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Contributed Paper

Antioxidant Properties of *Crocodylus siamensis* Blood Components on H_2O_2-induced Human Skin Fibroblast Cells

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ABSTRACT

To elucidate the antioxidant activity of *Crocodylus siamensis* (*C. siamensis*) blood components on BJ human skin fibroblasts, hydrogen peroxide (H_2O_2), a reactive oxygen species (ROS) causing cellular injury associated with the induction of numerous diseases, was selected as the oxidant in this research. Using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide solution (MTT) assay, *C. siamensis* blood components were found to exhibit no cytotoxicity on BJ cells. However, the oxidative damage induced by 500 µM H_2O_2 to BJ cells led to a significant reduction of cell growth. A pronounced protective effect against this damage was observed upon pre-incubation of the cells with 25, 50 and 100 µg/ml of each *C. siamensis* blood component for 1 h prior to H_2O_2-exposure. The highest potential to preserve cell viability was found for *C. siamensis* hemoglobin (cHb) (89.0%). When observed by visible light microscopy, BJ cells exposed to H_2O_2 displayed diverse morphological alterations, including decreased cell density, cell shrinkage and loss of typical fibroblast appearance. In contrast, BJ cells pre-incubated with *C. siamensis* blood components remained largely intact after exposure to H_2O_2. It could further be shown that treatment with *C. siamensis* blood components did not exhibit any cytotoxic effect on human peripheral blood mononuclear cells (PBMC). To evaluate if the protective effect of cHb against H_2O_2-induced damage to fibroblast cells is correlated to cellular apoptosis, annexin V-FITC/PI-staining flow cytometry analysis was conducted. The collected data clearly demonstrate that H_2O_2 induces apoptosis in human skin fibroblast cells, which was evidently decreased by pre-treatment with cHb. It is therefore concluded that *C. siamensis* blood components possess a great potential to inhibit H_2O_2-induced oxidative damage in BJ human skin fibroblast cells.

Keywords: antioxidant, apoptosis, crocodile, hydrogen peroxide, peripheral blood mononuclear cell, plasma, serum, leucocyte, fibroblast

1. INTRODUCTION

Reactive oxygen species (ROS) are a group of reactive molecules or free radicals derived from molecular oxygen [1]. ROS include not only free radicals, such as the superoxide anion ($O_2^{\bullet-}$) or hydroxyl radical ($^{\bullet}OH$), but also non-radical molecules, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [2]. Several ROS are continuously generated under normal physiological conditions during mitochondrial electron transport in organisms that rely on aerobic respiration or oxidoreductase-mediated metabolism [3]. Additionally, free radicals may be released during peroxisomal fatty acid metabolism and phagocytosis by phagocytic cells, such as macrophages [3]. During normal aerobic respiration, low levels of ROS are generated to regulate numerous signal transduction processes necessary for cellular homeostasis [2]. At excessive concentrations, however, ROS lead to elevated oxidative stress in cells, which may cause severe damage to DNA, proteins and lipids. Consequently, pronounced oxidative stress supports the pathogenesis of various degenerative disorders, including carcinogenesis, neurodegeneration, atherosclerosis, diabetes, inflammation and aging [3-6]. Moreover, numerous studies have identified ROS as a cause of several skin diseases [2, 7-8].

The skin is the largest interface body organ and acts as a guard to prevent harmful effects of temperature, damaging ultraviolet radiation from sunlight, exogenous chemicals and bacterial infections [2, 8]. Oxidative stress induced by excessive ROS production in the skin may induce a variety of clinical conditions, including skin aging, inflammatory skin disorders and carcinogenesis [7]. Thus, natural bioactive compounds that display antioxidant activity while showing relatively low incidences of detrimental side effects are of significant interest for the prevention and treatment of negative effects arising from oxidative stress.

