Original Paper

Effects of Three Kinds of Curcuminoids on Anti-Oxidative System and Membrane Deformation of Human Peripheral Blood Erythrocytes in High Glucose Levels

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Key Words

Diabetes • Hyperglycemia • Oxidative Stress • Cell Morphology

Abstract

Background/Aims: Curcuminoids are the main bioactive constituents of the rhizome of turmeric. Erythrocytes lesions in diabetes are probably related to hyperglycemia and protein glycation. It has been reported that curcumin prevent lipid peroxidation. However, reports on the effects of demethoxycurcumin and bis-demethoxycurcumin on human erythrocytes at high glucose levels are scarce. Our aim is to investigate the effect of curcuminoids on oxidative stress and membrane of erythrocytes exposed to hyperglycemic condition. **Methods:** In this study, the different blood samples were treated with two doses of glucose (10 or 30 mM) to mimic hyperglycemia in the presence or absence of three kinds of curcuminoids (5 or 10 µM) in a medium at 37 °C for 24 h (Each experiment consists of 20 blood samples from 10 male and 10 female volunteers). The malondialdehyde was checked by HPLC, antioxidase (GSH and GSSG) were measured by LC/MS, SOD was checked by WST-1 kit, morphology and phospholipid symmetry were detected by flow cytometry, confocal scanning microscope and scanning electron microscope. **Results:** The results illustrated that all three curcuminoids reduce oxidative stress damage on the membrane and maintain a better profile for erythrocytes. Furthermore, three curcuminoids had benefit effects on antioxidase. **Conclusion:** The three kinds of curcuminoids supplementation may prevent lipid peroxidation at different intensity and membrane dysfunction of human erythrocytes in hyperglycemia.

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Introduction

Turmeric is extracted from the roots of the *Curcuma longa* plant (Turmeric-Zingiberaceae family). *Curcuma longa* naturally contains a mixture of three kinds of curcuminoids (Curs), namely curcumin (Cur; diferuloylmethane, 85%), demethoxycurcumin (Dem; 15%), and bis-demethoxycurcumin (Bis; 5%) (Fig. 1 A-C). *Curcuma longa* plant is not only known for its use as food or food additive, but also for its therapeutic value in clinical treatment. Indeed, turmeric has been used in traditional ayurvedic and chinese medicine since 3000 B.C. for treating a wide variety of diseases, including coughs, diabetes, hepatic disorders, rheumatism and various types of cancer [1-3].

Curcumin is the most active component of turmeric. Many studies that have been carried out with laboratory animals and human study indicate that curcumin is able to prevents protein glycosylation and decrease lipid peroxidation in high glucose level, reduce hyperlipidemia, to delay cataract and treat the coronary artery disease [4-6]. Curcumin may have potential as an clinical anti-fibrotic agent in type 2 diabetes [7]. It has also been proven that curcumin is able to decrease blood glucose (Glu) levels in type 2 diabetic KK-Ay rats and streptozotocin-treated rats [8, 9]. Another study has reported that the dietary supplementation with curcumin is able to promote wound healing in streptozotocin-treated diabetic rats and genetically diabetic mice [2]. Our previous study (*in vivo*) has also demonstrated that curcumin is able to decrease malondialdehyde (MDA) level in the case of ethanol-induced oxidative damage in hepatocytes [10]. The two other curcuminoids (demethoxycurcumin and bis-demethoxycurcumin) have similar functions. For example, bis-demethoxycurcumin enhances tissue remodeling for diatetic wound healing [11]. Demethoxycurcumin is a promising ingredient of functional food for the prevention or amelioration of complications due to type-2 diabetes [11].

Oxidative stress is considered to be a common complication that is caused by diabetes mellitus. Numerous studies have already illustrated that severe malformation and high lipid peroxidation in erythrocytes are evident markers in diabetic animals and humans. Erythrocyte lesions are probably related to hyperglycemia, protein glycation and sorbitol accumulation [2, 12]. These pathological factors could easily induce serious oxidative stress that causes serious cellular dysfunctions as well as hematic and vascular complications in diabetic patients. Numerous morphological studies have shown that many acanthocytes emerged in peripheral blood and these abnormal cells cannot flow through the small blood vessels in diabetic patients [13]. Recent studies have shown that curcuminoids alleviate lipid damage and maintain the ability of the antioxidant system *in vitro*. For instance, curcuminoids are able to reduce lipid peroxidation, oxygen radical generation and protein glycosylation in erythrocytes with 100 μ M glucose [2, 12, 14]. However, studies investigating the protective effects of bis-demethoxycurcumin and demethoxycurcumin on human erythrocytes with high glucose levels are limited.

This study aims to use human peripheral blood erythrocytes to test the hypothesis that curcuminoids supplementation can reduce oxidative stress and protect membrane of erythrocytes exposed to high glucose levels *in vitro*.

