

## The effect of L-carnosine on the rheological characteristics of erythrocytes incubated in glucose media

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### Abstract

Hemorheological properties are easily modified by glucose-induced oxidation and glycation. Carnosine, a naturally occurring dipeptide ( $\beta$ -alanine-L-histidine), has been recently proposed to act as an antioxidant as well as a free-radical scavenger. In the present study, we investigate its protective and rejuvenating effects in erythrocytes that are exposed to glucose-rich plasma. Erythrocytes that were incubated in glucose solutions were treated with different concentrations of carnosine and for different incubation times. Their hemorheological alterations were examined. The results reveal that the presence of carnosine effectively prevented these rheological alterations in a concentration-dependent manner in glucose-rich media. It is proposed that moderate concentrations of carnosine might be further explored as potential therapeutic agents for pathologies that involve hemorheological modification.

**Keywords** : erythrocytes, carnosine, hemorheology, oxidation, glycation

### 1. Introduction

The rheological properties of RBCs' microstructure play important roles in microcirculation, and also blood flow in large arteries. Microscopic mechanisms can be connected to the macroscopic behaviors of the blood and transferred by means of a blood viscosity model based on blood structure to the macroscopic behaviors of the blood. Thus, modeling the transport of drugs, determining a drug local concentration, and using in many other medical fields can be possible (Yilmaz and Gundogdu, 2008).

RBC proteins are susceptible to reactive oxygen species, which induce protein modifications. Among the many modification mechanisms of protein, non-enzymatic glycosylation (glycation) has been found to produce free radicals, which subsequently increase the peroxidation of cell membranes (Mullarkey *et al.*, 1990). The increased glycation of proteins in hyperglycemia may accelerate the lipid oxidative modification of RBC membranes, which result in an alteration of hemorheological properties, such as the deformability and aggregation of RBCs. Iwata *et al.* (2004) measured the deformability of RBCs that were *in vitro* exposed to carbonyl compounds, such as glucose and glyceraldehydes, and found a significant decrease in deformability via a biochemical reaction between erythrocyte proteins and carbonyl compounds. Thus, hyperglycemia is a potent factor that accounts for the modification

of erythrocytic constituents that are composed of proteins and lipids and induces severe glucose-induced deterioration of the hemorheological properties of erythrocytes (Babu and Singh, 2004; Riquelme *et al.*, 2005; Shin *et al.*, 2008). Consequently, the impaired erythrocytes are associated with microvascular complications (Schmid-Schonbein and Volger, 1976), which are frequently observed in diabetes mellitus (Brown *et al.*, 2005).

Carnosine has been proposed to act as an antioxidant as well as a free-radical scavenger, which can protect and stabilize the cell membrane from non-enzymatic glycosylation and oxidation. Carnosine is a naturally-occurring dipeptide that consists of two amino acids, -alanine and L-histidine, and performs various remarkable functions, such as pH buffering and the chelation of divalent metal cations (Tamba and Torreggiani, 1999; Hipkiss and Brownson, 2000). In addition, carnosine also prevents protein glycation by using an enzyme that catalyses the splitting of interior peptide bonds in a protein (Quinn *et al.*, 1992). Furthermore, the ability of carnosine to disintegrate the readily glycosylated protein was observed through the hydration and unfolding of deleterious reactions, such as the cross-linking of proteins (Seidler *et al.*, 2004).

Recently, Aydogan *et al.* (2008) investigated the effect of carnosine on RBC deformability under  $H_2O_2$ -induced oxidative stress and demonstrated the protective functions of carnosine on RBC membranes in high oxidative-stress environments. Even though many potential functions of carnosine have been proposed, not much is known about the effects of carnosine on hemorheology. Therefore, the

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objective of the present study is to examine the protective and rejuvenating function of carnosine in the context of hyperglycemia-associated oxidative stress. In the present study, RBCs were incubated in glucose-rich media with different concentrations of carnosine. Also, RBCs that were impaired due to hyperglycemia were incubated in autologous plasma with different concentrations of carnosine. For these treated RBCs, the deformability and aggregability were measured as hemorheological properties.

