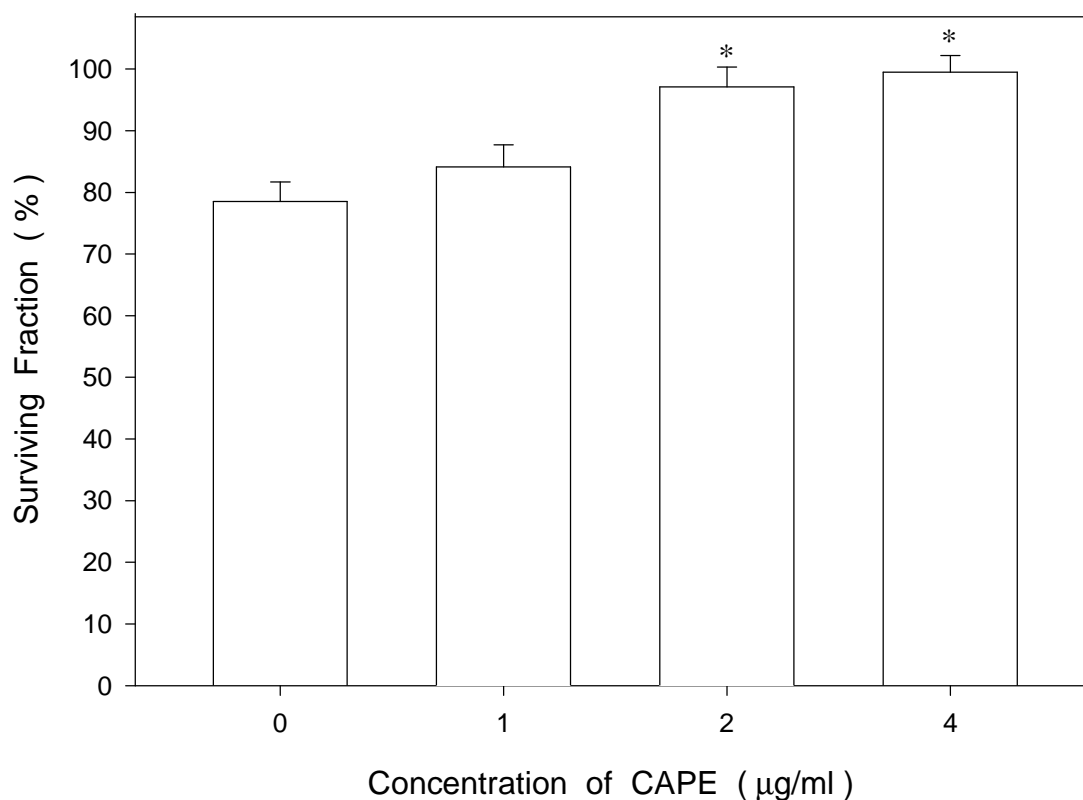


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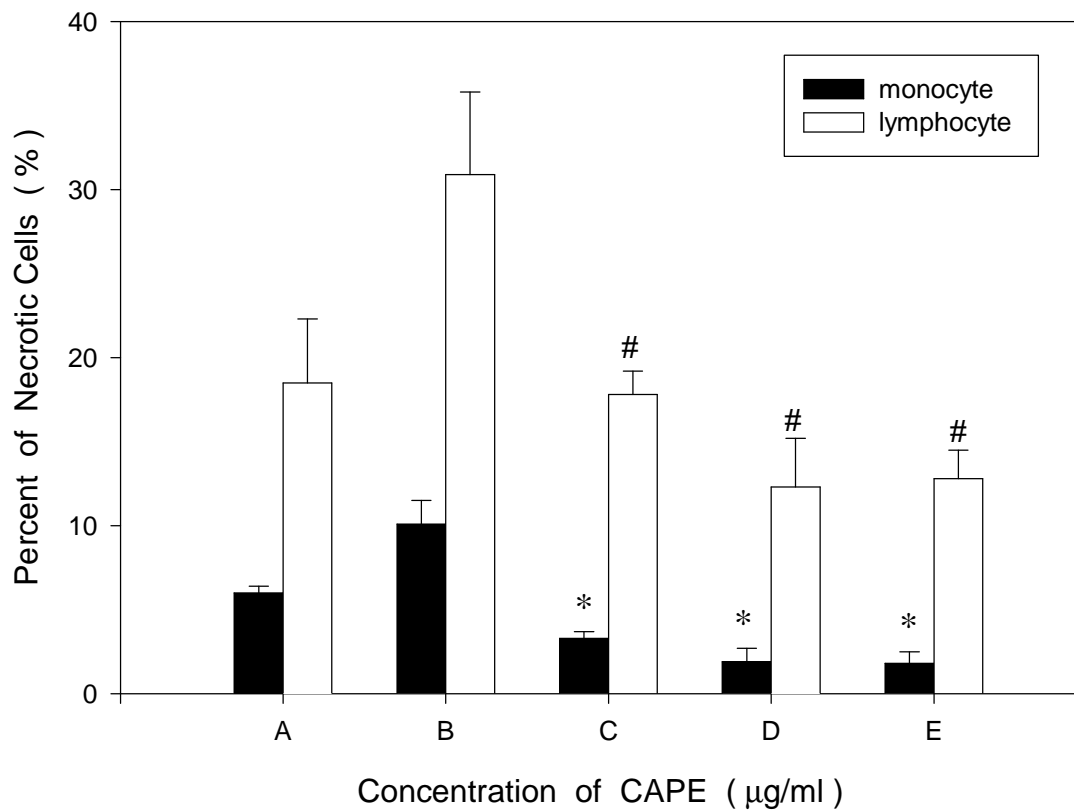