Materials and Methods

Study population and study design

This is an *in vitro* study. The study consists of a series of four experiments. For each of the three curcuminoids (curcumin, demethoxycurcumin and bis-demethoxycurcumin) effects have been tested on lipid peroxidation by MDA, GSH/GSSG, SOD and cell morphology. The MDA was checked by HPLC, antioxidantase (GSH and GSSG) were measured by LC/MS, SOD was checked by WST-1 kit, morphology and phospholipid symmetry were detected by flow cytometry, confocal scanning microscope and scanning electron microscope.

Study population: Volunteers recruited from the clinical department of the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). Different blood samples were from 80 healthy and non-

Fig. 1. A-C Chemical structures of three kinds of Curcuminoids. A: Chemical structure of Curcumin: (E, E)-1, 7-bis(4-Hydroxy-3-methoxyphenyl)-1, 6-heptadiene-3, 5-dione. B: Chemical structure of Demethoxycurcumin: (1E, 6E)-1-(4-hydroxy-3-methoxyphenyl)-7-(4-hydroxyphenyl) hepta-1, 6- diene-3, 5-dione. C: Chemical structure of Bisdemethoxycurcumin: (E, E)-1, 7-Bis (4-hydroxyphenyl)-1, 6-heptadiene-3, 5-dione.

smoking volunteers (40 males aged 25.1 ± 2.6 years old and 40 females aged 26.2 ± 1.9 years old) and blood samples were collected in different tubes containing ethylenediamine tetraacetic acid (EDTA) (The sample size is based on our total sample number. The each experiment consists of 20 blood samples from 10 male and 10 female volunteers). The fasting blood glucose levels in volunteers were 5.2 ± 1.13 mmol/l (40 males) and 5.1 ± 1.77 mmol/l (40 females) respectively. An informed consent was obtained from each participant. All procedures were approved by the Tongji Medical Ethics Committee.

Study design (Sample treatment and setting): In this part, our aim is to set up curcuminoids concentration and incubation time for human erythrocytes.

Human blood samples were treated with for each experiment. Blood sample was centrifuged at 1800 r/min for 10 min. The clear plasma and buffy coat layers were carefully discarded.

The erythrocytes were suspended in phosphate-buffered saline (PBS, 0.01 mol/l, pH = 7.4) with 10% hematocrit. An erythrocytometer (Aibao Biocompany, Shanghai, China) determined the hematocrit and the hemolysis [2]. The fraction of hemolysis was lower than 1% in all incubations. All incubations contained a 10 μ l penicillin-streptomycin/ml cell suspension in order to vitiate any microbial growth during the overnight incubations. The working solution of penicillin-streptomycin contained 300 mg of penicillin G and 500 mg of streptomycin in a buffer.

Aliquots of the cell suspension were placed in Erlenmeyer flasks. The erythrocyte suspension was pre-incubated with the three kinds of curcuminoids for 4 h before the glucose was added [1]. After pre-incubation, the two concentrations of glucose were added in different groups, the cell suspension was continually incubated for 20 h in a shaking water bath at 37°C. The positive and negative control groups were incubated with PBS and two doses of glucose from 0 to 24 h [2]. Subsequently, a freshly prepared stock solution of glucose or curcuminoids was added. The concentrations were expressed in terms of the total cell suspension. The effects of the three kinds of curcuminoids (5 and 10 μ mol/l) on the antioxidative status or on the erythrocyte morphology in 10 and 30 mmol/l glucose were examined (*Curcumin*: 5 μ mol/l Curcumin + 10 mmol/l glucose, 5 μ mol/l Curcumin + 30 mmol/l glucose, 10 μ mol/l Curcumin + 10 mmol/l glucose, 10 μ mol/l Curcumin + 30 mmol/l glucose. *Demethoxycurcumin*: 5 μ mol/l Demethoxycurcumin + 10 mmol/l glucose, 5 μ mol/l Demethoxycurcumin + 30 mmol/l glucose, 10 μ mol/l Demethoxycurcumin + 10 mmol/l glucose, 10 μ mol/l Demethoxycurcumin + 30 mmol/l glucose. *Bis-demethoxycurcumin*: 5 μ mol/l Bis-demethoxycurcumin + 10 mmol/l glucose, 5 μ mol/l Bis-demethoxycurcumin + 30 mmol/l glucose, 10 μ mol/l Bis-demethoxycurcumin + 10 mmol/l glucose, 10 μ mol/l Bis-demethoxycurcumin + 30 mmol/l glucose) [2]. Solutions of 10 and 30 mmol/l glucose without the three kinds of curcuminoids were used as positive control groups (erythrocytes + 10 mmol/l glucose and erythrocytes + 30 mmol/l glucose are positive control respectively). Otherwise, The PBS (0.01 mol/l, pH = 7.4) served as the negative control groups.

