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Inhibitory Effect of Carnosine and Anserine on DNA Oxidative Damage Induced by Fe^{2+}, Cu^{2+} and H_2O_2 in Lymphocytes

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ABSTRACT

The effects of carnosine and anserine on oxidative DNA damage in human lymphocytes induced by Fe^{2+}, Cu^{2+} or H_2O_2 were investigated using single cell gel electrophoresis (comet assay). Carnosine and anserine induced a slight DNA damage in human lymphocytes after incubation with cells for 30 min. Carnosine and anserine caused a tail DNA % from 4.7% to 7.3% and 6.1% to 10.0% respectively, at a concentration of 5-100 mM. The cell viability of carnosine and anserine to human lymphocytes was more than 85%. Fe^{2+} and Cu^{2+} caused a marked cytotoxicity and DNA damage in human lymphocytes with a concentration dependent manner. At a concentration of 100 μM, carnosine and anserine possessed 60-70% inhibitory effect on DNA damage in human lymphocytes when they reacted with Fe^{2+} or Cu^{2+} for 30 min before incubated with human lymphocytes. Fe^{2+}, Cu^{2+}, and H_2O_2-induced DNA damage in human lymphocytes were inhibited by carnosine and anserine in a concentration dependent manner (5-100 μM). However, the inhibitory effect of carnosine and anserine on oxidative DNA damage in human lymphocyte was 46.4% and 49.3%, respectively, when reacted simultaneously with H_2O_2. Carnosine and anserine possessed more marked inhibitory effect on oxidative DNA damage in human lymphocyte induced by Fe^{2+} and Cu^{2+} than that induced by H_2O_2. This result might be due to carnosine and anserine did not possess significantly scavenging effect on H_2O_2, thus, H_2O_2 entered the cell and caused DNA damage.

Key words: carnosine, anserine, oxidative DNA damage, comet assay, human lymphocytes

INTRODUCTION

Free radicals (FR) and reactive oxygen species (ROS) have recently attracted increasing attention in the fields of biology and medicine. They are generated from normal metabolism or exposure to various oxidative sources such as transition metals, exogenous materials, contaminants and radiation. Halliwell and Gutteridge[1] and Ames *et al*[2] indicated that FR and ROS play important roles in clinical diseases, aging and oxygen-mediated food deterioration[3]. Generation of FR and ROS causes the so-called oxidative stress, and contributes to lipids, proteins and DNA damage which further influence food quality and biological tissue. Moreover, they are associated with the mechanism of mutagenesis and carcinogenesis. Sahu[4] suggested that oxidative damage leads to DNA mismatch or misrepair which presents the onset of carcinoma. To replenish the antioxidants is an effective way to reduce oxidative stress. Synthesized antioxidants was long term questioned their safety problems[5]. Natural antioxidants protect bioorganisms from the invasion of free radicals, and therefore have attracted broad attention recently[6].

FR and ROS also result in DNA oxidative damage. Distinct cells have individual DNA damage rates that depend on the amount of reactive oxygen and capability of anti-oxidation in cells[7]. When oxidative stress is larger than the anti-oxidation defense system, it follows cell aging, apoptosis or DNA mutant-mediated carcinoma. For elucidating the oncogenetic process and mechanism, it is important to quantify the DNA oxidative damage. According to recent epidemiology studies, researchers are seeking some biomarkers, such as DNA adducts, sister chromosome exchanges and DNA lesions. However, there is no effective way for evaluating single cell DNA damage so far. The measurements of DNA damage at present resort to analysis of the oxides of modified DNA bases in HPLC such as 8-hydroxy-2-deoxyguanine; or examining sister chromosome exchange. Nonetheless, extra damage during analysis makes a precise estimate of DNA damage in cells unfeasible, and does not clearly indicate whether the damage is from the free radicals or improper experiment procedures[7]. Therefore, a new rapid and precise method for estimating DNA oxidative damage was developed, called single cell gel electrophoresis (SCGE) or Comet assay. This method, developed by östling and Johansson[8] is a rapid, simple, easy, economic and sensitive assay for measuring DNA breakdown in mammalian cells[9]. It was extensively used in the investigation of DNA repair mechanism, radiation mechanism, genotoxicity and apoptosis. A great deal of recent research uses Comet assay to investigate DNA damage in human lymphocytes. When DNA damages in human lymphocytes, genetic materials breakdown into fragments and migrates under subjection of elec-

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trophoresis. After staining, it forms a comet shape from which DNA damage can be judged by distance between the comet head and its dragged tail. This technique obtains the DNA damage difference between the smokers and non-smokers. It also gives clues on individual DNA damage information from different eating habits, physiologic stress and genotoxic therapeutic drugs.

