Biochemical Integrity of Stored Blood: Preliminary Investigation into Protective Effects of Cinnamic Acid

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Abstract
Oxidative stress is a critical contributor to the development of blood lesions during blood storage. It damages lipids and proteins, including hemoglobin, and can impair the function and viability of blood both during and after transfusion. This research sought to evaluate the effect of cinnamic acid on oxidative stress induced in whole blood withdrawn from rats and stored for 28 days.

Rats were divided into control and experimental groups. Blood was collected and preserved at 4 ^{o}C for 28 days. The control group was stored without any antioxidants. The experimental group was treated with cinnamic acid at concentrations of 50 µg and 100 µg. On days 0, 14, and 28, blood was collected for assessment of oxidative stress indicators, antioxidant enzymes, and protein oxidation byproducts.

Results showed that blood storage for 28 days in the control group caused a decrease in the superoxide dismutase (SOD) and catalase (CAT) activities ($P>0.05$) compared to day 0. There was a significant rise in lipid peroxidation ($P<0.0001$), and an elevation of protein sulfhydryl level ($P<0.05$) compared to day 0. Advanced oxidation protein products (AOPP) were significantly lowered during the storage period when compared to day 0. However, the addition of cinnamic acid to the stored blood significantly increased the protein sulfhydryl level on the 14th day and slightly reduced it on the 28th day ($P>0.05$), in contrast to the control. The activities of superoxide dismutase (SOD) and catalase were increased, and the lipid peroxidation level was notably lower, especially on the 14th day, compared to the group without an antioxidant ($P<0.05$).

In conclusion, cinnamic acid increased the antioxidant capacity of whole blood stored and protected against oxidative stress. Our data showed that cinnamic acid failed to inhibit the production of advanced oxidation protein products and did not protect against the formation of protein sulfhydryl groups.

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Introduction

Blood and its components play an essential biochemical role in sustaining life and restoring physiological balance, and ensure the survival of patients over the years (Carl *et al*., 2016; Barzegar *et al*., 2022). The storage of blood is essential to meet the requirements for the demand for blood in blood transfusion (Li *et al*., 2010). Some metabolic, biochemical, molecular and structural changes such as altered properties of hemorheology, increased rigidity of erythrocyte membrane, loss of nitrous oxide (NO) bioactivity, ATP depletion, loss of 2,3-diphosphoglycerate (2,3-DPG), glutathione (GSH), and nicotinamide adenine dinucleotide (NADH/NADPH) depletion with subsequent oxidation of hemoglobin and exhaustion of the endogenous antioxidants (Li *et al*., 2010), (Sanford *et al*., 2017) occur during the *ex-vivo* storage of red blood cells (RBCs). These changes are collectively called storage lesions (Barzegar *et al*., 2022).

Oxidative stress constitutes one of the processes by which major storage lesions occur in blood storage. It is referred to as a situation that occurs due to a physiological imbalance between the levels of antioxidants and oxidants (reactive oxygen species and reactive nitrogen species), with the level of oxidants exceeding the level of antioxidants (Barzegar *et al*., 2022). According to Adeyanju *et al*. (2021), the susceptibility of rat erythrocytes deteriorating is faster compared to human erythrocytes and the storage lesion observed in rat erythrocytes stored for 1 week can be compared to the storage lesions in human erythrocytes stored for 4 weeks, which makes the use of rat a good model that can provide valuable insights into oxidative stress conditions during blood storage.

Preserving blood for transfusion with **antioxidants** is important because stored blood undergoes biochemical changes over time. These changes known as **storage lesions** can reduce the quality and safety of blood used for transfusion. Therefore, antioxidants help to slow or prevent these harmful effects. Studies have shown that the use of antioxidants has potential in reducing oxidative stress in the erythrocytes of blood during blood storage. Under normal conditions, the

erythrocyte antioxidant system and free radicals are in balance, but the incidence of oxidative stress reduces the antioxidant system of the erythrocytes. Different antioxidants have been studied for mitigating oxidative stress caused by blood storage. Barzegar *et al*. (2022) explored the effect of antioxidant nanoparticles, and it was found that these nanoparticles can improve the quality of erythrocytes. In another study by Sanford *et al*. (2017), it was discovered that vitamin C partially protects erythrocytes from oxidative stress.