## 2. Materials and methods

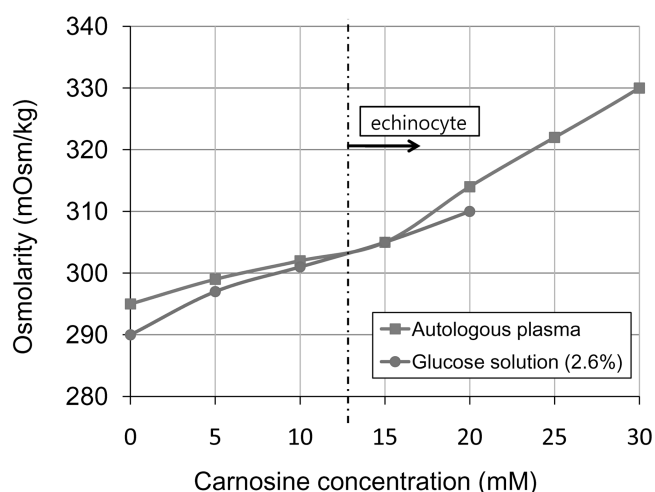
### 2.1. Blood sample preparation

Blood was drawn from the antecubital vein of healthy adult donors through a 21-gauge butterfly infusion set into vacutainers (BD, Franklin Lakes, NJ, USA) that contained  $K_2$  EDTA as an anticoagulant. None of the donors had taken any medication in the preceding one week. After centrifugation of the whole blood at 800 g for 12 min and the removal of plasma and the buffy-coat, the remaining erythrocytes were washed three times with a phosphate buffer saline (PBS, pH 7.4) at room temperature.

The RBC samples were divided into two groups. One sample in the first group was the control. The remaining three samples in the first group were treated with glucose solutions with different concentrations. The glucose solutions were prepared by diluting 5% (w/v) of a glucose solution (Dextrose 5% in distilled water, 300 mOsm/kg  $H_2O$ , Choongwae Pharma Co., Korea) with 0.9% of NaCl solution (295 mOsm/kg  $H_2O$ , DC Chemical Co., Korea) to yield three different concentrations (2.4%, 2.6%, and 2.8%) of glucose solution. Each concentration of the glucose solution was divided into four aliquots. Each aliquot was prepared with four different concentrations of L-carnosine (Sigma Aldrich, USA): 2 mM; 5 mM; 10 mM; and 20 mM, respectively. For the second group, RBC samples were incubated with 2.4% of glucose solution and RBC samples were washed to remove the oxidizing agent. The RBC samples were resuspended in autologous plasma that had the same concentration of carnosine as the first group. All samples were incubated at 37°C for 30 min at a hematocrit level of 10% in a shaking water bath. The osmotic pressures of the prepared glucose-rich media that contained carnosine were kept in the range of 290–310 mOsm/kg  $H_2O$ .

### 2.2. Rheological measurements

The deformability of erythrocytes was determined through a microfluidic ektacytometer (Rheoscan-D, Rheo-Meditech, Korea). Details on the measurement can be found elsewhere (Shin *et al.*, 2007; 2009). Three l of RBCs were resuspended in 0.6 ml of the highly-viscous polyvinylpyrrolidone (PVP, Sigma, MO, USA) solution. As a measure of the deformability, RBC elongation index (EI) was measured at shear stresses that ranged from 0.5 Pa to 20 Pa.



**Fig. 1.** The effect of carnosine concentrations on osmotic pressure and RBC deformability in glucose-rich media.

Erythrocyte aggregation was measured by the use of a microchip-stirring system that was based on a light transmission technique (Shin *et al.*, 2007). Six ml of whole blood were poured in a disposable microchip and RBC aggregates were disaggregated through a magnetic-driven rotating stirrer. The rotating shear mechanism was large enough to disperse the RBC aggregates but not enough to cause any mechanical hemolysis of cells. After 10 s of stirring that was followed by an abrupt halt of the stirring, the intensity of the light that was transmitted through a microchip was measured by a photodiode with respect to time. Through an analysis of the graph (sylllectogram) of the light intensity over time, conventional aggregation indices were immediately determined. All measurements were conducted at room temperature.