Carnosine (N-β-alanyl-*L*-histidine) and anserine (N-β-alanyl-3-methyl-*L*-histidine) are dipeptides, which mainly exist in most vertebrate muscle tissue[10-11]. Carnosine is more than 20 mM content in many tissues[12]. Reports show that carnosine can inhibit lipid peroxidation in many lipid systems[13-14]. In metal-catalyzed deoxyribose system, carnosine was shown to be capable of cleaning the hydroxyl radicals. In liposome and ground beef homogenate, carnosine prevents lipid peroxidation[15]. Carnosine scavenges singlet oxygen better than histidine, and efficiently inhibits peroxidative effect induced by ferric ions, hemoglobin, lipoxygenase and hydroxyl radicals[16]. The antioxidant mechanism of carnosine is attributed to its chelating effect against metal ions, SOD-like activity, ROS and FR scavenging ability[17-19; 13-14]. Chan and Decker[16] pointed out that carnosine spread in muscle tissues with pervasive anaerobic glycolysis serve as a lactic acid-neutralization buffer. Therefore, white muscle fiber, which always uses anaerobic glycolysis, contains more carnosine and anserine than red muscle fiber. These carnosines and anserines increase muscle antioxidation. Many reports showed the addition of carnosine and anserine on meat products not only increases the shelf life but also maintains good color, constitution and flavor and is a replacement for artificial antioxidant[13]. Also, carnosine can selectively kill tumor cells via inhibition of the formation of pyruvate, oxaloacetate and α-ketoglutarate[20]. Hipkiss[21] indicated that carnosine could effectively inhibit acetaldehyde-mediated damage of DNA, proteins and lipids. Thereby, carnosine is potent to prevent disease and aging.

Although carnosine may serve as an antioxidant in many systems and is capable of chelating metal ions, little research has focused on its real effect on human cells or whether it is true that carnosine can prevent human cells from oxidative damage. Therefore, the main purpose of this study was to investigate the effects of carnosine and anserine on DNA oxidative damage in human blood lymphocytes caused by different inducers such as Fe^{2+}, Cu^{2+} and H_2O_2 by using Comet assay, and to estimate their potentials to serve as a biological antioxidant.

MATERIALS AND METHODS

I. *Materials*

(I) *Samples*

Carnosine and anserine were purchased from Sigma (St. Louis, MO, USA).

(II) *Reagents*

Histopaque 1077 was purchased from Pharmacia Biotech (Pharmacia Biotech Co., Sweden). Carnosine, anserine trypan blue, triton X-100, ethidium bromide (EtBr), and N-lauroyl sarcosinate were obtained from Sigma (St. Louis, MO, USA). RPMI 1640 medium, ultrapure low melting point agarose (LMA), and normal melting point agarose (NMA) were purchased from Gibco BRL (Gibco BRL Co, Grand Island, NY). Iron (II) chloride tetrahydrate, copper (II) sulfate pentahydrate, and hydrogen peroxide (H_2O_2) were obtained from Wako Pure Chemical Inc. (Wako Pure Chemical Industries Ltd., Osaka, Japan). Dimethyl sulfoxide (DMSO) and sodium chloride were purchased from Merck (Darmstadt, Germany). Human blood was donated by volunteers.

II. *Methods*

(I) *Cytotoxicity and DNA Damage Study in Lymphocytes Induced by Different Concentrations of Carnosine, Anserine, Fe^{2+}, or Cu^{2+}*

1. Cell collection

Human blood was homogeneously mixed with RPMI 1640 followed by adding Histopaque 1077, then centrifuged at 1600 rpm for 10-15 min. Buffy coat was taken out, then added serum free RPMI 1640 and mixed homogeneously followed by centrifuged at 1600 rpm for 10 min. Neubauer Improved Haemocytometer was applied to count cells and examine the cell viability. Then cells with viability over 90% were applied to examine the cytotoxicity and DNA damage in lymphocytes induced by carnoine, anserine, Fe^{2+}, and Cu^{2+}.

Incubated cell suspensions with different concentrations of samples ranged from 0 to 100 μM at 37˚C for 30 min, then centrifuged at 800-900 rpm. Cell pellets were reconstituted with RPMI 1640, and cell viability was determined by trypan blue (0.4%) exclusion method.

2. DNA damage in lymphocytes induced by carnosine, anserine, Fe^{2+}, and Cu^{2+}[22, 23]

Lymphocyte suspensions were incubated with different concentrations of carnosine (0-100 μM), anserine (0-100 μM), Fe^{2+} (0-1000 μM), or Cu^{2+} (0-200 μM) at 37˚C for 30 min, then centrifuged at 800-900 rpm. Cell pellets were resuspended with 75 μL LMA (Low melting gel agarose). 75 μL of NMA (Normal melting gel agarose, 50˚C) was applied on a slide, then NMA was allowed to be solidified (first layer) at room temperature. Another 75 μL of cell suspension in LMA was applied over the first layer, then allowed to be solidified at 0˚C to form second layer. Another 75 μL of LMA was applied over the second layer, then allowed to be solidified at 0˚C to form a third layer. After applying three layers of gel, the slide was dipped into lysing solution at 4˚C for 1h to destroy cell membranes, then washed with electrophoresis buffer, followed by incubation with basic elec-

trophoresis buffer for 20 min. Electrophoresis was applied under 300 mA, 25 V, then the slide was incubated with tris buffer for 3 times, 5 min each time. DNA was stained with ethidium bromide (20 µg/mL) and observed under a fluorescence microscope (Nikon EFD-3, excitation filter BP 543/10 nm, emission barrier filter 590 nm). Duplicated samples were prepared and examined.