Cinnamic acid, a phenolic compound and an active component of cinnamon (*Cinnamomum cassia*) (Babaeenezhad *et al*., 2021) and its derivatives have been assessed to possess high antioxidant activity with low toxicity. Previous research has explored the properties of cinnamic acid, with antioxidant activity being one of the identified properties. The antioxidant efficacy of cinnamic acid and its derivatives is often assessed by their capacity to impede lipid oxidation or free radical scavenging activity. The antioxidant activity of cinnamic acid and its derivatives is well known and it has been found that they have strong antioxidant activity due to the phenolic hydroxyl group, which reacts with oxidants and free radicals (Sova, 2012).

However, a review of the literature indicates that the antioxidative effects of cinnamic acid during blood storage remain unexplored to date. Therefore, this study attempts to analyze its role in blood storage.

Materials and Methods

Chemicals and Reagents

Cinnamic acid (CA), trichloroacetic acid (TCA), potassium iodide, acetic acid, adrenaline, hydrogen peroxide (H_2O_2), sodium chloride (NaCl), sodium dodecyl sulphate (SDS), 5,5-dinitrobis-2-nitro benzoic acid (DTNB), thiobarbituric acid (TBA), hydrochloric acid (HCl).

Animals

In this study, 12 female wistar rats weighing about 250g to 300g were obtained and acclimatized for two weeks before the experiment commenced. The rats were placed in a room inside plastic cages, with a 12-hour light and 12-hour dark cycle. They were fed a regular pellet diet with free access to water. Rats were randomly grouped into control and experimental groups. The rats were sacrificed through cervical dislocation and blood was withdrawn by cardiac puncture and stored in CPDA-1 bottles in a ratio of 6:1 (Gilson *et al*., 2009) at 4°C in the refrigerator for 28 days.

Animal Ethics

The animals received human care in compliance with the standard guidelines set up for the care and use of laboratory animals (NRC 2011) for animal experiments. An ethical approval was given by UI-ACUREC (Animal Care and Use Research Committee) with an approval code UI-ACUREC/006-0125/07.

Experimental Design

At day 0, 14, and 28, whole blood (1 mL) was aliquoted from the stored blood and suspended in phosphate buffer (pH 7.4) for biochemical evaluation of its antioxidant status. Cinnamic acid at two different concentrations of 50 µg and 100 µg was added to blood samples of the experimental group. The control group had no antioxidant.

Experimental Procedure

Evaluation of the Antioxidant Status of Stored Blood

Superoxide Dismutase

Superoxide dismutase activity was measured by the method of Misra and Fridovich (Gilson *et al*., 2009). Plasma was added to 1.25ml of carbonate buffer and 0.15ml of adrenaline. The spectrophotometric measurement was at 480 nm. SOD activity was measured as the amount of enzyme that inhibited epinephrine oxidation by 50%.

Catalase

Catalase activity was determined by the method of Aebi (Aebi, 1984). Decrease in absorbance was measured at 240nm. An extinction coefficient of 43.6M/cm was used for the calculation.

Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric acid reactive substances were determined by the method of Bar-Or *et al.* (Bar-Or *et al*., 2001). The absorbance was taken at 532 nm.

Advanced Oxidation Protein Products

Spectrophotometric determination of advanced oxidation protein product levels was assayed as an index of dityrosine-containing cross-linked protein

products by Witko and Descamps-Latscha (Witko *et al*., 1992). An extinction coefficient of 26/ (mM $_{s}$ cm) was used to calculate AOPP.

Protein Sulfhydryls

The level of protein sulfhydryls (PSHs) was measured as described by Habeeb (Habeeb, 1972). Absorbance was measured at 412nm.