Before the biochemical analyses, the erythrocytes were washed twice with a 1:10 dilution of 0.15 mol/L NaCl.

Materials

Thiobarbituric-acid (TBA), reduced L-glutathione (GSH; 99%), oxidized L-glutathione (GSSG; 98%), trifluoroacetic acid, dimethyl sulphoxide, and curcumin ($C_{21}H_{20}O_6$; FW: 368.38, purity: 98%, CAS Number: 458-37-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Demethoxycurcumin ($C_{20}H_{18}O_5$; FW: 338.35, purity: 96%, CAS Number: 33171-16-3) and Bis-demethoxycurcumin ($C_{19}H_{16}O_4$; FW: 308.32, purity: 97%, CAS Number: 33171-05-0) were obtained from Tauto Biotech (Shanghai, China) (Supplementary data. 2). The superoxide dismutase (SOD) kit was obtained from Dojindo Molecular Technologies, Inc. (Tokyo, Japan). The annexin V-fluorescein isothiocyanate (FITC) kit was obtained from Keygen Biotech Company (Nanjing, China).

MDA assay detected by high-performance liquid chromatography (HPLC)

Lipid peroxidation was assessed by measuring the TBA reactivity with MDA. The method was based on a previous report with some modifications [2]. Blood samples were obtained from 10 males and 10 females.

After incubation, erythrocytes (0.4 ml) were suspended in 0.8 ml PBS and 0.025 ml butylated hydroxytoluene (88 mg/10 ml absolute alcohol). Trichloroacetic acid (0.5 ml, 30%) was added. The tubes were vortexed, allowed to stand on ice for at least 2 h, and centrifuged at 1800 r/min for 15 min. For each sample, supernatant (1 ml) was transferred to a new tube, and 0.25 ml of 1% TBA in 0.05 N NaOH was added. The tubes were mixed and placed in a boiling water bath for 1 h. The concentration of the MDA-TBA complex was assessed by HPLC after its separation by ion exclusion using a reverse-phase C-18 column (5.0 mm $\times$ 250 mm, Waters, Milford, MA, USA) with the UV/vis detector set at 532 nm. All data were collected and analyzed by an Empower Workstation 2.0 (Waters, Milford, MA, USA).

GSH and GSSG detection by liquid chromatography/mass spectrometry (MS)

GSH and GSSG contents were determined according to a method presented in a previous report with minor modifications [15]. Blood samples were obtained from 10 males and 10 females.

After the incubation, the blood samples were centrifuged immediately at $600 \times g$ for 5 min. The packed erythrocytes were washed twice with equal volumes of ice-cold isotonic saline solution. Erythrocytes (400 μ l) in ice-cold KCl solution (1.15%) were added. Then, the cells were lysed by freezing and thawing three times to ensure complete lysis [16]. About 50 μ l of the cell lysis were transferred to a 1.5 ml snap cap conical bottom centrifuge vial, to which glutathione ethyl ester (20 ml, 0.01 mg/ml) was added as an internal standard. 2-Nitrobenzoic acid (100 ml, 10 mmol/l; Sigma, St. Louis, MO, USA) was added as well. Then, an MS analysis using positive ion electrospray ionization (Agilent 6404, Santa Clara, CA, USA) was carried out. The HPLC eluate was introduced into the stainless steel electrospray capillary spray held at 2.3 kV. The source and detector voltages were 35 and 655 V, respectively. The low- and high-mass resolutions were set at 12.5 during analysis. Ion monitoring was set to simultaneously monitor the ions with 505 and 613 m/z , which corresponded to the protonated molecular ions of GSH and GSSG, respectively. HPLC was performed using a Phenomenex C18 column (5.0 mm $\times$ 500 mm; 411 Madrid Avenue, Torrance, CA, USA), mobile phase A [aqueous solution with 0.1% (v/v) trifluoroacetic acid], and mobile phase B (acetonitrile). At time zero, the mobile phase A was pumped isocratically for 2 min. Mobile phase B was increased from 0% to 60% from 2 min to 12 min, and held at 60% for an additional 2 min. All flow rates were 0.6 ml/min. The injection volume was 20 μ l. All data were expressed as nmol/mg Hemoglobin (Hb) [17].

Erythrocyte SOD activity measurements with an SOD assay kit

Erythrocyte SOD activity was assayed by using a water-soluble tetrazolium salt, WST-1 kit [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulphophenyl)-2H-tetrazolium monosodium salt]. The procedure was performed strictly in accordance with the manufacturer's instructions (Dojindo, Tokyo, Japan). Blood samples were obtained from 10 males and 10 females.