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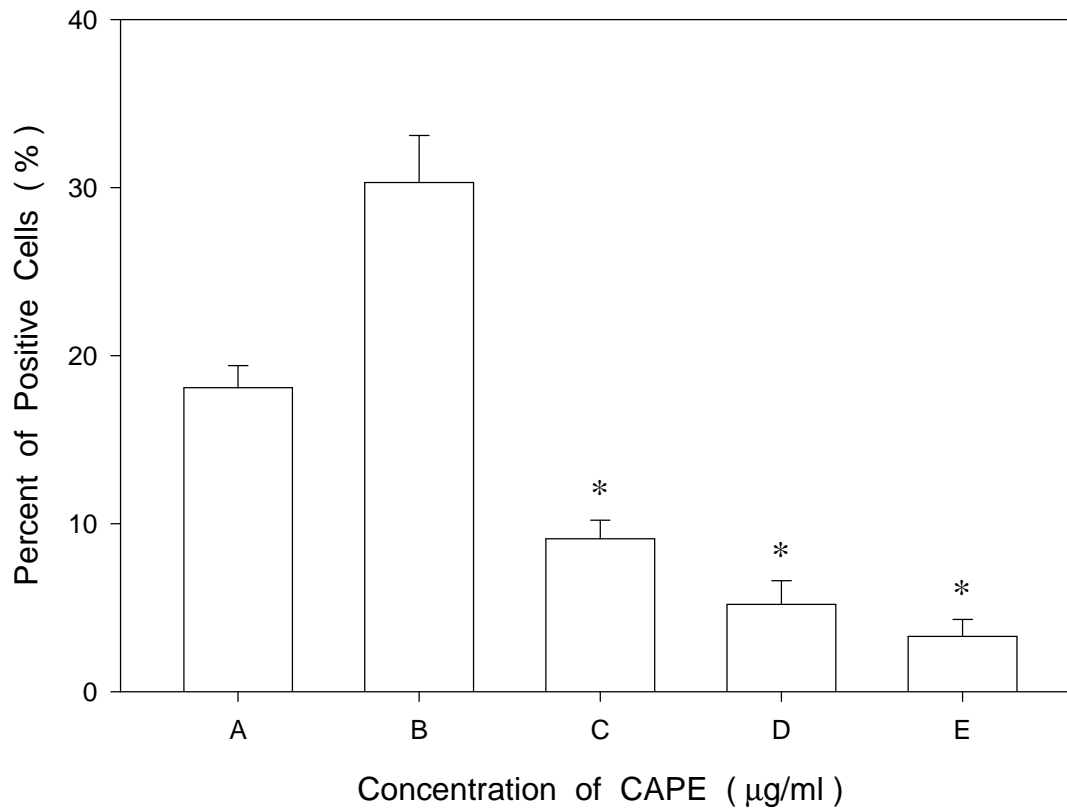