Data Analysis

The results were presented as the mean ± standard error of mean. The statistical analysis was conducted using one-way analysis of variance with the Statistical Package for Social Sciences software for Windows version 16 (SPSS, Inc., Redmond, WA). Post hoc testing was done for intergroup comparisons using the least significant difference

Results

SOD Activity

The activity of SOD in blood stored for 28 days without cinnamic acid is depicted in Figure 1. There was a slight reduction in the activity of SOD in blood stored over 28 days when compared to day 0 (P>0.05).

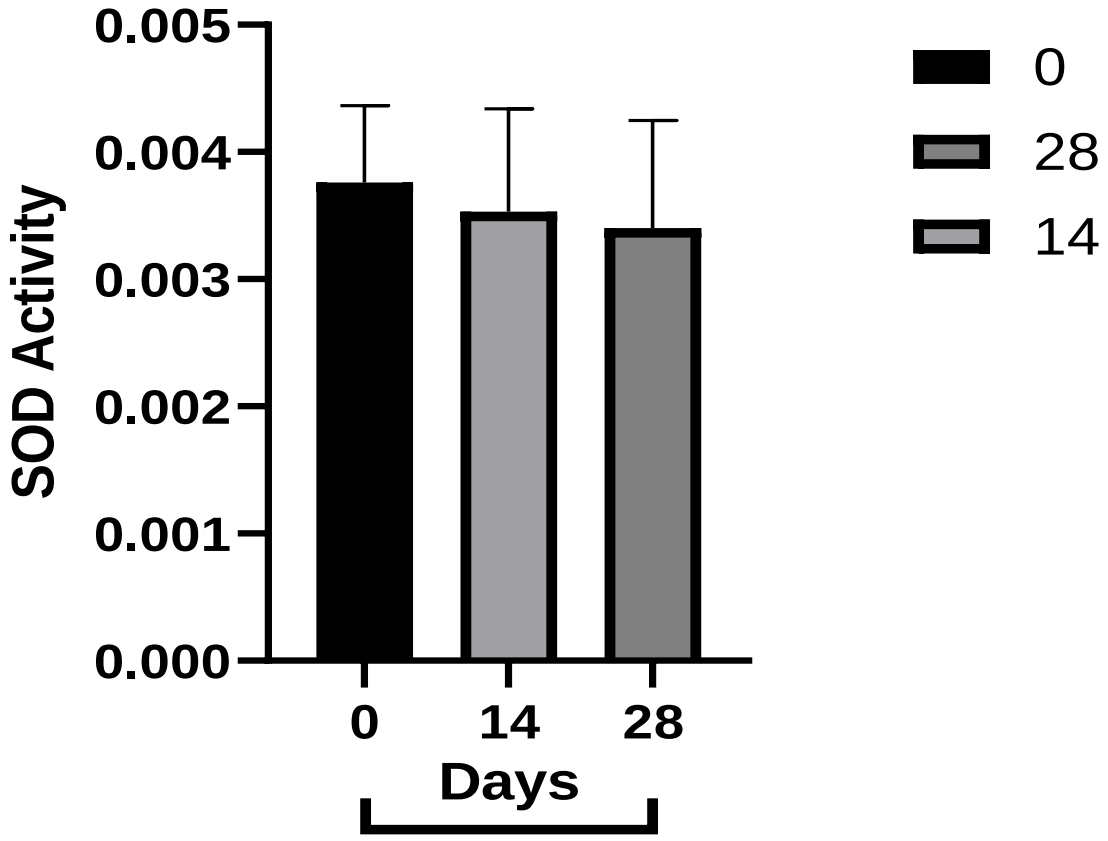

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Fig. 1: Evaluation of SOD activity in blood stored for 28 days. Values are mean ±SE; P > 0.05 when compared to day 0.

The effect of cinnamic acid on SOD activity at day 14 of blood storage is shown in Figure 2. There was a slight elevation in the activity of SOD when 50µg and 100µg of cinnamic acid were added compared to the control, although there was also no statistical significance.