After the incubation, 0.5 ml blood cells were centrifuged at $600 \times g$ for 10 min at 4°C, and the supernatant was removed. The pellet was suspended with the same volume of saline and centrifuged at $600 \times g$ for 10 min at 4°C. The packed cells were re-suspended in 4 ml of distilled water, 1 ml ethanol, and 0.6 ml chloroform. The mix was vigorously shaken in a shaker for 15 min at 4°C. Subsequently, the mixture was centrifuged at $600 \times g$ for 10 min at 4°C. The upper water-ethanol phase was transferred into a new tube. The samples were reacted by using a WST-1 kit and incubated at 37°C for 20 min. All samples were scanned by their absorbance at 450 nm using a microplate reader (Synergy 2 Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA). The enzyme activity was reported as U/mg Hb in erythrocyte lysate.

Analysis of the protection conferred to the erythrocyte membrane by the three kinds of curcuminoids using flow cytometry, laser confocal scanning microscopy and ultra scanning electron microscopy (USEM)

The protective effects of three kinds of curcuminoids on the erythrocyte membrane were assessed as described in a previous report with some modifications [18]. Blood samples were obtained from 10 males and 10 females.

In cell biology, flow cytometry is a laser-based, biophysical technology employed in cell counting, cell sorting, biomarker detection and protein engineering, by suspending cells in a stream of fluid and passing them by an electronic detection apparatus. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second. After incubation, the cell suspension (4×10^5 cells) was washed twice with PBS. Then, the cells were suspended by a binding buffer kit (300 μ l) in a tube and 5 μ l Annexin V-FITC (Keygene, Nanjing, China) was added for cell labeling. The samples were examined by FACSCalibur flow cytometry (Becton-Dickinson, San Jose, CA, USA). The CellQUEST softwares (Becton-Dickinson, San Jose, CA, USA) were used for data analysis. Forward and sideward scatter profiles were used to define the region of the intact red cell population. The percentage of Annexin V-FITC positive erythrocytes was determined from the fluorescence signal in excess of that obtained with respect to a negative (unlabeled) aliquot of the control sample. The fluorescence signal of each sample was determined to assess the auto-fluorescence properties of the cells after the incubation period.

Furthermore, confocal laser scanning microscopy (CLSM or LSCM) is a technique for obtaining high-resolution optical images with depth selectivity. The key feature of confocal microscopy is its ability to acquire in-focus images from selected depths, a process known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically complex objects. The morphological assay procedure was performed in accordance with the manufacturer's instructions. After incubation, the erythrocytes (1×10^4 cells) were labeled by probe (Annexin V-FITC) in a Petri dish for 30 min in darkness at room temperature. Then, the samples were removed at 488 nm for an observation under a laser confocal scanning microscope (Carl Zeiss LSM510 META, Oberkochen, Germany). Tests were performed in CELLview Glass Bottom Dish (35 $\times$ 10 mm, Cat No. 627 899, Greiner Bio-One GmbH, Maybachstreet 2, Frickenhausen, Germany) in triplicate.

Meanwhile, a scanning electron microscope (SEM) is a type of electron microscope that produces images of a sample by scanning it with a focused beam of electrons. The electrons interact with atoms in the sample, producing various signals that can be detected and that contain information about the sample's surface topography and composition. The electron beam is generally scanned in a raster scan pattern, and the beam's position is combined with the detected signal to produce an image. Therefore, the deformity of erythrocyte morphology was assayed by ultra scanning electron microscopy (USEM). The sample slides were prepared according to a method that had been previously described [19]. Three drops of blood (5×10^6 cells) were directly dripped into a solution with 2% glutaraldehyde in a phosphate buffer (0.05 mol/l, pH = 7.2). The test tube was gently inverted three times, and the blood cells were fixed for 1 h. The erythrocytes were settled by gravity. One drop (1×10^6 cells) from the erythrocyte layer was then mounted onto a piece of glass (2 cm $\times$ 2 cm). Cells that adhered to the section were dehydrated with 50%, 70%, 90%, and 100% ethanol until they were dry. After dehydration, the slides were coated with gold vapor (Eiko IB-3 Ion Coater, Tokyo, Japan). All sections of the photographs were magnified from 5000 $\times$ to 25000 $\times$ by USEM (EDAX FEI QUANTA 200, Amsterdam, Holland). Tests were performed on ultra thin slide in triplicate.

Statistical analysis

All statistical procedures were carried out with the SPSS 11.0 software for Microsoft Windows (SPSS Inc., Chicago, IL, USA). All data were presented as mean $\pm$ S.D. One-way ANOVA followed by the least significant difference point test was used to identify significantly different groups. $P < 0.05$ indicates significant differences between the different groups.