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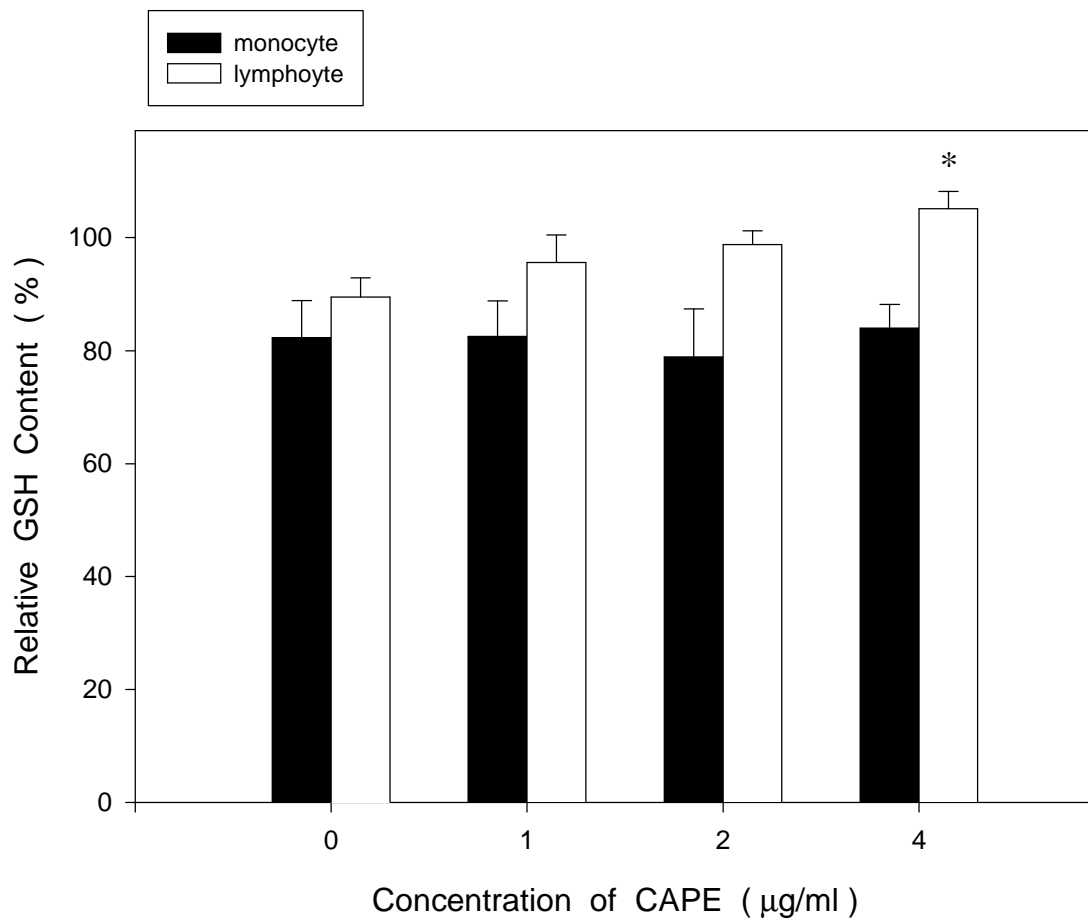


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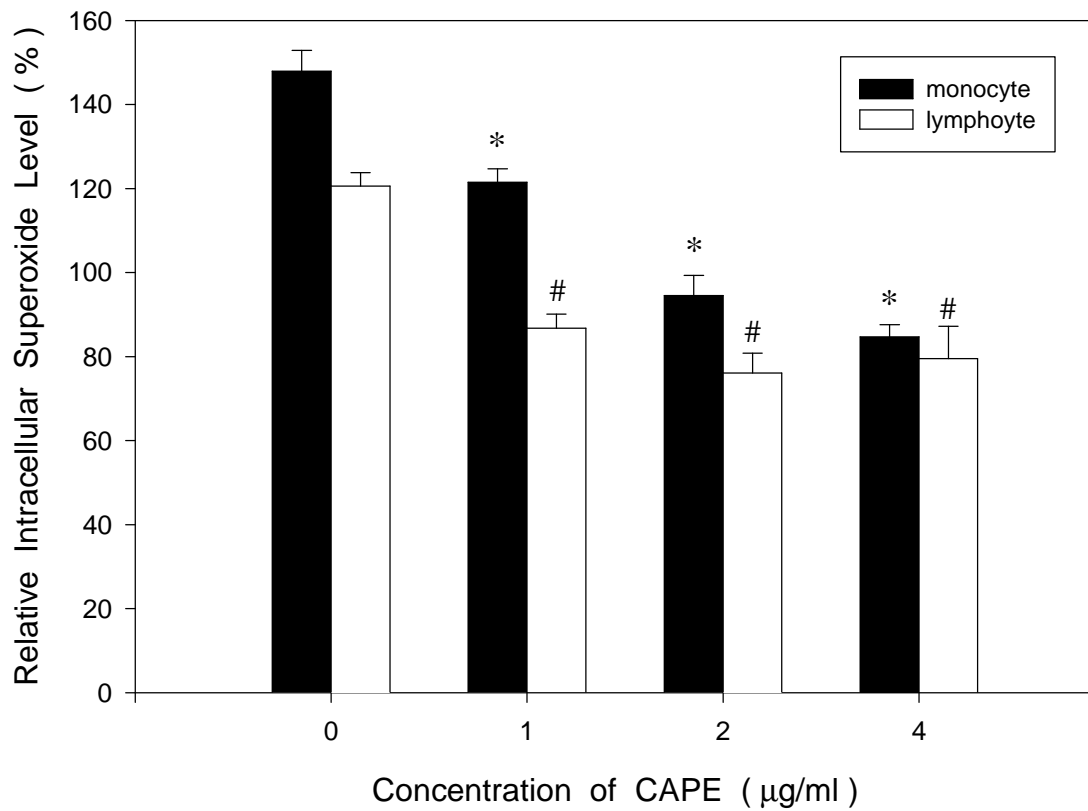


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