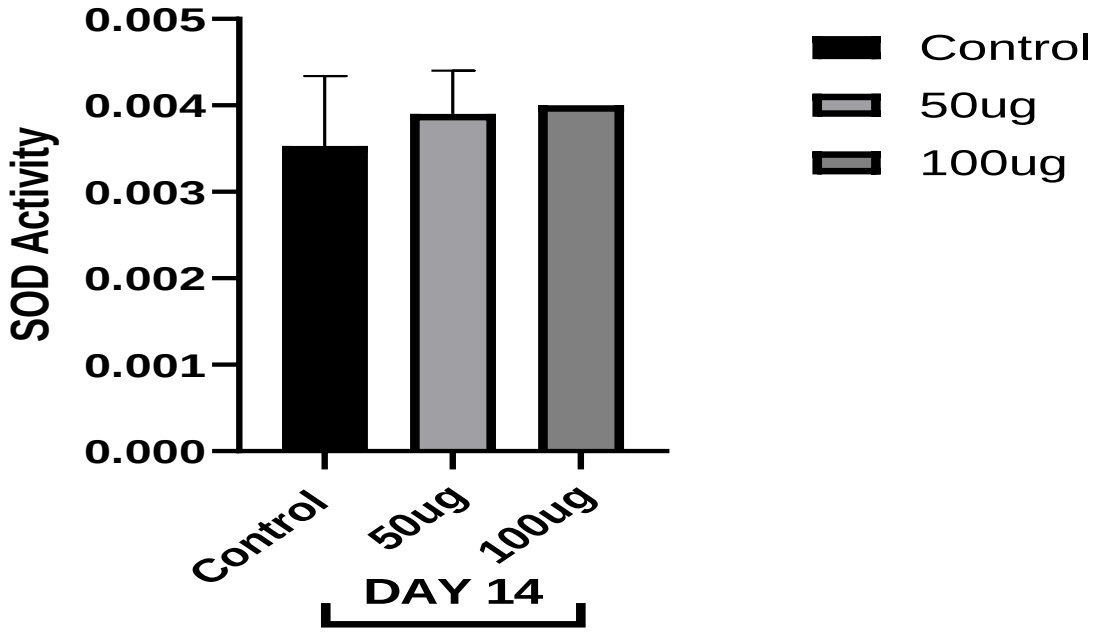

Fig. 2: The effect of cinnamic acid on the activity of SOD at 50µg and 100µg on day 14 compared with the control. Values are mean ±SE; p>0.05 when compared to the control

The effect of cinnamic acid on SOD activity at day 28 of blood storage is shown in Figure 3. The activity of SOD increased slightly in a dose-dependent manner compared to the control without an antioxidant (P>0.05).