(II) *Influence of Carnosine or Anserine on DNA Damage in Lymphocytes Induced by Fe^{2+}, and Cu^{2+} or H_2O_2*

Cell suspensions were incubated with 50 μM H_2O_2 for 30 min together with different concentrations of carnosine or anserine, then DNA damage in lymphocytes was examined by Comet assay.

(III) *Quantification of Oxidative DNA Damage*

Comet analysis system (Komet 3.1, Kinetic Imaging Ltd., Liverpool, UK) was applied to quantify the DNA damage. Extent of DNA damage was expressed as the percentage of tail DNA. Tail DNA% = [Tail DNA/(Head DNA + Tail DNA)] × 100.

(IV) *Statistical Analysis*

All experiments were repeated triplicate. Data was analyzed by SAS (statistical analysis system). Variance analysis was performed by PROC ANOVA and Duncan's multiple range tests.

RESULTS AND DISCUSSION

I. *Cytotoxicity and Genotoxicity of Human Blood Lymphocytes Induced by Carnosine and Anserine*

The carnosine and anserine treated group, which carnosine and anserine reacted with lymphocyte cells under 37˚C for 30 mins, has more than 85% cell viability when compared with the control group (Figure 1). When examining the DNA-damaging effect of carnosine and anserine on human lymphocytes, we found that comet-like tails from damaged DNA appeared after electrophoresis. The longer ones implied severe damage of DNA. Tail DNA of control group is 4.3%, whereas carnosine and anserine are 4.7-7.3% and 6.1-10.0% respectively, within the concentration of 5-100 μM. When the concentration of samples goes up, it is followed by a mild DNA breakdown event.

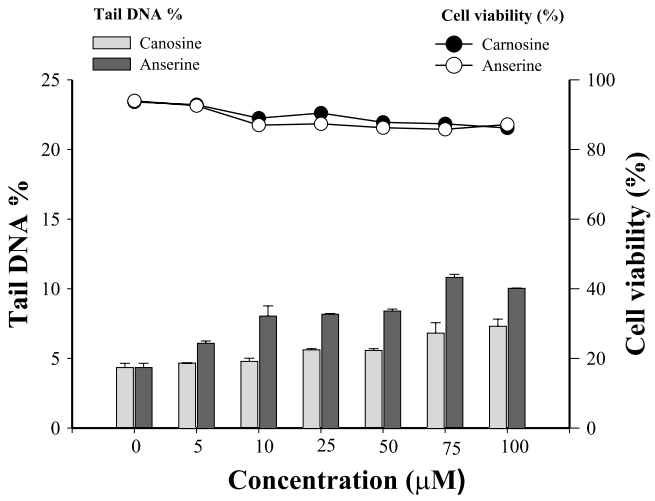


Figure 1. Cytotoxicity and genotoxicity of carnosine and anserine in human blood lymphocytes. Results are mean ± SD for n≥3.

II. *Cytotoxicity and DNA Damage Study in Lymphocytes Induced by Different Concentrations of Fe^{2+}, or Cu^{2+}*

(I) *Cytotoxicity in Lymphocytes Induced by Fe^{2+} or Cu^{2+}*

Figure 2 shows the effect of Fe^{2+} and Cu^{2+} on cell viability in human lymphocytes at 37˚C for 30 mins. The results showed increasing cytotoxicity with the increasing Fe^{2+} concentration, which is obvious and significant ($P < 0.05$). When Fe^{2+} concentration went up to 1000 μM, cell viability remained at only 26.8%. There was also increasing cytotoxicity with an increasing Cu^{2+} concentration which is also sig-