## 3. Results

Prior to a discussion of the measured hemorheological properties, it is necessary to examine the variation of the osmolarity of blood samples with the carnosine concentration. Fig. 1 shows the variation of the osmolarity with the concentration of carnosine in 2.6% glucose solution and in plasma. For both media, the osmolarity almost linearly increased with the concentration of carnosine; as a result, red cells may change their morphological shapes and become echinocytes due to the difference in the osmotic pressure. Additionally, RBC morphology was also examined via microscopic observation of RBCs. When the carnosine concentration exceeded 10 mM, in both solutions, the osmolarity surpassed 305 mOsm/kg and echinocytic RBCs were found through microscopic observation. For instance, at 20 mM of carnosine in glucose solution, about 30% of erythrocytes changed their shape. Furthermore, when the carnosine concentration in plasma was 25 mM, the corresponding osmolarity was 322 mOsm/

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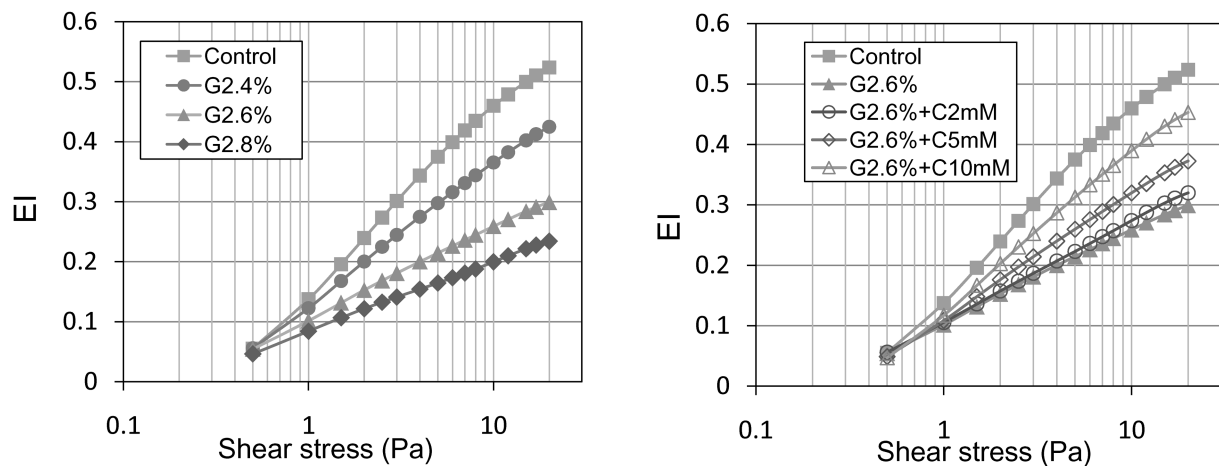


Fig. 2. (a) The effect of glucose concentration on RBCs. (b) The effect of carnosine concentration on RBC deformability in a glucose-rich medium. G: glucose, C: carnosine.