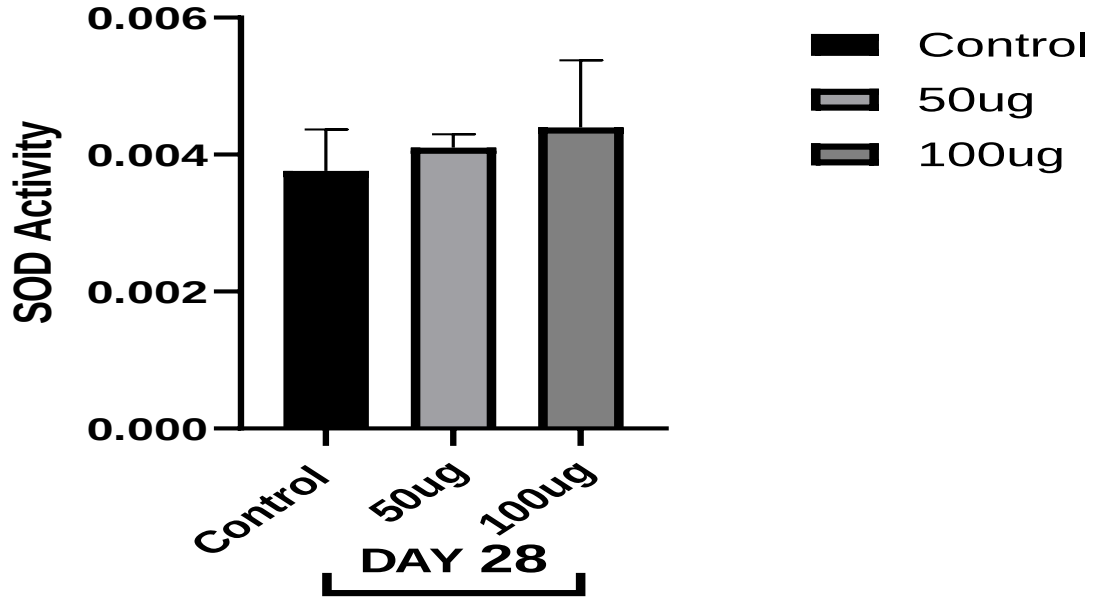

Fig. 3: The effect of cinnamic acid on the activity of SOD at 50µg and 100µg on day 28 compared with the control group. Values are mean ±SE; P>0.05 when compared to the control.

Catalase Activity

The activity of catalase in blood preserved for 28 days without cinnamic acid is shown in Figure 4. There was a progressive decrease in catalase activity of blood stored over 28 days when compared to day 0 ($p>0.05$).

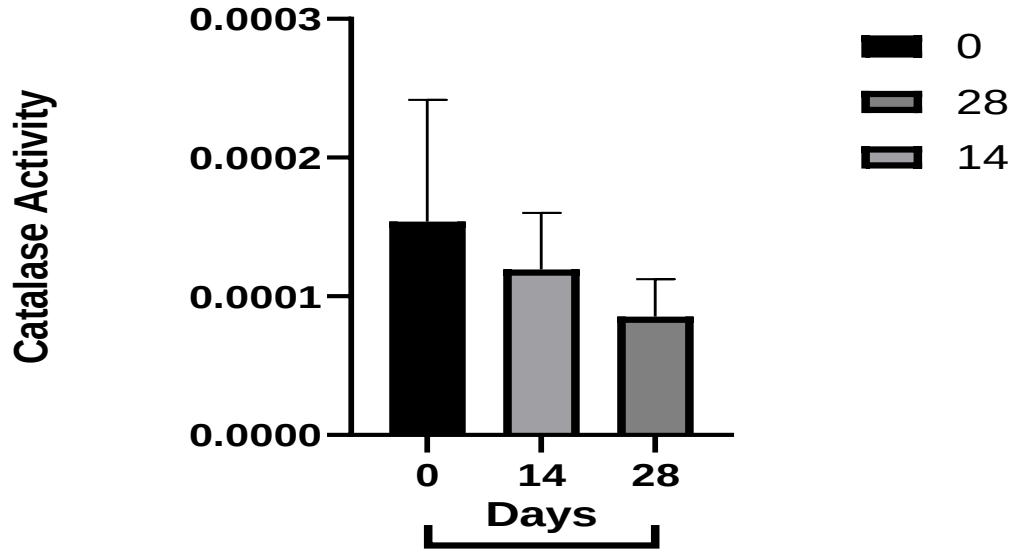

Fig. 4: The activity of catalase in blood stored for 28 days. Values are mean ±SE; P > 0.05 when compared to day 0.

The effect of cinnamic acid on catalase activity at day 14 of blood storage is represented in Figure 5. A modest increase in the activity of catalase was observed with both concentrations of cinnamic acid when compared to the control ($p>0.05$).