Crocodile blood represents a rich and valuable source for bioactive compounds of natural origin. Scientific research covering potential applications for crocodile blood is predominantly focused on the utilization as treatment or supplement for human health. Notable examples are the application as alternative antibiotics and artificial blood products [9]. Moreover, traditional Chinese medicine uses crocodile blood and other components, such as oil, bile and gall bladder to treat a broad variety of ailments in patients, ranging from bronchitis, coughing, allergy, skin problems, high blood pressure and cancer [9]. With regard to our previous studies about blood of the Siamese crocodile (*C. siamensis*), we found potent antimicrobial property in all investigated components, including serum [10], plasma [11-12], hemoglobin (Hb) [13] and white blood cell extract (WBC) [14]. In addition, these blood components were shown to exhibit promising anti-cancer, anti-inflammatory and antioxidant activity [15-18]. With respect to their antioxidant properties, *C. siamensis* blood components displayed high activity in ABTS (2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) radical scavenging, DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging, nitric oxide scavenging, hydroxyl radical scavenging and linoleic peroxidation assays. Among the investigated blood components, cHb and WBC were found to possess the highest antioxidant activity. Furthermore, we discovered that *C. siamensis* blood components

contain several proteins or peptides with antioxidant activity [13, 15, 18-19], which strongly suggests a potential application as novel natural antioxidants, especially useful as skin care products.

Therefore, to the best of our knowledge, the present work constitutes the first report covering antioxidant effects of *C. siamensis* blood components against H_2O_2-induced oxidative stress in BJ human skin fibroblast cells.

2. MATERIALS AND METHODS

2.1 Crocodile Blood Collection

Crocodile (*C. siamensis*) blood was purchased from Sriracha Moda Farm., Ltd., Chon Buri, Thailand. As previously described, crocodile blood samples were withdrawn from the supravertibral branch of the internal jugular vein of crocodiles aged between 1-3 years. In order to preserve the crocodile whole blood, one fraction of crocodile blood was transferred into a bottle containing EDTA (8 mg/ml) while the other fractions were transferred into 15-ml conical tubes without any anticoagulant for crocodile serum collection. Afterwards, all blood samples were stored overnight at 4 °C. Serum was obtained after induction of complete blood clotting by centrifugation at 2,000 × *g* for 10 min in a refrigerated centrifuge. Plasma, white blood cells (WBCs) and red blood cells (RBCs) were collected individually after the cells in the whole blood sample were allowed to settle in the top (liquid), middle (buffy coat) and bottom layers of the bottle, respectively. The obtained plasma and serum were kept at -70 °C until further use [15, 17-18], while RBCs and WBCs were used as starting material for the extraction of the other *C. siamensis* blood components.

2.2 Extraction of Crocodile Hemoglobin

Crocodile hemoglobin (cHb) was extracted from RBCs according to previously reported methods [13, 17-19]. Briefly, centrifugation at 3,000 × *g* for 5 min was performed after RBCs were washed with ice-cold phosphate buffer saline (PBS), pH 7.0, for three times. After centrifugation, the pellet cells were lysed by adding ice-cold distilled water and incubated for 10 min. cHb in the supernatant was obtained after centrifugation at 10,000 × *g* for 20 min and then kept at -70 °C until use.

2.3 Extraction of Crocodile White Blood Cells

White blood cells (WBCs) were extracted as previously described [14-15]. Briefly, WBCs were washed at least three times with PBS buffer, pH 7.0. After centrifugation at 5,000 × *g* for 10 min, the pellets were re-suspended in 10% acetic acid solution (v/v). The suspended WBCs were extracted by homogenization on ice for 5 min. After that, the homogenate was centrifuged at 12,000 × *g* for 30 min in a refrigerated centrifuge. The supernatant was collected and the acetic acid was removed by a SpeedVac concentrator. The WBC extract was finally dissolved in PBS buffer to yield cWBC.

2.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The protein profile of crocodile blood components, including plasma, serum, cHb and cWBC, was analyzed by 13.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Therefore, equal concentration (5 μg/μl) of each blood component was loaded onto the gel. A low molecular weight kit was run alongside as a standard marker containing six proteins: Phosphorylase b, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa and α-lactalbumin, 14.4 kDa (GE Healthcare,

Sweden). The proteins were visualized as a pronounced band on SDS-PAGE with Coomassie brilliant blue R-250.

2.5 Cell Culture

Human skin fibroblast (BJ) cells were purchased from ATCC CRL-2522, Rockville, MD, USA. BJ cells were cultured in an Eagle's Minimum Essential Medium (EMEM), supplemented with fetal bovine serum (FBS) and antibiotic:antimycotic (Gibco, USA) with a final concentration of 10% and 1%, respectively, in order to create an appropriate culture medium. Cell incubation was performed in a humidified atmosphere of 5% CO_2 at 37 °C.