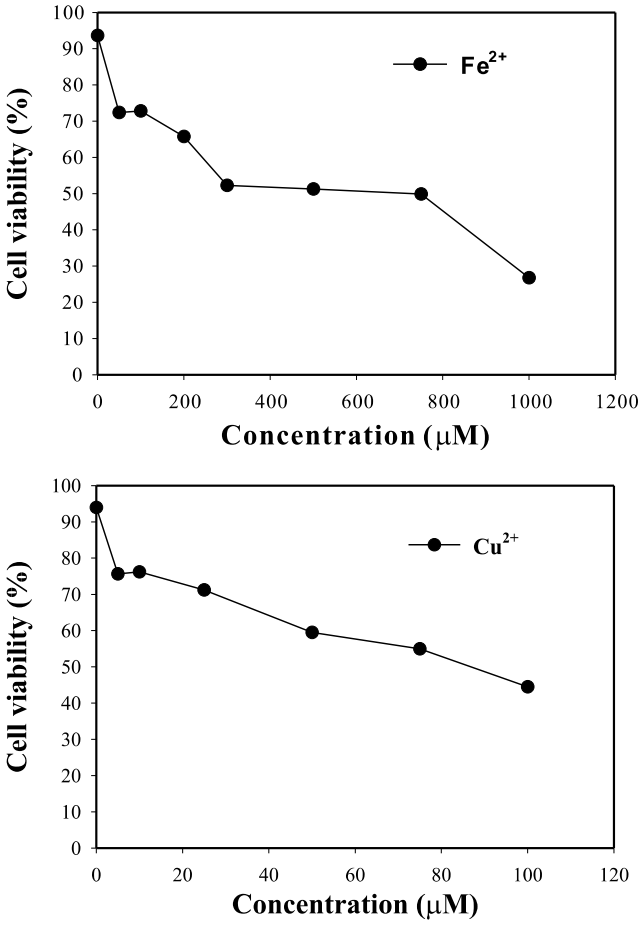


Figure 2. Effect of Fe^{2+} and Cu^{2+}on cell viability in human lymphocytes.

nificant ($P < 0.05$). When Cu^{2+} concentration went up to 1000 μM, cell viability remained at only 44.5%. Copper ions are transition metal ions, which are prone to induce FR generation; therefore their existence in organisms lead to oxidative damage of proteins[24].

(II) *DNA Damage in Lymphocytes Induced by Fe^{2+} or Cu^{2+}*

Figure 3 shows the effect of Fe^{2+} and Cu^{2+} on DNA damage in human lymphocytes, which was determined by Comet assay. The addition of increasing Fe^{2+} resulted in raising DNA damage in lymphocytes. In Fe^{2+} concentration of 750 μM, tail DNA was as low as 36.7%, which has significant difference from groups in 50-500 μM ($P < 0.05$). Therefore, in the following experiments, we took Fe^{2+} 750 μM as the optimal concentration to induce DNA damage on lymphocytes and inquired into the carnosine or anserinein effect on DNA damage in lymphocytes through various concentrations. This study showed Fe^{2+} did cause DNA damage on lymphocytes. It was reported that the ferrous ion is one of the catalysts for TBARS[15]. In liquid solution ferrous ion will interact with endogenous and exogenous H_2O_2 to form • OH[25] through Fenton reaction, and cause DNA oxidative damage in lymphocytes. When Fe^{2+} or Fe^{2+} content compound was added into liposomes or lipoprotein, lipid peroxidation occurred[26]. The freezing and cooking process may increase the dissociation of heme containing compound and therefore evoke increasing levels of free ferrous, ferric ions and low molecular weight ferric compounds which produces lipid peroxidation[27]. Furthermore, from Figure 3, we can see the severe DNA damage on lymphocytes is related to the increasing copper ion concentration. In 25 μM, the tail DNA was already 38.1%, implying very severe DNA damage. Therefore, in the following experiments, we took Cu^{2+} 25 μM as the optimal concentration to induce DNA damage on lymphocytes and further investigated the effect of carnosine or anserine on DNA damage in lymphocytes. Transition metal ions, including ferrous and copper ions, will promote deoxyribose degradation[15]. Transition metal ions such as ferrous ions, copper ions, and heme-containing compound will highly contribute to lipid oxidation which causes non-lipid molecule damage. Thus, the existence of ferrous and copper ions did cause damage to human lymphocytes.