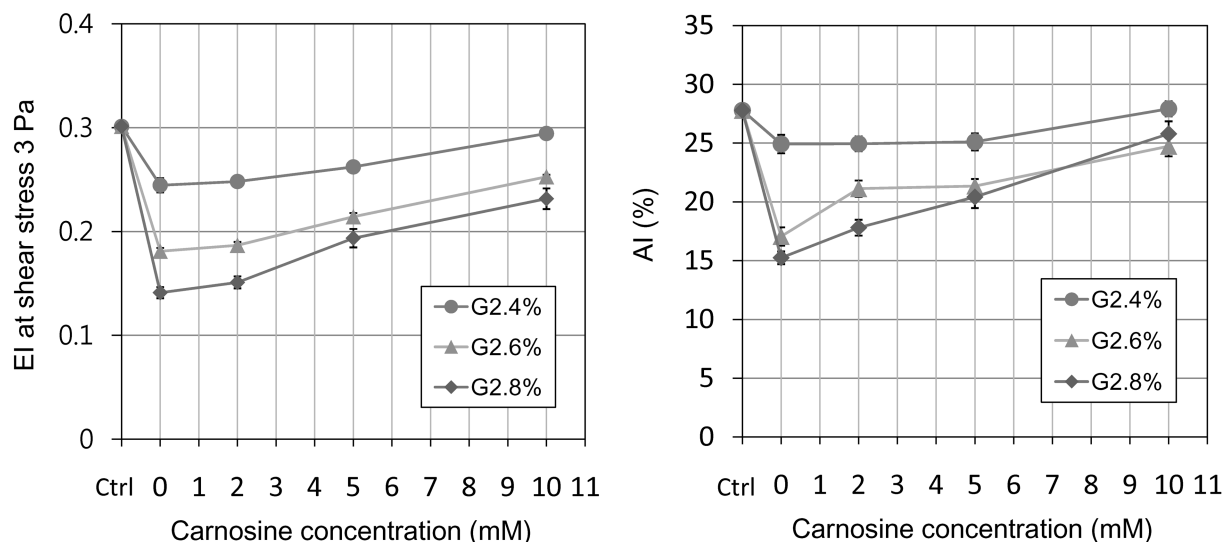


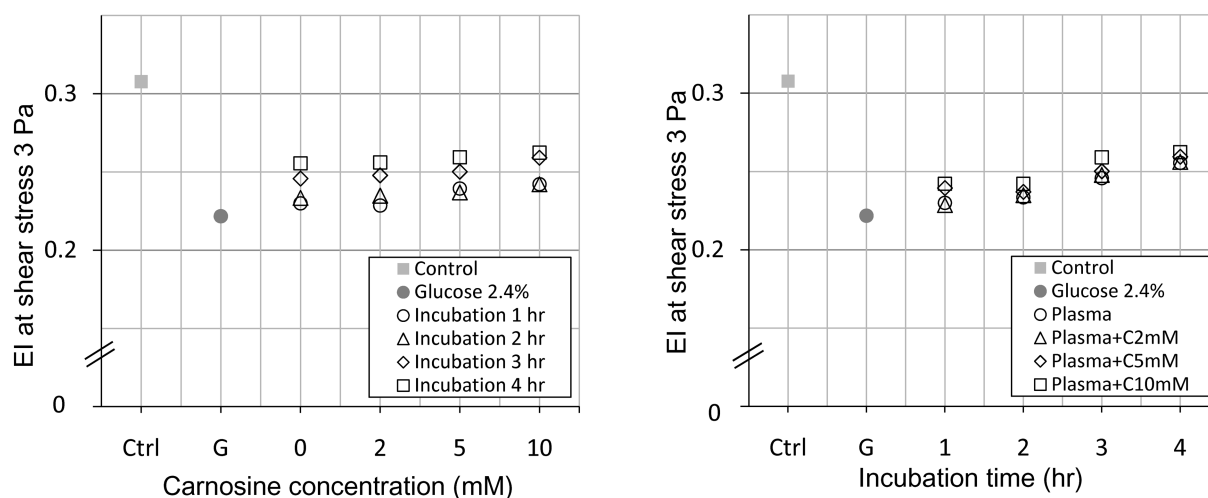
Fig. 3. (a) The effect of the concentration of carnosine premixed in glucose media on RBC deformability. (b) The effect of the concentration of carnosine premixed in glucose media on RBC aggregability. G: glucose.

kg and plural echinocytes were observed. Thus, the present study used the range of 293~305 mOsm/kg H<sub>2</sub>O for the osmolarity, which corresponded to 2~10 mM of carnosine concentration in either glucose solution or autologous plasma. In this range of carnosine concentration, no echinocytes were found through microscopic observation.