Results

In presence of curcuminoids the MDA concentration in human erythrocytes at high glucose level was lower

As displayed in Fig. 2, the MDA rates of the two positive control groups were approximately 14-fold and 15-fold higher than the PBS group respectively (4.17 ± 0.63 and 5.88 ± 0.77 nmol

Fig. 2. MDA level in erythrocytes when blood samples of 10 male and 10 female subjects were treated with curcuminoids (Cur, Dem and Bis, 5 or 10 µmol/l) under addition of glucose (10 and 30 mmol/l). Results are expressed as means $\pm$ SD. ## indicates a significant difference from the 10 mmol/l glucose group, ** indicates a significant difference from the 30 mmol/l glucose group and && indicates a significant difference from the 10 and 30 mmol/l glucose groups ($P < 0.01$).

MDA/ml in the 10 and 30 mmol/l glucose groups versus 0.28 ± 0.02 nmol MDA/ml in the PBS group; $P < 0.01$). The erythrocytes were treated with 5 or 10 µmol/l curcuminoids that significantly attenuate the MDA levels comparing with two positive control groups (10 and 30 mM glucose) ($P < 0.01$). Among the three curcuminoids, bis-demethoxycurcumin was the substance that was least able to attenuate the lipid peroxidation. The statistics about the age of participants are 10 males aged 25.6 ± 1.3 years old and 10 females aged 26.2 ± 1.7 years old.

In presence of curcuminoids the GSH concentration in human erythrocytes at high glucose level was higher

Fig. 3 illustrates the levels of GSH (Fig. 3A) and GSSG (Fig. 3B) in the erythrocytes. Fig. 3A presents the GSH levels in the different groups. It demonstrated that the GSH levels of two positive control groups were significantly lower than in the PBS group (2937.6 ± 118.4 nmol GSH/mg Hb in the PBS group versus 988.14 ± 77.65 and 641.01 ± 53.28 nmol GSH/mg Hb in the 10 and 30 mmol/l glucose groups; $P < 0.01$). In the three groups of curcuminoids, the GSH levels were obviously higher than the 10 and 30 mmol/l glucose groups ($P < 0.01$).

Fig. 3B demonstrates that the GSSG level in the PBS groups was lower than two positive control groups (10.52 ± 2.19 nmol GSSG/mg Hb in the PBS group versus 127.64 ± 10.75 and 180.33 ± 17.92 nmol GSSG/mg Hb in the 10 and 30 mmol/l glucose groups; $P < 0.01$). The GSSG levels in the three kinds of curcuminoids groups were also remarkably lower than the 10 and 30 mmol/l glucose groups respectively ($P < 0.01$). Among the three curcuminoids, bis-demethoxycurcumin was the least able to reduce the decrease of the GSH level. The statistics about the age of participants are 10 males aged 24.8 ± 1.1 years old and 10 females aged 25.4 ± 1.4 years old.

In presence of curcuminoids the SOD concentration in human erythrocytes at high glucose level was higher

The levels of SOD are summarized in Fig. 4. The SOD activity in the PBS group was higher than in the two positive control groups (1420.17 ± 90.34 U/mg Hb in the PBS group versus 425.74 ± 33.78 and 304.59 ± 22.65 U/mg Hb in the 10 and 30 mmol/l glucose groups; $P < 0.01$). All three curcuminoids groups also were higher than the 10 mmol/l and the 30 mmol/l glucose groups ($P < 0.01$). Among the three kinds of curcuminoids, bis-demethoxycurcumin

Fig. 3. Illustrated curcuminoids (Cur, Dem, Bis) maintained the GSH levels and reduced the GSSG levels (A and B) in erythrocytes. Results are expressed as mean $\pm$ SD (Males = 10, Females = 10). ## indicates significant a difference from the 10 mmol/l glucose group, ** indicates a significant difference from the 30 mmol/l glucose group and && Indicates significant difference from the 10 and the 30 mmol/l glucose groups ($P < 0.01$).

had the worst ability to maintain the enzyme levels. The statistics about the age of participants are 10 males aged 26.4 ± 1.8 years old and 10 females aged 27.2 ± 1.9 years old.

The three kinds of curcuminoids protected the integrity of erythrocyte membranes in high glucose concentrations

The results of the flow cytometry are summarized in Fig. 5A. The three curcuminoids protected the membrane of the erythrocytes. The labling percentage of the erythrocytes in PBS group was lower than in the 10 and 30 mmol/l glucose groups respectively ($0.57\% \pm 0.2\%$ in PBS groups versus $5.34\% \pm 0.81\%$ and $8.86\% \pm 1.02\%$ in 10 and 30 mmol/l glucose groups, $P < 0.01$). The three curcuminoids groups had similar effects in this assay. However, bis-demethoxycurcumin had a lower ability for protecting the membrane of the erythrocytes than curcumin and demethoxycurcumin.