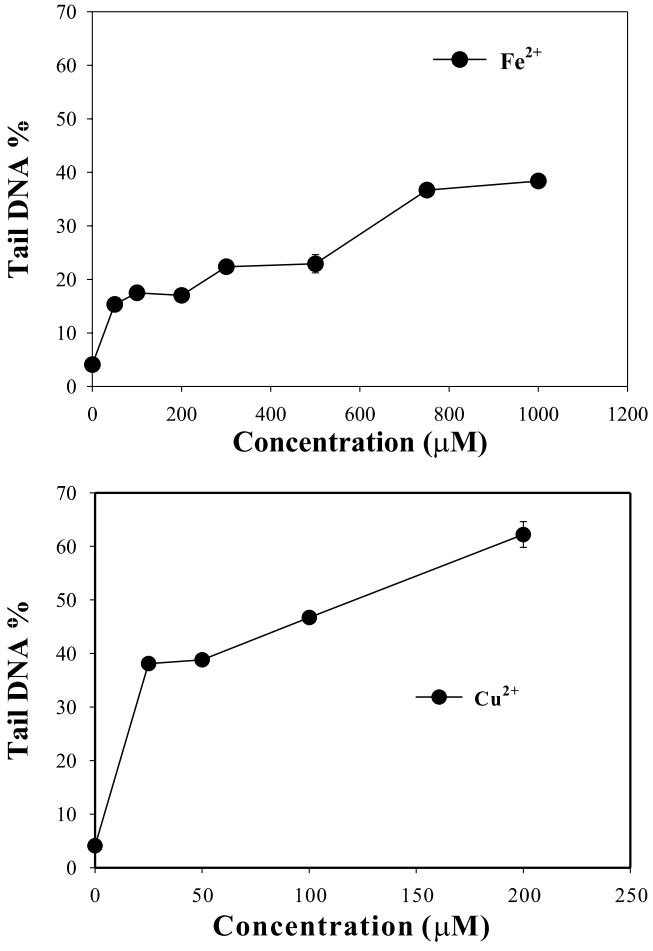


Figure 3. Effect of Fe^{2+} and Cu^{2+}on human lymphocytes DNA damage.

III. *Influence of Carnosine or Anserine on DNA Damage in Lymphocytes Induced by Fe^{2+}, Cu^{2+} or H_2O_2*

(I) *Influence of Carnosine or Anserine on DNA Damage in Lymphocytes Induced by Fe^{2+}*

Lipid oxidation in muscle involves peroxidation of unsaturated fatty acids on membrane. Transition metal ions such as ferrous ions, copper ions, and heme containing compound increase the oxidation effect. Muscle tissues contain abundant ferrous ions and the excess ferrous ions contribute to lipid peroxidation.

Carnosine and anserine are endogenous dipeptide compounds, which mainly exist in vertebrate muscle and have 10-70 μM content in beef, pork and chicken. Research has shown that carnosine inhibited lipid peroxidation in many lipid systems[13-14], whose major antioxidant effect is supposed to be from its metal ion chelating and free radicals scavenging ability[14, 18]. However, the chelating effect varies with ion forms[13].

Figure 4 shows the 750 μM ferrous ions react with the various concentrations of carnosine or anserine for 30 minutes prior to addition of lymphocytes at 37˚C for another 30 minutes. The carnosine or anserine reacts with 750 μM for 30 minutes in advance for the anticipating chelating event. It was shown that elevating carnosine and anserine all present higher inhibition effect on DNA oxidative damage in lymphocytes induced by ferrous ions. At 100 μM, inhibition effect of carnosine and anserine are 79.6% and 69.3% respectively. Figure 5 showed the DNA damage of lymphocytes before and after ferrous ions treatment. It has been reported that the inhibitory effect of carnosine on ferrous-induced lipid peroxidation might result from the radical effect during ferrous ions scavenging rather than its chelating function on ferrous ions[13]. In the presence of ferrous ion, a higher carnosine concentration is required to inhibit TBARS formation, which implies that carnosine is involved in the hydrox-

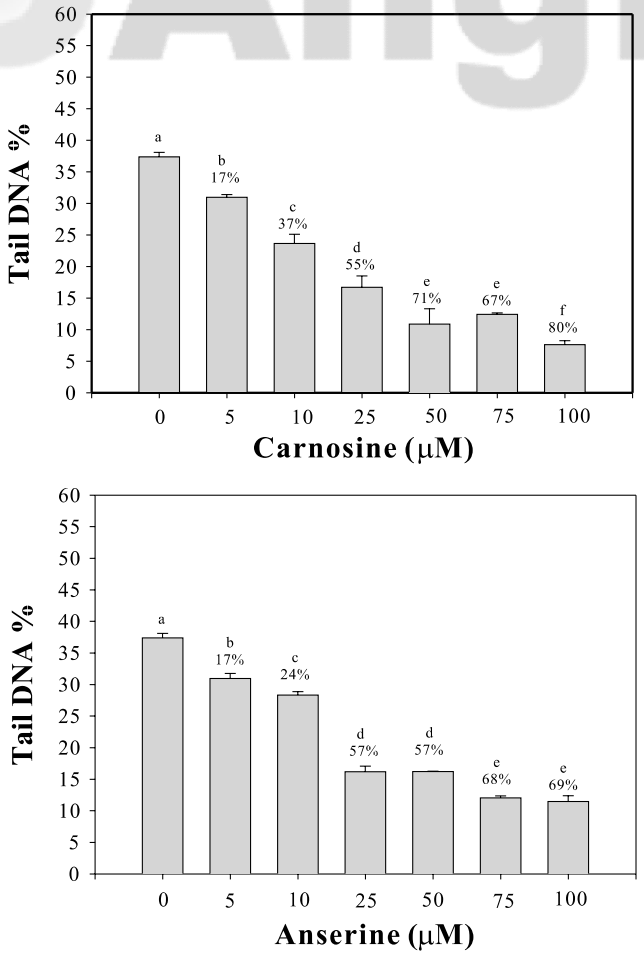


Figure 4. Effect of carnosine and anserine on human lymphocytes DNA damage induced by Fe^{2+} (750 μM). Results are mean ± SD for n≥3. Values in each column with different letters are significantly different ($P < 0.05$).