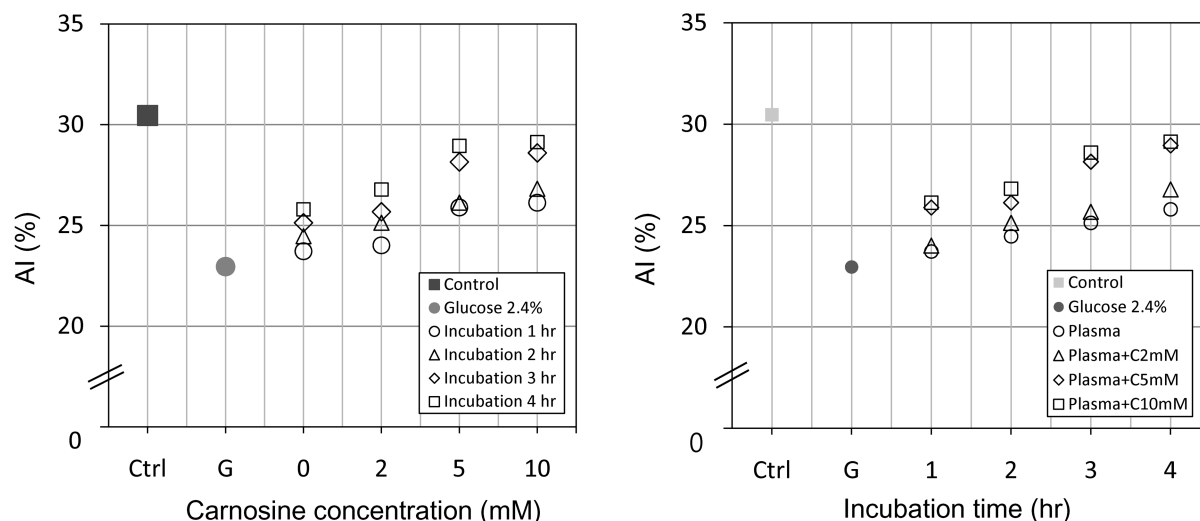
Fig. 2(a) shows the variation of the deformability of RBCs that were incubated in three different concentrations of glucose solution for 30 min. The elongation index, EI, as measure of deformability decreased as the glucose concentration increased. The RBC deformability decreased in a concentration-dependent manner for all shear regions. However, the deformability of RBCs that were incubated in glucose solutions that were supplemented with carnosine shows rather different results, as shown in Fig. 2(b) For a fixed concentration (2.6%) of the glucose solution, three different concentrations (2 mM, 5 mM, and 10 mM) of car-

nosine were prepared and RBCs were incubated in the three different solutions for 30 min. As the carnosine concentration increased, RBC deformability increased in a whole range of the shear stress. The increase in the RBC deformability was dependent on the concentration of carnosine. In fact, further increase in the carnosine concentration above 10 mM might result in the same RBC deformability as in the control. However, due to echinocytic changes in RBCs under high concentrations of carnosine, these results were excluded from the present study.

Fig. 3 shows the results on the EI at 3 Pa and the aggregation index (AI) for various concentrations of carnosine in glucose-rich media. When there was no carnosine (0%) in glucose-rich solution, both the RBC deformability and aggregability significantly decreased. The degree of impairment in RBC deformability and aggregability was proportional to the glucose-concentration. However, through the



**Fig. 4.** (a) The effect of carnosine concentration on the deformability of impaired RBCs in high-glucose media. (b) The effect of incubation time in carnosine-reinforced plasma on the deformability of impaired RBCs.



**Fig. 5.** (a) The effect of carnosine concentration on the aggregability of impaired RBCs in high-glucose media. (b) The effect of incubation time in carnosine-reinforced plasma on the aggregability of impaired RBCs.

addition of carnosine to glucose solutions, the impairment of RBC deformability and aggregability gradually diminished. Subsequently, at 10 mM of carnosine in 2.4% glucose solution, there was nearly no decrease in RBC deformability. Similar result was also observed in RBC aggregability for 10 mM of carnosine in 2.4% glucose solution, as shown in Fig. 3(b).

To examine the rejuvenating function of carnosine, RBCs, which were impaired by hyperglycemia-associated oxidative stress, were incubated in plasma that was supplemented with carnosine. For hemorheological impairment, RBCs were initially incubated in 2.4% glucose solution and then resuspended in four different concentrations (0 mM, 2 mM, 5 mM, and 10 mM) of carnosine in plasma. Fig. 4 shows the results on the EI at 3 Pa of RBCs vs. the carnosine concentration. As the carnosine concentration

increased, the deformability of impaired RBCs slightly increased. However, the same degree of increase in RBC deformability was also found in pure plasma (0 mM carnosine). In fact, the increase in RBC deformability seems to be more dependent on the incubation time than on the carnosine concentration. For instance, as shown in Fig. 4(b), four-hour incubation in carnosine-free plasma yielded greater deformability than two-hour incubation for a 10mM concentration of carnosine. However, even though the deformability of impaired RBCs increased, it was still much smaller than that for the control. By increasing the incubation time, the deformability could be further increased. However, an increase in the carnosine concentration could cause echinocytic change in the shape of RBCs due to hyper-osmolarity.