2.6 Isolation of Peripheral Blood Mononuclear Cells (PBMC)

Whole blood from healthy humans was obtained from Blood Transfusion Center Faculty of Medicine Khon Kaen University (ethical approval number HE601010). PBMCs were isolated from whole blood using Ficoll-Paque (GE Healthcare, Sweden). Blood was diluted with PBS (1:1) and carefully layered on Ficoll medium. Then, centrifugation was performed at 400 × *g* for 40 min at 22 °C. The layer containing the PBMC was carefully transferred to new 15-ml conical tubes. The remaining red blood cells were lysed with lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). Afterwards, centrifugation was performed at 400 × *g* for 10 min at 22 °C and the supernatant was discarded carefully without disturbing the resulting cell pellet. PBMCs were then washed twice with PBS and re-suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 1% antibiotic:antimycotic (Gibco, USA). The cell suspension was adjusted to contain 2×10^5 cells/well for subsequent MTT analysis.

2.6 MTT Cell Viability Assay

The BJ cells were plated at a density of 1×10^4 cells/well into 96-well tissue culture plates and allowed to adhere by culturing in EMEM supplement with 10% FBS. Cell incubation was performed in a humidified atmosphere containing 5% CO_2 at 37 °C. In cell viability experiments, the culture medium was replaced with the same medium supplemented with (0-500 µg/ml) of *C. siamensis* plasma, serum, cHb or cWBC. In the case of PBMC, cells at a density of 2×10^5 cells/well were immediately plated into 96-well tissue culture plates after cell isolation, followed by adding 25, 50, and 100 µg/ml of each blood component (*C. siamensis* plasma, cHb, or cWBC). After 24 h incubation, BJ or PBMC cell viability was evaluated using the MTT assay by addition of 150 µl of MTT solution (MTT, 0.5 mg/ml in EMEM) to each well. Incubation for 4 h at 37 °C in a humidified atmosphere was performed to ensure that living cells with active metabolism are able to convert MTT into a purple colored formazan product, precipitating inside the cells. Afterwards, the formazan product was solubilized by addition of 100 µl of dimethyl sulfoxide (DMSO) and gently tapping or shaking for 1 - 2 min. Then, the absorbance of each well was determined at 550 nm using a micro plate reader, and the cell viability was evaluated by comparing the measured absorbance with the absorbance of untreated cells. All experiments were repeated at least three times.

2.7 H_2O_2-induced Oxidative Stress Assay

The assessment of H_2O_2-induced oxidative stress on BJ human skin fibroblast cells was carried out according to methods reported previously [20-21] with slight modifications. BJ cells were seeded into 96-well plates at a density of 1×10^3 cells/

well. The pre-treatment with crocodile blood components was initiated 24 h after seeding, employing 25, 50, and 100 µg/ml of each blood component (plasma, serum, cHb, and cWBC). After pre-treatment with defined concentrations of crocodile blood components for 1 h, the cells were exposed to 500 µM of H_2O_2 for 24 h and their viability was assessed by the MTT assay as described earlier.

A microscopic technique was used to assess the antioxidant activity of the crocodile blood components. Therefore, BJ cells (1×10^5 cells/well) were plated into 24-well plates and allowed to attach overnight. Afterwards, the cells were pre-treated with defined concentrations of crocodile blood components for 1 h, followed by incubation in the presence of 500 µM H_2O_2 for 24 h. Then, the medium was emptied and washed twice with PBS, and the cells were fixed with 10% neutral buffered formalin for 10 min. The cell morphology was examined after staining with Wright-Giemsa under a light microscope. Untreated cells and cells treated with H_2O_2, which were cultured under the same condition, were used as controls.

2.8 Flow Cytometric Apoptosis Assays

Apoptotic rates were evaluated using a fluorescein isothiocyanate (FITC)-conjugated annexin V/propidium iodide (PI) double staining method (BioLegend, San Diego, CA) in conjunction with a flow cytometer [22]. The fibroblast cells were seeded at a density of 1×10^5 cells/well in 12-well plates and allowed to attach overnight. Afterwards, pre-treatment with cHb at concentrations of 25, 50 and 100 µg/ml for 1 h was performed, followed by exposure to 500 µM of H_2O_2 for 24 h. Adherent cells were collected and washed twice with ice-cold PBS before resuspension in binding buffer at a concentration of 1×10^5 cells/ml. Then, 100 µl of cell suspension were transferred into a 5-ml polystyrene round-bottom tube and labeled with 5 µl of annexin V-FITC and 10 µl of PI. The cell solution was gently stirred and incubated for 15 min at room temperature in the dark. Immediately, after addition of 400 µl binding buffer to each tube the mixture was analyzed on a BD FACSCanto II flow cytometer (BD Biosciences, USA).