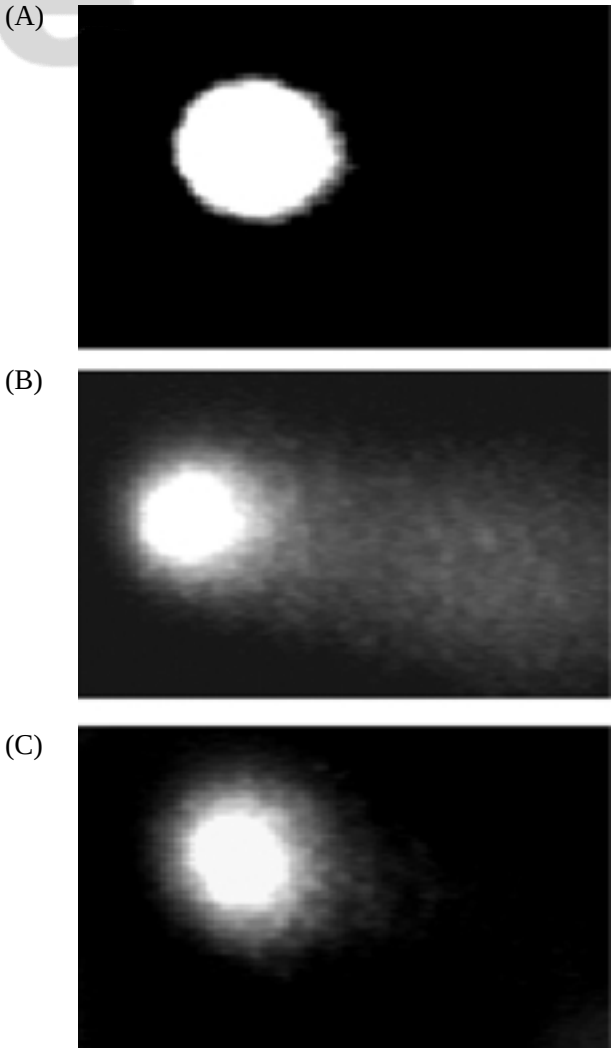


Figure 5. DNA comet images of lymphocyte after treatment with Fe^{2+} (750 μM) and carnosine (50 μM). (A) untreated lymphocytes; (B) lymphocytes treated with Fe^{2+} and (C) lymphocytes treated with carnosine and Fe^{2+}.

yl FR scavenging pathway directly rather than inhibiting the hydroxyl FR generation step[15]. So it is valid that carnosine and anserine possess the inhibition potency to lymphocytes DNA damage induced by ferrous ions. In this study, carnosine and anserine with concentration from 5 to 100 μM, lymphocytes DNA damage was reduced to 31-7.6% and 31-11.5%, respectively.

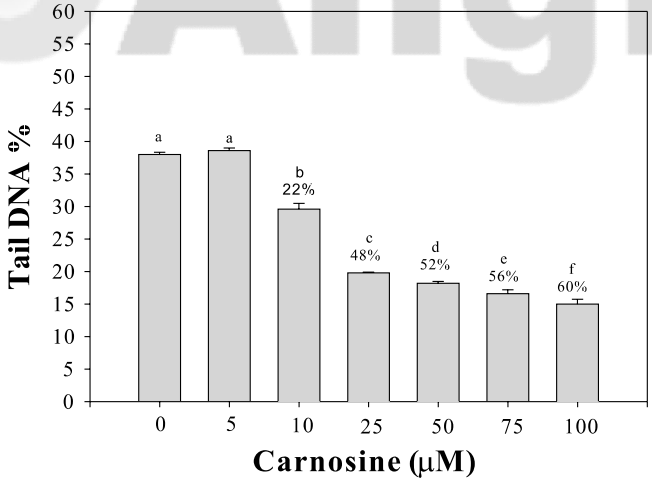
(II) *Influence of Carnosine or Anserine on DNA Damage in Lymphocytes Induced by Cu^{2+}*

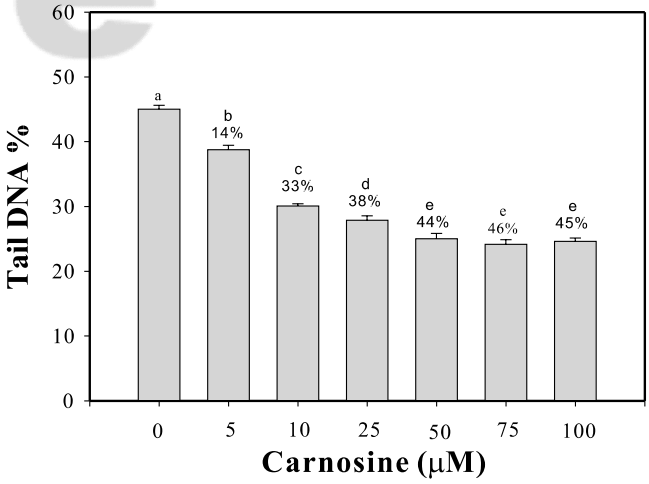
The addition of 10 μM copper ion into the ascorbic acid-containing system caused degradation of deoxyribose[15]. Copper ion, referring to as a transition metal ion, leads oxidative damage of non-lipidic and lipidic molecules.