Similarly, Fig. 5 shows the results on the aggregability of

impaired RBCs, which were resuspended in various concentrations of carnosine in plasma. As the carnosine concentration increased in plasma, the AI of impaired RBCs increased in a concentration-dependent manner. Again, an increase in RBC aggregability was also found in pure plasma but the presence of carnosine apparently results in a greater increase in RBC aggregability in comparison with pure plasma. In addition, the increase in RBC aggregability was also strongly dependent upon the incubation time. Furthermore, the degree of the increase in RBC aggregability was quite large; the values were only slightly smaller – or the same as – those for the control. For instance, for four-hour incubation in a 10 mM carnosine-plasma solution, the AI of 29.2% was almost the same as that for the control (30.6%). Thus, longer incubation periods for RBCs might increase RBC aggregability up to the value of the control.

#### 4. Discussion

Hyperglycemia has been known to produce glycation (or non-enzymatic glycosylation), which can be characterized by the binding of glucose to amino groups of proteins. This glycation leads to the formation of chemically complex compounds, viz., advanced glycation end-products (AGEs). The highly cross-linked AGEs are often deleterious to cells because they can provoke a hyperoxic response in those cells that bear appropriate receptors (scavenging or RAGEs) (Thornalley, 1998). Thus, the exposure of red blood cells to hyperglycemia can induce hemoglobin glycation and stimulate protein degradation, lipid peroxidation, and hemolysis (Riquelme *et al.*, 2005; Snyder *et al.*, 1985). Coincidentally, the structure of carnosine closely resembles that of the preferred glycation sites in proteins, which can react with many potential glycating agents, thereby inhibiting their ability to react with and modify polypeptides (Hipkiss and Brownson, 2000). Subsequently, carnosine in hyperglycemia may play an important role as an anti-glycant and an oxygen free-radical scavenger (Crush, 1970). Through the present hemorheological study of RBCs, these potential functions of carnosine have been confirmed. Carnosine, when premixed in glucose-rich media, prevents hemorheological degradation in a concentration-dependent manner. In glucose-rich media, carnosine apparently affects the deformability and aggregability of RBCs. Thus, it may be confirmed that carnosine is an effective anti-glycant and a free-radical scavenger, even in hyperglycemia.

A recent hemorheological study (Aydogan *et al.*, 2008) of RBCs that were incubated in H<sub>2</sub>O<sub>2</sub> showed similar results with the addition of carnosine. These results demonstrated the rejuvenating effect of carnosine on the deformability of RBCs that were already impaired by oxidative stress. In this study, the rejuvenated deformability of RBCs with carnosine was even greater than that for the

control. Similar experiments were conducted in our study. RBCs were incubated in glucose-rich media and then re-incubated in carnosine-reinforced plasma. There was an apparent, rejuvenating effect of carnosine on both the deformability and aggregability of impaired RBCs, even though the effect was not significantly large. As discussed above, since the structure of carnosine closely resembles that of the preferred glycation sites in proteins, the glycated sites may be occupied by carnosine, which, in turn, results in the rejuvenation of hemorheological characteristics. It is commonly known that most anti-oxidants prevent free radicals from binding the proteins but have no effect after protein binds with the free radicals. However, carnosine performs an apparent rejuvenating function for hemorheologically damaged cells in hydrogen peroxide and glucose-rich media. However, it has not been fully validated yet whether reversible chemical changes associated with the rejuvenating function of carnosine may occur. Thus, it may be worth examining the rejuvenating effect for varying incubation times in glucose-rich media. Also, it is worthwhile to further study possible roles of carnosine on regulating blood glucose in-vivo (Nagai *et al.*, 2003), even though the present study is limited to in-vitro study.

#### 5. Conclusion

Hemorheological properties, which are easily modified by glucose-induced oxidation and glycation can be prevented by carnosine, a naturally occurring dipeptide ( $\beta$ -alanine-LFull-size image-histidine). Carnosine acts as an anti-oxidant as well as a free-radical scavenger. The results reveal that the presence of carnosine effectively prevented these rheological alterations in a concentration-dependent manner in glucose-rich media. Furthermore, carnosine reveals its rejuvenating effects in erythrocytes that are exposed to glucose-rich plasma. Since the present results were from in-vitro study, it is required to confirm the effect of carnosine on hemorheological alteration through in-vivo study. It is also proposed that moderate concentrations of carnosine might be further explored as potential therapeutic agents for pathologies that involve hemorheological modification.

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