2.9 Statistical Analysis

All results are expressed as means ± standard deviation (SD). Analysis of variance was performed for the MTT-based assay of H_2O_2-induced oxidative stress, which was conducted with 3 replications and the least significant difference (LSD) was used to compare means. The difference between groups of data from flow cytometry assay (apoptosis) was analyzed by one-way analysis of variance (ANOVA followed by Dunnett's test). Statistical significance was accepted at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Protein Profile of *C. siamensis* Blood Components

In order to elucidate the molecular mass distribution profile of *C. siamensis* blood components (plasma, serum, cHb and cWBC), 13.5% SDS-PAGE was used for the measurement (Figure 1). The derived results were consistent with previous reports stating that most protein bands of crocodile plasma are associated with a molecular mass of over 27 kDa. Major protein bands were found at around 66 and 120 kDa, which correspond to albumin and gamma globulins, respectively [18]. Moreover, the profile of crocodile serum was found to be very similar to that of crocodile plasma, although the serum contained more protein bands between 14 kDa and 15 kDa. In addition, the protein

profile of cHb also coincided with earlier observations [18, 19]. Two major bands of α- and β-cHb were observed in the cHb profile around 14 and 15 kDa, respectively, while additional faint bands representing dimeric forms of cHb were found at around 28 kDa and 30 kDa. As described by Theansungnoen *et al.* and Phosri *et al.* [15, 18], cWBC contains several proteins that can be divided into two groups with respect to protein size: The first group containing proteins of more than 14 kDa, and another group with a molecular mass lower than 14 kDa. The collected data further indicate that the derived *C. siamensis* blood components contained all major proteins as previously reported, even though they were obtained from different of batches (lots). Furthermore, it was determined that all blood components may still retain their normal biological functions, allowing the use of the samples in further studies.

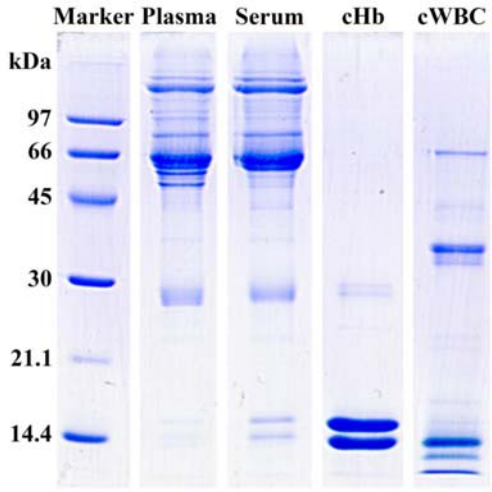

Figure 1. Protein profile of *C. siamensis* blood components derived by 13.5% SDS-PAGE. Equal concentrations of each sample at 5 µg/ml were used for this experiment. lane 1 (Marker): low molecular weight protein marker, lane 2: plasma, lane 3: serum, lane 4 (cHb): hemoglobin, lane 5 (cWBC): crude white blood cell extract.

3.2 Cell Viability/Cytotoxicity

In order to test the anticipated protective properties of *C. siamensis* blood components against H_2O_2-induced oxidative damage in BJ cells, the MTT assay was used for the screening of potential effects on the viability of the cells, as well as for cytotoxicity assessment. The MTT assay constitutes an enzyme-based technique widely used in both cell viability and cytotoxicity tests, which relies on the conversion of MTT into a purple colored formazan product, mediated by a specific mitochondrial reductase only present in living cells. Consequently, a complete loss of MTT conversion ability is observed upon cell death, resulting in clear distinction between living and dead cells and thus allowing an assessment of cell viability by measurement of the formazan-specific absorbance [23]. Herein, we provide evidence that none of the *C. siamensis* blood components displayed considerable cytotoxic effects toward BJ cells after treatment with defined concentrations for 24 h, as visible from the high percentage of cell viability (Figure 2). Cell viability was calculated in comparison to untreated BJ cells, which were considered to represent 100% viability. The highest reduction in

cell viability was found for crocodile serum, with values not exceeding 20%, while other blood components showed only negligible reductions in cell viability, ranging from 5-9%. These results guaranteed that none of the *C. siamensis* blood components in a range of 25-100 µg/ml exhibited cytotoxic effects toward BJ cells, and, in consequence, similar doses were used in the following experiments.