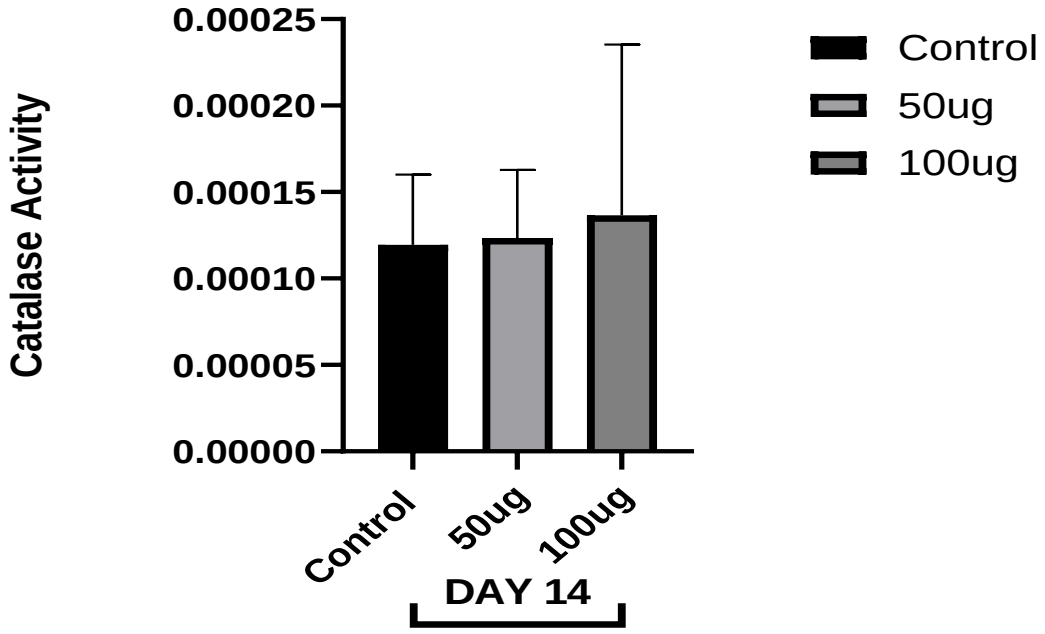

Fig. 5: The effect of cinnamic acid on catalase activity at 50µg and 100µg on day 14 compared with the control group without an antioxidant. Values are mean ±SE; p>0.05 when compared with the control.

The effect of cinnamic acid on catalase activity at day 28 of blood storage is represented in Figure 6. There was a concentration-dependent increase in the activity of catalase in the 50µg and 100µg concentrations of cinnamic acid used relative to control (p>0.05).

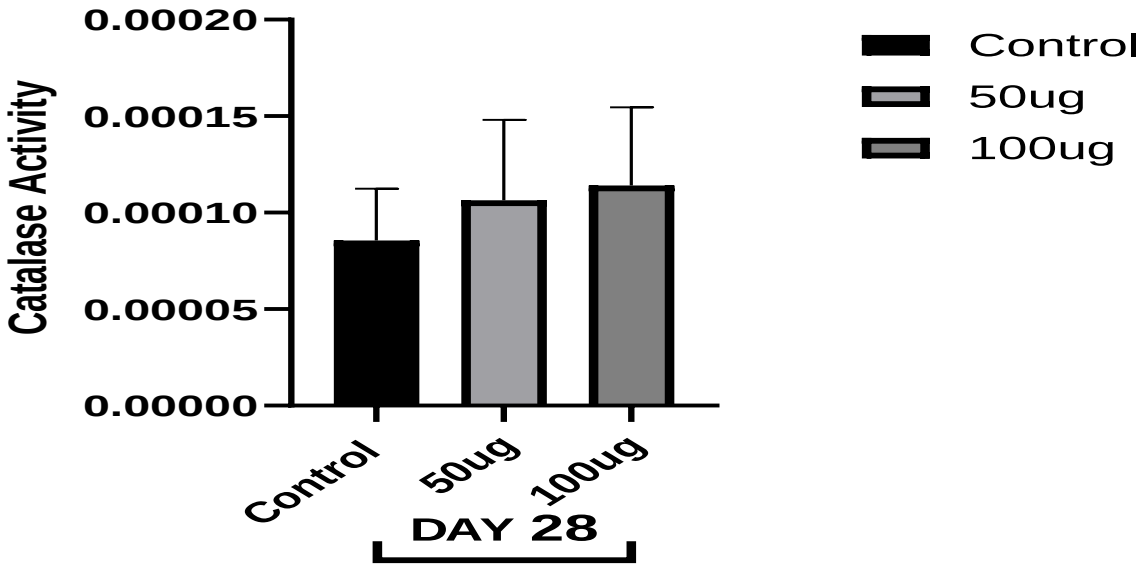

Fig. 6: The effect of cinnamic acid on catalase activity at 50µg and 100µg on day 28 compared with control group. Values are mean ±SE; p>0.05 when compared with the control.