Fig. 5B presents the image of the flow cytometry, the curcuminoids (Fig. 5B₁) and PBS (Fig. 5B₃) groups were less labeled by the fluorescents probe than the positive control group (Fig. 5B₂). Figs. 5C and 5D present the two channels in the laser confocal scanning microscopy (fluorescent and scanning channels). Fig. 5C demonstrated that the probe signal in the positive control group (Fig. 5C₂) was clearly stronger than in the curcuminoids and

Fig. 4. Displayed curcuminoids (Cur, Dem, Bis) reduced the decrease of SOD activity in the erythrocytes. Results are expressed as mean $\pm$ SD (Males = 10, Females = 10). ## indicates a significant difference from the 10 mmol/l glucose group, ** indicates a significant difference from the 30 mmol/l glucose group and && indicates a significant difference from the 10 and the 30 mmol/l glucose groups ($P < 0.01$).

the PBS groups (Figs. 5C₁ and 5C₃, respectively). In the scanning channel, many acanthocytes appeared in the glucose group. The erythrocytes gradually lost their normal shape (Fig. 5D₂). However, Fig. 5D₁ and 5D₃ demonstrate that the curcuminoids were able to partially reverse the damage on the membrane that had been caused by high glucose levels, and that the erythrocytes maintained a normal shape in the PBS group.

The Curcuminoids maintained the normal shape of the erythrocytes

The representative images from the USEM analysis are summarized in Fig. 6. The Fig. 6A demonstrates that the erythrocytes had a normal shape when the glucose concentration was low (portion of acanthocytes: $10.43 \pm 0.97\%$, 10 mmol/l glucose + curcuminoids). The red arrows depict the protection conferred by the curcuminoids to the erythrocyte membrane, as it contains only a small damage. In the group with a high glucose concentration (portion of acanthocytes: $37.69 \pm 2.54\%$, 30 mmol/l glucose + curcuminoids, Fig. 6B), the erythrocytes gradually became rough and the cells became flat due (red arrows). The erythrocytes in the PBS group (Fig. 6C) still maintained their original shape (portion of acanthocytes: $1.75 \pm 0.12\%$). However, in the 30 mmol/l glucose group (portion of acanthocytes: $78.44 \pm 5.12\%$, Fig. 6D), the erythrocytes became disk-like (yellow arrows) and many acanthocytes appeared (red arrows). Fig. 6D displays that the cells lost their function and shape when this high dose of glucose was applied. These figures clearly demonstrate that the curcuminoids are able to protect the membrane in case of high glucose levels. The statistics about the age of participants are 10 males aged 23.7 ± 1.2 years old and 10 females aged 25.8 ± 1.9 years old.

Discussion

The present study demonstrates that the oxidative stress of human erythrocytes was attenuated in the presence of three curcuminoids in hyperglycemia.

Hyperglycemia combined with a prolonged intake of a diet with a high proportion of carbohydrates affects membrane fluidity and the structure of red blood cells (patients with severe exhibited blood glucose levels as high as 45 mmol/l). This phenomenon induces the autoxidation of glucose, the glycation of proteins and the activation of polyol metabolism. Hyperglycemia also accelerates the generation of reactive oxygen species (ROS) and the increases in the oxidative chemical modification of lipids, DNA and proteins in various tissues.

Fig. 5. Demonstrated curcuminoids (Cur, Dem, Bis) protect the membrane of the erythrocytes. Results are expressed as mean $\pm$ SD (Males = 10, Females = 10). ## indicates a significant difference from the 10 mmol/l glucose group, ** indicates a significant difference from the 30 mmol/l glucose group, and && indicates a significant difference from the 10 and the 30 mmol/l glucose group ($P < 0.01$). B: Representative flow cytometry figures. The erythrocytes were labeled with Annexin V-FITC in the curcuminoids groups (B), the positive group (glucose and without curcuminoids) (B2), and the PBS group (B3). C: Representative fluorescent channel images under laser confocal scanning microscopy. The erythrocytes were labeled by Annexin V-FITC (400 $\times$). The probe signals of the curcuminoids (C1, 400 $\times$) and PBS (C3, 400 $\times$) groups were lower than those of the positive group (glucose and without curcuminoids) (C2, 400 $\times$). D: Three representative scanning channel images under laser confocal scanning microscopy. In the curcuminoids group (D1, 200 $\times$), the erythrocytes were deformed (white arrows). In the PBS group (D3, 200 $\times$), the arrows indicate that the cells maintained their appearance. In the positive group (glucose and without curcuminoids) (acanthocytes, white arrows, D2, 200 $\times$).