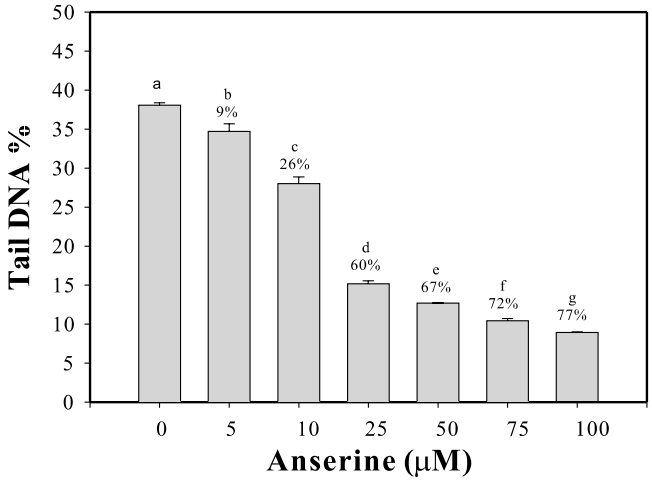
Figure 6 shows that the copper ions react with the various concentrations of carnosine or anserine for 30 minutes prior to the addition of lymphocytes at 37˚C for 30 minutes. It was shown that increasing the concentration of carnosine or anserine attenuated the DNA damage of lymphocyte caused by copper ion. The inhibition of carnosine and anserine with 100 μM copper ion was 60.4% and 76.5%, respectively. When compared with the control group, there was a significant ($P < 0.05$) difference in each concentration. Human skeletal muscle is rich in copper as well as has a high concentration of carnosine, which is able to prevent oxidative stress. One of the protective mechanisms is its ability of chelating transition metal ions out of a Fenton reaction. It has been proved that carnosine and anserine are chelating agents which chelate other transition metal ions[19]. Some research has showed that carnosine has strong inhibition effect. Inhibition of TBRAS formation of phosphatidylcholine liposome increases to 57, 73, 80, 82 and 87% with the addition of 1, 2.5, 5, 10, 15 mM carnosine, respectively[15]. Therefore, carnosine and anserine were much like an effective chelater preventing metal-induced damage in skeleton muscle.

(III) *Influence of Carnosine or Anserine on DNA Damage in Lymphocytes Induced by H_2O_2*

H_2O_2 is one of the oxygen metabolites. The accumula-







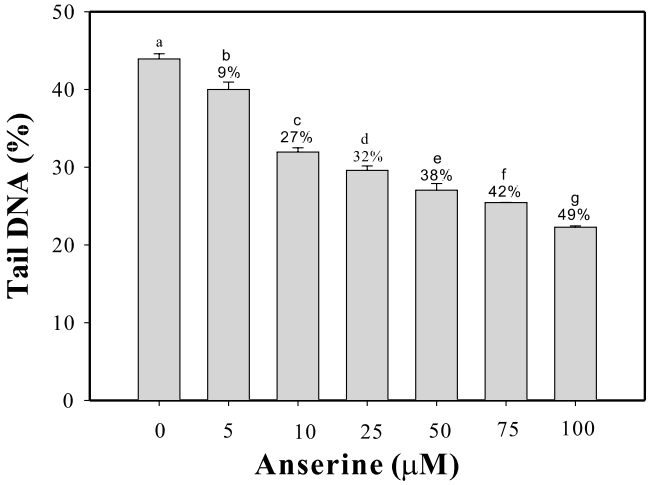


Figure 6. Effect of carnosine and anserine on human lymphocytes DNA damage induced by Cu^{2+} (25 μM). Results are mean ± SD for n≥3. Values in each column with different letters are significantly different ($P < 0.05$).