Thiobarbituric Acid Reactive Substances Level. The level of TBARS in blood preserved for 28 days without an antioxidant is illustrated in Figure 7. There was significant increase in TBARS level in blood stored for 28 days when compared to control on day 0 (P<0.0001).

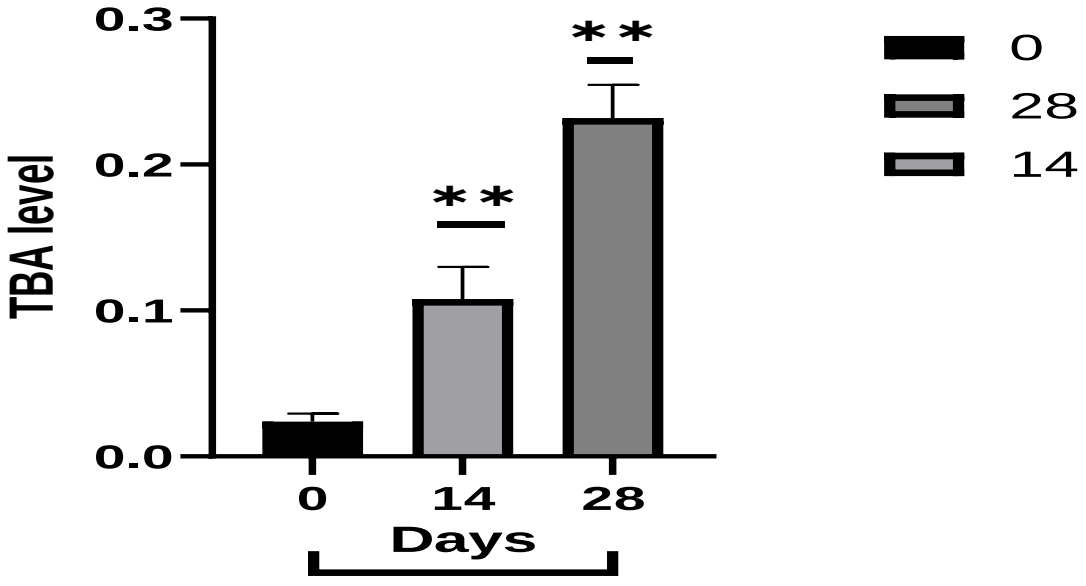

Fig. 7: The TBARS level in blood stored for 28 days. Values are mean ±SE; **P <0.0001 when compared to control.

The effect of cinnamic acid on TBARS level in blood storage over 14 days is shown in Figure 8. There was a decrease in the TBARS level when 50µg and 100µg of cinnamic acid were added. This was significant compared to the control ($p>0.01$).