SOD plays an essential role in reducing oxidative stress in the human body. In the current study, the three curcuminoids groups maintained a higher SOD activity than the two

Fig. 6. Representative erythrocyte images under scanning electron microscopy. (A) In the low glucose concentration group (10 mmol/l glucose + curcuminoids, 25000 $\times$). (B) The high glucose levels (30 mmol/l glucose + curcuminoids). Red arrows indicate that many deformations emerged on the membrane (25000 $\times$). (C) PBS group (25000 $\times$). (D) Blood cells in the positive group (30 mmol/l glucose and without curcuminoids, leptocytes: yellow arrows; acanthocytes: red arrows; 25000 $\times$).

positive control groups. It may relate that β -diketone structure plays an important role in the anti-oxidative mechanism of curcuminoids, because C - C bond cleavage was observed in β -diketone moiety during the anti-oxidative process (β -diketone might act as the free radicals scavenger) [1, 20]. Meantime, our data in positive control groups were similar with earlier studies that the blood of diabetic patients contains a higher amount of acanthocytes and lower SOD activity in acanthocytes than the blood of healthy people [20], these abnormal cells (sickle cells and acanthocytes) have a very low SOD activity and approximately 50% of SOD is glycated in erythrocytes of diabetic patients. Besides, other studies were also similar with our finding in three curcuminoids group that curcumin and curcuminoids could attenuate oxidative damage and maintain antioxidase level *in vivo* or *in vitro* [10, 11]. Therefore, our data support the hypothesis that the mechanism was related to the prevention of excessive SOD exhaustion by three curcuminoids and to progressive glycation of enzymes by high levels of glucose. GSH is the major intracellular non-protein thiol compound [16].

Erythrocytes contain more than 95% of the total blood glutathione. Our data demonstrate that the three curcuminoids are able to maintain higher GSH levels than the two positive control groups and prevent the excessive consumption of GSH. We suppose that the three curcuminoids could attenuate free radicals that attack the membrane in order to prevent the activation of redundant glucose via the polyol pathway [21]. Meanwhile, earlier clinical study has demonstrated a similar result with our data in positive control group that low GSH levels in erythrocytes usually occurred in diabetic, obese diabetic and hypertensive patients

[21]. This phenomenon may be due to a relative depletion of NADPH in order to activate the aldose reductase activation and to reduce its production via the pentose phosphate pathway. This factor impairs the GSH regeneration and leads to the depletion of GSH [22, 23]. On the other hand, based on the present data, the three kinds of curcuminoids significantly reduced the levels of GSSG than positive control groups. We suggest this result that high GSSG concentrations may be caused by glucose penetration into the cell which leads to an increased flux via the polyol pathway in positive control group [21-25].

MDA is a critical index for evaluating the oxidative status of the membrane. MDA molecules may be cross-linked to membrane proteins and membrane phospholipids to form polymers, thereby enabling the cells to show abnormal morphology and malfunction [26]. In our data, three kinds of curcuminoids could obviously reduce MDA levels in human erythrocytes. The previous clinical study has also verified significant data from a study with human subjects. Patients took capsules of curcumin (1 or 4 g/day) for 6 months that exhibited a mean level of curcumin concentration of 0.49 $\mu\text{mol/l}$ in plasma and the high dose curcumin (4 g/day) has not toxic effects on human body [3]. Our results also were similar with previous publications that curcumin was able to significantly reduce the MDA level and oxygen radical production at concentrations of 0.01, 0.1, 0.5 and 1 $\mu\text{mol/l}$ [2]. Some earlier laboratory animal studies have also shown that curcumin reduces the generation of cellular reactive oxygen species [26]. Turmeric, turmerin, and curcumin reduce H_2O_2 -induced injury of renal epithelial cells (LLC-PK1) [27]. Thus, according to our data, the three kinds of curcuminoids were able to decrease the production of oxygen radicals and to counteract lipid peroxidation at high glucose levels. The symmetry of phospholipids is also a critical factor in evaluating membrane damage (membrane symmetry). Our results of the flow cytometry and laser confocal scanning microscopy illustrated that the three curcuminoids protect the membrane and maintain the original cell profiles at high glucose levels. The USEM analysis also indicated that the erythrocytes in three curcuminoids had normal shapes than those from the positive control group. The normal shapes are attributed to the three curcuminoids that attenuate the decrease of the antioxidase activities and alleviate the oxidative damage to the membrane. Many previous studies have also demonstrated that extracellular glucose is able to penetrate directly into (influx) the intracellular matrix due to oxidative stress which inhibits the membrane kinetic and leads to the phospholipid asymmetry of erythrocytes in hyperglycemia [14, 17]. We suppose that the three curcuminoids are able to counteract lipid peroxidation to enhance the fluidity and to prevent phosphatidylserine externalization for the membrane of human erythrocytes [17, 28, 29]. Therefore, the morphology and the flow cytometry analyses further indicated that the three curcuminoids attenuated oxidative damage to the antioxidative system and alleviate peroxidation in order to protect the erythrocyte membrane in high glucose levels.