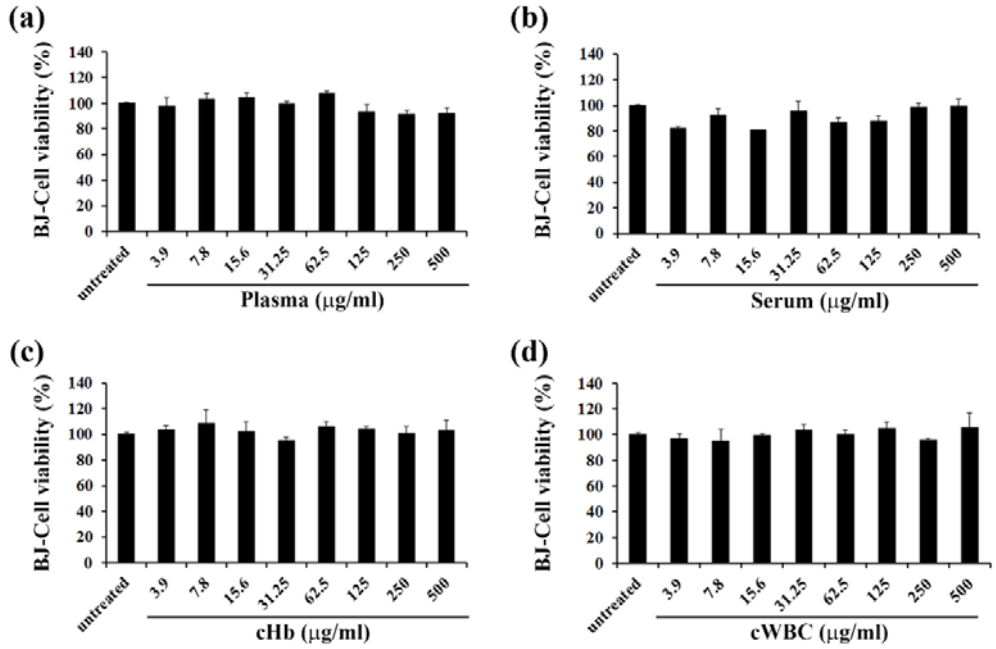

Figure 2. Cell viability/cytotoxicity of *C. siamensis* blood components analyzed using the MTT assay. BJ cells at a density of 1×10^4 cells/well were cultured overnight in a 96-well plate and then co-incubated with *C. siamensis* blood components at defined concentrations ranging between 0-500 µg/ml for 24 h before assessment by the MTT assay. (a) plasma, (b) serum, (c) hemoglobin (cHb) and (d) crude white blood cells extract (cWBC). Each result was expressed as mean ± SD.

3.3 Protective Effects of *C. siamensis* Blood Components on H_2O_2-Induced Oxidative Stress in BJ Cells

H_2O_2-mediated induction of oxidative stress in cell culture models has been utilized in numerous studies as a powerful methodology for the discovery of novel, naturally derived antioxidant compounds [6, 20-21, 24]. H_2O_2 is generally considered the most important ROS and acts further as a precursor for numerous other ROS with the potential to induce severe cellular injury, consequently leading to the occurrence of a variety of diseases related to free radicals [20, 25]. In recent research, statistical tests revealed that a concentration of 500 µM H_2O_2 is sufficient to induce significant cytotoxicity to BJ cells. Hence, a reduction of cell viability by approximately 50% was observed for BJ cells in the MTT assay (Figure 3). However, pre-treatment with 25, 50 and 100 µg/ml of each *C. siamensis* blood component for 1 h before exposition to H_2O_2 decreased the observed cell toxicity significantly (Figure 3a, c, d) in all cases, except for serum treated cells (Figure 3b). The elevated cell viability of

BJ cells after pre-treatment was found to range between 60.7-66.0% for plasma (Figure 3a), 48.5-52.9% for serum (Figure 3b), 83.4-89.0% for cHb (Figure 3c), and 67.2-71.8% for cWBC (Figure 3d), respectively. Consistent with earlier reports [18], the highest potential to elevate cell viability was observed for cHb-treated cells (89.0%). In contrast, serum treatment exhibited the lowest protective effects against H_2O_2-induced oxidative damage (48.5%), indicating that *C. siamensis* serum has little to no potential to enhance cell viability with respect to H_2O_2-induced cytotoxicity.