Figure 7. Effect of carnosine and anserine on lymphocytes DNA damage induced by H_2O_2 (50 μM). Results are mean ± SD for n≥3. Values in each column with different letters are significantly different ($P < 0.05$).

tion of H_2O_2 has high toxicity to cells, which not only modifies base pairs to produce DNA breakdown but also activates cell apoptosis. There is no doubt that H_2O_2 has cytotoxicity and causes DNA breakdown. *In vivo*, H_2O_2 is from the degradation of superoxide anion in macrophage, mitochondria or from an autoxidation endogenous compound. Guidarelli *et al.*[28] suggested that the generation of H_2O_2 will promote karyoplasmatic Ca^{2+} accumulation and directly activates endonucleolytic enzymes which leads to DNA breakdown. Moreover, Ca^{2+} also results in electron loss of reductive type and semiquinone type coenzyme Q. Liberating excess calcium ions in blood will fail the Ca^{2+}-Na^{+} pump control, prompting Ca^{2+} into the cell, making chromatin aggregation and promote the cell apoptosis in the subsequent chromatin breakdown[29]. Despite carnosine and anserine have metal ion chelating and FR scavenging functions, it is doubted if they are still capable to inhibit cell oxidative damage under excess FR and ROS.

Admixing carnosine or anserine in different concentrations with 50 μM H_2O_2 and lymphocytes simultaneously at 37˚C for 30 minutes, the effect of carnosine and anserine on lymphocytes DNA damage was determined by Comet assay (Figure 7). The control group without any dipeptide inside was established by DNA oxidative damage under 50 μM H_2O_2, which possessed a tail DNA 44.4%. From Figure 7, in the study of carnosine on DNA damage in lymphocytes induced by H_2O_2, there was no significant ($P > 0.05$) inhibitory effect among 50-100 μM carnosine. It was also observable that 100 μM anserine could achieve 49.3% inhibition when compared with the control group; nevertheless it was still lower than the inhibitory effect of carnosine and anserine on DNA oxidative damage induced by ferrous and copper ions. It is possible that H_2O_2 has gotten into the cell and caused DNA breakdown before effective scavenging of H_2O_2 by carnosine or anserine.

CONCLUSION

Experiment results showed that carnosine and anserine within the concentrations 0-100 μM have a slight cytotoxicity and genotoxicity. Fe^{2+} and Cu^{2+} caused cytotoxicity and DNA damage in lymphocytes. However, carnosine and

anserine had antioxidant effect on the DNA damage assay in lymphocytes induced by transition metal ions and H_2O_2. This probably resulted from the chelating and scavenging effects of carnosine and anserine on free radicals which are generated from Fe^{2+}, Cu^{2+} and H_2O_2. Presently, most of the dietary natural antioxidants are from plants. And studies on carnosine are mostly *in vitro*. From this study, we know carnosine and anserine have protective effects on cells and are potential biological antioxidants.

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Carnosine 與 anserine 對於亞鐵離子、銅離子及過氧化氫誘導淋巴球細胞 DNA 氧化損傷之影響

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摘　要

本研究以彗星試驗法探討carnosine 及anserine 對不同誘發劑造成人體淋巴球細胞DNA 氧化傷害之影響。結果顯示carnosine 與anserine 在100 μM 對人體淋巴球細胞有輕微的基因毒性及細胞毒性，但細胞與carnosine 與anserine 混和反應30 分鐘後，控制組（0 μM）細胞平均tail DNA 為4.3% ，carnosine 與anserine 在濃度5-100 μM 的範圍內 tail DNA 分別為4.7-7.3% 及6.1-10.0% 且細胞存活率皆在85% 以上。在加入誘發淋巴球細胞DNA 氧化傷害之試劑－Fe^{2+} 及Cu^{2+} 的試驗中，結果顯示Fe^{2+} 及Cu^{2+} 對淋巴球細胞有明顯的細胞毒性及DNA 氧化傷害，且隨著劑量的增加對淋巴球細胞DNA 損傷之情形愈嚴重。Fe^{2+} 濃度為750 μM 時tail DNA 高達36.7% ，明顯的與濃度50-500 μM 時的tail DNA（15.3-22.9% ）有顯著性的差異（$P < 0.05$）。在Cu^{2+} 的試驗中，結果顯示濃度為25 μM 時tail DNA 已達38.1% 。在carnosine 與anserine 對不同誘發劑，Fe2+ 、Cu^{2+} 及H_2O_2 ，對淋巴球細胞DNA 氧化傷害的試驗中發現，carnosine 或anserine 先與Fe^{2+} 或Cu^{2+} 反應30 分鐘後再與淋巴球細胞反應，結果顯示有抑制細胞DNA 氧化傷害的效果，在100 μM 的濃度下約有50% 的抑制率。然而carnosine 或anserine 與H_2O_2 同時與淋巴球細胞反應其抑制效果分別為46.4% 及49.3% 。Carnosine 及anserine 抑制Fe^{2+} 及Cu^{2+} 所誘導的淋巴球DNA 氧化傷害較H_2O_2 所誘導的明顯，其可能的原因是carnosine 或anserine 來不及清除H_2O_2 ，而H_2O_2 已進入細胞造成DNA 的氧化傷害。

關鍵詞：Carnosine ，Anserine ，DNA 氧化傷害，彗星試驗，人類淋巴球