The three curcuminoids had similar abilities and only minor differences between them were discovered in the present studies. Previous studies had already compared the effects of curcumin, demethoxycurcumin, bis-demethoxycurcumin using different endpoints. The presence of hydroxyl groups at the ortho-positions of the aromatic ring and β -diketone moieties were found to be essential for the quinone reductase activity of natural and synthetic curcuminoids. These structural requirements are met by curcumin, demethoxycurcumin and by bis-demethoxycurcumin, so that they only differ in terms of the extent of the presence of methoxy group(s) [30]. Huang et al. [31] have demonstrated that curcumin was more potent than bis-demethoxycurcumin in the inhibition of tumor promotion in mouse skin. However, it has also been reported that bis-demethoxycurcumin was a more potent antimutagenic agent than curcumin and that it may be more active in the induction of heme oxygenase-1 [32]. Cohly et al. [27] have demonstrated that curcumin inhibits lipid peroxidation more effectively than the aqueous extract turmerin in H_2O_2 -induced renal epithelial cells. Ma et al demonstrated that 30 $\mu\text{mol/l}$ of curcumin activated HSP25 signaling and extreme concentrations of curcumin (100 $\mu\text{mol/l}$) induced the death of podocytes. However, lower concentrations of curcumin (30 $\mu\text{mol/l}$) could better protect cytoskeleton and avoid cytoskeletal disruption [33]. We suggest that extreme concentrations of curcumin reduce

cell viability [33]. This could activate the p53 signaling pathway and block the cell cycle in the G2/M phase (down-regulation of PI3K/Akt and NF- κ B pathways) [34]. Therefore, these studies have illustrated that the activities of curminoids depend on the examined biological action and the cell type.

Otherwise, in this present work, human blood samples pretreated with three curcuminoids for 4 hours with different glucose concentrations, because previously publication has illustrated that pretreatment of curcumin from 0 to 6 h (0.1, 1 and 10 μ M,) could maintain normal symmetry of phospholipids of erythrocytes(pretreatment time = 6 h, $\leq$ 60 % Annexin V binding) [35]. Mehdi et al. demonstrated that pretreatment with curcumin for 4 h could activate signaling of mitogen-activated protein kinase for maintaining differentiation and survival in chondrocytes [1]. We suppose that extreme or higher concentration and incubation time of the three analyzed curcuminoids were able to change the osmotic pressure of erythrocytes dramatically and thereby induce hemolysis [2].

Interestingly, the present results demonstrated that a low concentration of three curcuminoids (5 μ mol/l) leads to different results than a high concentration (10 μ mol/l). This could be due to the fact that in an red blood cell study, the actual concentrations of three kinds of curcuminods entering the cell may be much lower and differences in the kinetics for glucose metabolism and lipid peroxidation or to a lack of sensitivity of the lipid peroxidation assay in comparison to the glucose assay [36]. Previous publication suggested this point that high concentration curcumin triggered a certain degree of eryptosis [35]. Eryptosis could be triggered by increase in the cytosolic Ca^{2+} activity, which leads to cell membrane vesiculation and stimulates cell membrane scrambling resulting in phosphatidylserine exposure at the cell surface [35]. Ca^{2+} further stimulates the cysteine endopeptidase calpain, which degrades the cytoskeleton and thus facilitates cell membrane blebbing (Curcumin could favour the entry not only of Ca^{2+} but as well of Na^+). At adequate energy supply the Na^+ would be eliminated by the Na^+/K^+ ATPase and the stimulating effect of Ca^{2+} on K^+ channels would lead to the expected KCl loss and cell shrinkage [35]. Meanwhile, Flora *et al* also illustrated nanocurcumin (15 mg/kg, orally) could restore GSH level and decrease reactive oxygen species better than normal curcumin in Swiss albino mice [37]. This data indicated that low molecule structure or low concentration of curcumin could enhance chelating property or bioavailability in tissue [37, 38].

Conclusions

In summary, these results had led to hypothesize that supplementation of the regular diet with the three curcuminoids could benefit effects on the antioxidant defense system, increases the activities of antioxidant enzymes, alleviates lipid peroxidation and acts against the deformation of the cellular membrane at high glucose levels.

Abbreviations

Bis-demethoxycurcumin (Bis); Curcuminoids (Curs); Curcumin (Cur); Demethoxycurcumin (Dem); Ethylenediamine tetraacetic acid (EDTA); Glucose (Glu); High performance liquid chromatography (HPLC); Hemoglobin (Hb); L-glutathione, reduced (GSH); L-glutathione, oxidized (GSSG); Liquid chromatography/mass spectrometry (LC/MS); Malondialdehyde (MDA); Phosphate-buffered saline (PBS); Superoxide dismutase (SOD); Thiobarbituric acid (TBA); Ultra scanning electron microscopy (USEM).

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Disclosure Statement

The authors declare that they have no conflict of interests.

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