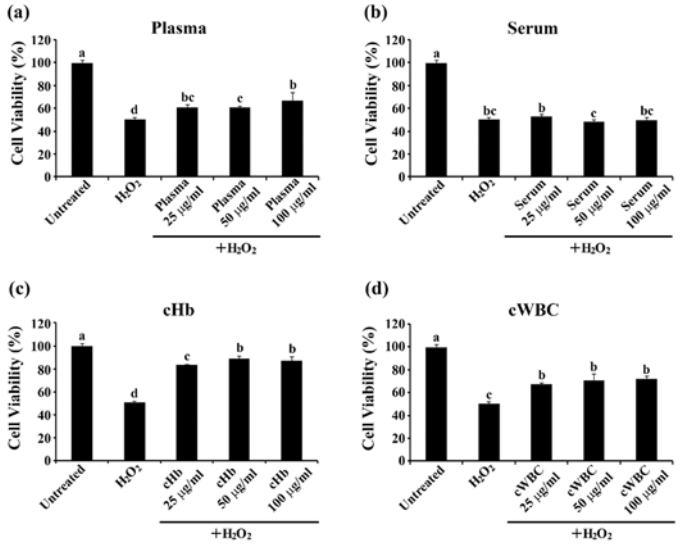

Figure 3. Effects of *C. siamensis* blood components on H_2O_2-stimulated oxidative stress in BJ cells. Data were reported as viability of cells either pre-incubated with defined concentrations of each *C. siamensis* blood component only, or co-cultured with H_2O_2. (a) plasma, (b) serum, (c) hemoglobin (cHb) and (d) crude white blood cells extract (cWBC). Each value is shown as the mean ± SD. Different letters on the top of the bar represent statistically significant differences ($P < 0.05$).

A number of examples for *in vitro* antioxidant activity of *C. siamensis* blood components have recently been reported [13, 15, 18-19]. Crocodile plasma was found to be potent in suppressing ABTS (13.62%) and hydroxyl (57.27%) radicals [18], while cHb and cWBC inhibited linoleic peroxidation (68.7% for cHb and 78% for cWBC) and displayed antioxidant activity against DPPH (47.3% for cHb and 38% for cWBC), ABTS (24.88% for cHb and 40.18% for cWBC) and NO radicals (40.58% for cHb and 33.6% for cWBC) [15, 18-19]. However, no data for antioxidant activity of crocodile serum have been reported up to date. According to this information, it is plausible to conclude that cHb and cWBC bear a high potential to alleviate H_2O_2-induced intracellular damage of BJ cells, owing to their excellent capability to inhibit a variety of *in vitro* free radicals.

Zilinskas et al. [26] reported a higher antioxidant capacity for blood plasma in patients with periodontitis than for plasma in healthy individuals, while the antioxidant capacity of serum was found to be lower. Therefore, the antioxidant properties of *C. siamensis* plasma might be related to the total antioxidant capacity of several enzymes

(superoxide dismutase, catalase, and glutathione peroxidase) present within the plasma. Previous work covering cHb indicated that the antioxidant activity of proteins might be exerted by specific domains or functional groups in each subunit that could act as electron donors, eventually leading to the termination of radical chain reactions. Examples include aliphatic and aromatic hydrophobic amino acid residues (Valine, Leucine, Proline, Histidine or Tyrosine) [13, 19]. In analogy, Theansungnoen *et al.* revealed that cWBC contain several peptides which were presumed to be responsible for their antioxidant properties [15]. The activity of these peptides was attributed to the presence of basic amino acids, such as Lysine and Arginine, and protonated amino acids, including Asparagine, Glutamine and Threonine [15], which were proposed to inhibit free radicals by proton donation [15, 27].

Morphological changes of BJ cells were observed under a light microscope after pre-treatment with defined concentrations of *C. siamensis* blood components, followed by incubation with H_2O_2 (Figure 4). Compared with healthy BJ cells (untreated), cells exposed to 500 µM H_2O_2 displayed morphological changes similar to previously reported observations [20-21, 28]. The alterations of cell morphology included a decrease of cell density, cell shrinkage and loss of typical fibroblast appearance. However, upon pre-incubation with *C. siamensis* blood components, the BJ cells remained intact and retained the typical fibroblast shape after H_2O_2-exposure. Furthermore, the distribution of cells on a 24-well culture plate was found to be normal and homogeneous. It is noteworthy that serum treatment resulted in the lowest observable cell density, thus supporting our previous assertion that *C. siamensis* serum does not have the potential to elevate cell viability toward H_2O_2-induced cytotoxicity.

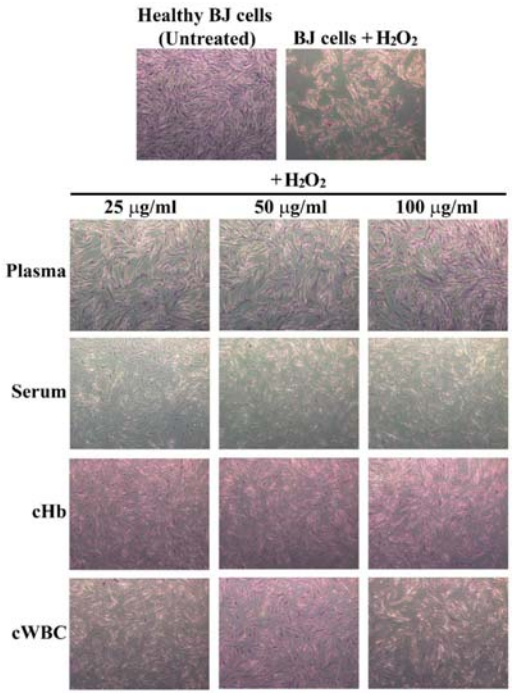

Figure 4. Light microscope photographs of BJ cells under H_2O_2-induced oxidative stress. Investigation of the ability of individual *C. siamensis* blood components to prevent oxidative damage to BJ cells was carried out by pre-incubation with defined concentrations of each *C. siamensis* blood component for 1 h and subsequent co-culturing with H_2O_2. Healthy (untreated) cells and cells treated with 500 µM H_2O_2 were used as controls.

3.4 Cytotoxicity of *C. siamensis* Blood Components on Human PBMC

Peripheral blood mononuclear cells (PBMCs) belong to a population of immune cells characterized by a round nucleus, such as lymphocytes, monocytes or macrophages. These blood cells are an important component of the immune system and help combatting infection and adapting to pathogenic intruders. Extraction of PBMCs from whole blood can be performed using

Ficoll, which facilitates the separation of the individual blood layers formed by gradient centrifugation. Using this technique, the blood will be partitioned into a top layer consisting of the plasma, followed by a lower layer of PBMCs and a fraction of polymorphonuclear cells (such as neutrophils and eosinophils) and, finally, a bottom layer containing the erythrocytes [29]. PBMCs are widely used in research as well as for clinical or toxicological applications [30]. Therefore, a cytotoxicity assay was performed in order to elucidate safe treatment concentrations of *C. siamensis* plasma, cHb and cWBC by which no toxic effects on PBMC cells were observed after 24 h of incubation. The cytotoxicity results demonstrated that all *C. siamensis* blood components were safe within the investigated concentration range and induced no toxicity to human PBMC immune cells. Moreover, the PBMC population was found to exhibit enhanced cell proliferation in response to 25-100 µg/ml of either *C. siamensis* plasma, cHb or cWBC (Figure 5). Taken collectively, these results imply that the investigated *C. siamensis* plasma, cHb and cWBC contain substances with antioxidant activity, which were considered non-toxic for human PBMC cells over a broad range of concentrations.

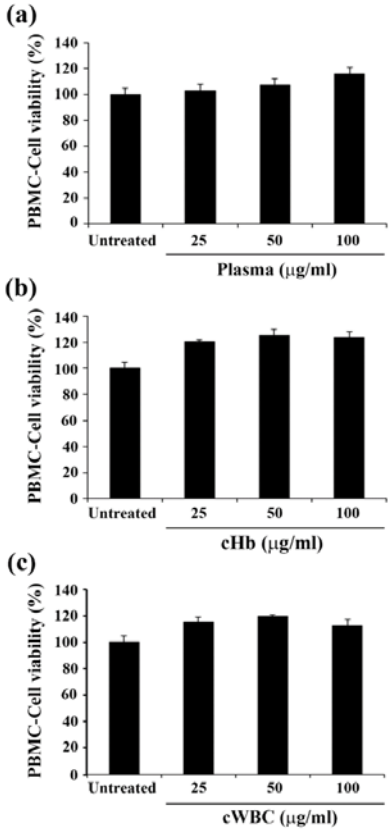

Figure 5. PBMC cells cytotoxic assay. PBMC cells were isolated from whole blood of healthy humans using Ficoll-Paque (GE Healthcare, Sweden). A cell suspension of 2×10^5 cells/well was seeded in 96-well plates and different quantities (25-100 µg/ml) of *C. siamensis* blood components were added. The impact of the individual *C. siamensis* blood components on the cell viability was assessed after treatment for 24 h using the MTT assay. (a) plasma, (b) hemoglobin (cHb) and (c) crude white blood cells extract (cWBC).

3.5 Apoptosis

Apoptosis refers to the process of programmed cell death, which ensures effective cellular disposal in response to development anomalies and specific environmental conditions. The ability of cells to undergo apoptosis can be induced by diverse stimuli, common signaling mediators and ROS (i.e. H_2O_2). In this context, H_2O_2 has been shown to be capable of inducing apoptosis [24, 31]. Hence, inhibition of apoptosis was considered as a plausible mechanism explaining the protective effects of *C. siamensis* blood on H_2O_2-induced oxidative stress in human skin fibroblast cells. Among all crocodile blood components, cHb represents the most abundant constituent and showed the strongest ability to protect cells from H_2O_2-induced oxidative damage. Therefore, cHb was chosen to represent *C. siamensis* blood in the apoptosis assay used in the present study. The results demonstrated that 500 µM H_2O_2 induced significant oxidative stress-related apoptosis as shown in Figure 6. The induction of early apoptosis, as defined by Annexin V positive but PI-negative staining, was determined as 14.5% increase compared to untreated cells. Late apoptosis (both Annexin V and PI positive) was significantly increased by 32.3%, exceeding the degree of early apoptosis. Notably, no significant increase in either early or late apoptosis could be seen when human skin fibroblast cells were pre-treated with cHb at a concentration of 25, 50 or 100 µg/ml. Therefore, the data demonstrate that *C. siamensis* blood has the potential to protect cells from oxidative damage induced by H_2O_2 via inhibition of cellular apoptosis.

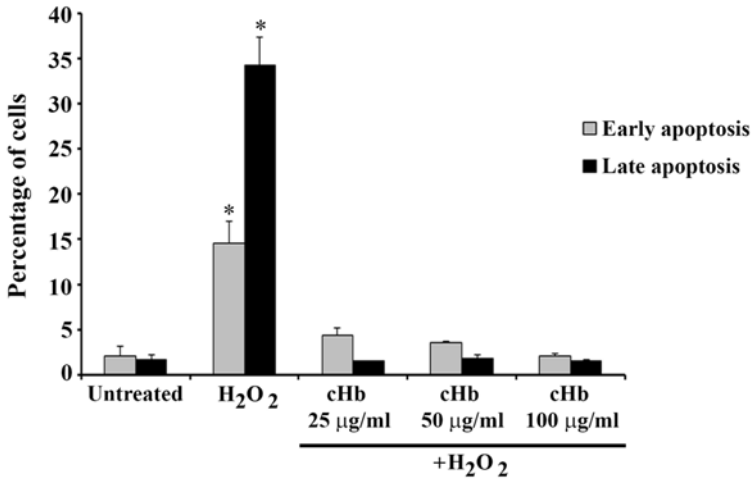

Figure 6. Protective effects of cHb on H_2O_2-induced apoptosis analysed by Annexin V-FITC/PI flow cytometry. A cell suspension of 1×10^5 cells/well was seeded in 12-well plates and different concentrations (25-100 µg/ml) of cHb were added. After a 1 h incubation period, cells were exposed to 500 µM H_2O_2 for 24 h and labeled with annexin V-FITC/PI. The apoptosis rate was measured immediately after H_2O_2-stimulation by flow cytometry. Results are presented as mean ± SD. *, $P < 0.05$, significantly different from control by one-way ANOVA.

4. Conclusion

The present study provides strong evidence that *C. siamensis* blood components confer a pronounced protective effect against oxidative cellular stress, while showing no adverse effects on BJ skin fibroblast cells. All collected data clearly demonstrate that pre-treatment with *C. siamensis* plasma, cHb or cWBC 1 h prior to H_2O_2 exposure attenuates H_2O_2-induced cytotoxicity in BJ cells. Moreover, incubation with *C. siamensis* plasma, cHb and cWBC in over a broad concentration range for 24 h did not exert any toxic effect on human PBMC immune cells. Flow cytometry measurements proved that H_2O_2 induces the apoptosis of human skin fibroblast cells, which could be evidently attenuated by pre-treatment with cHb. Therefore, the collected results in this study provide a well-founded rationale for the utilization of *C. siamensis* blood components as valuable natural resource in the development of skin therapeutic products.

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