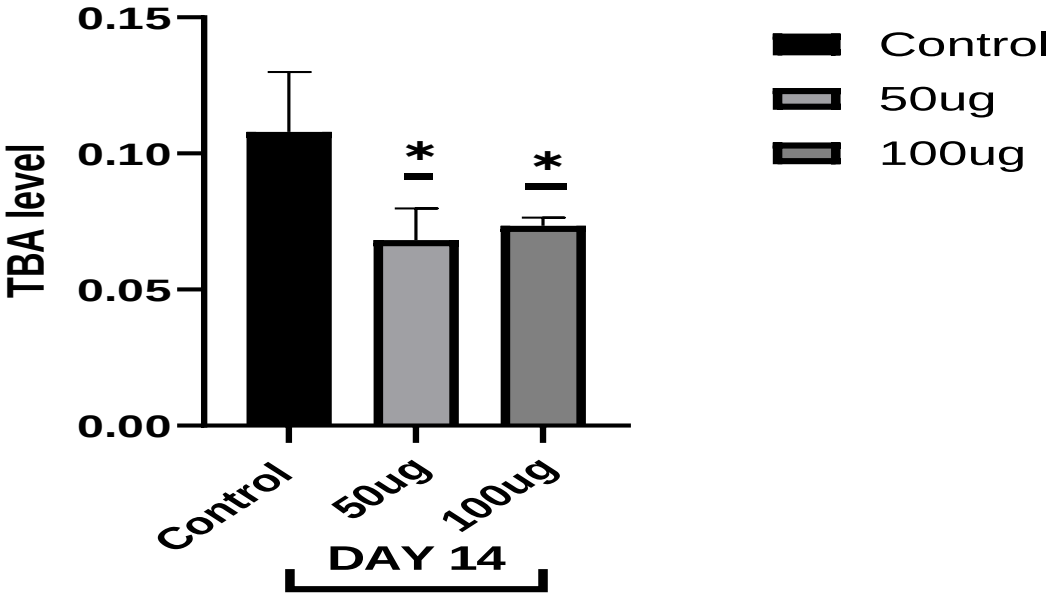

Fig. 8: shows the effect of cinnamic acid in the level of TBARS at 50µg and 100µg on day 14 compared with control group without antioxidant. Values are mean ±SE; *P<0.05 when compared to control.

The effect of cinnamic acid on TBARS level in blood storage over 28 days is shown in Figure 9. There was no notable change in TBARS at the 50µg and 100µg concentrations of cinnamic acid compared to the control ($p>0.05$).

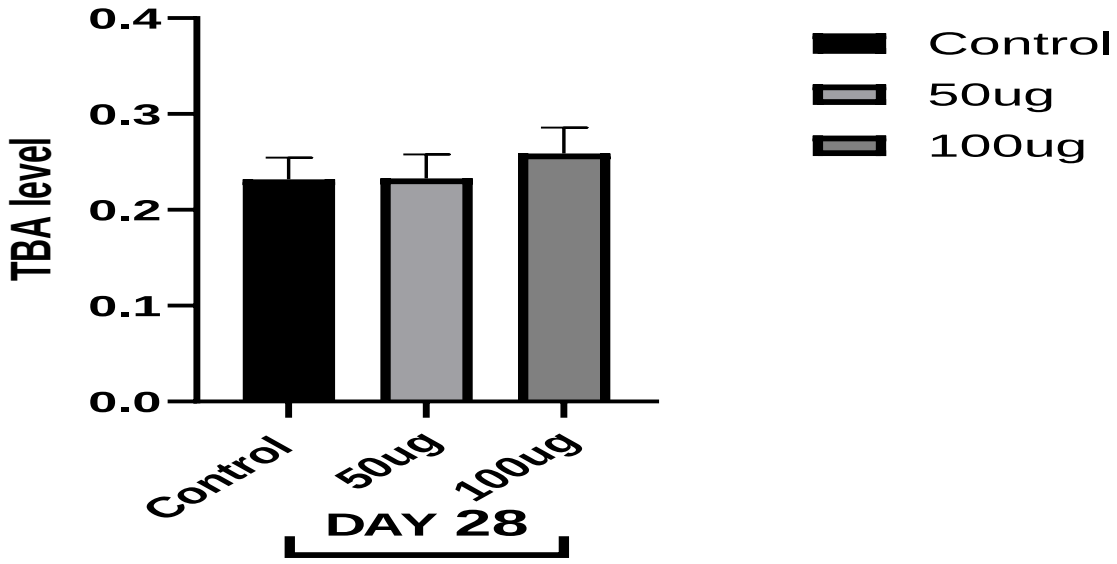

Fig. 9: The effect of cinnamic acid on the level of TBARS at 50µg and 100µg on day 28 compared with the control. Values are mean ±SE; P>0.05 when compared to the control.

Protein Sulfhydryl Level

The level of protein sulfhydryl in 28 days of blood storage without an antioxidant is shown in Figure 10. There was an increase in the level of protein sulfhydryl over 28 days compared to day 0. Protein sulfhydryl level on day 14 was higher and significant when compared to day 0.

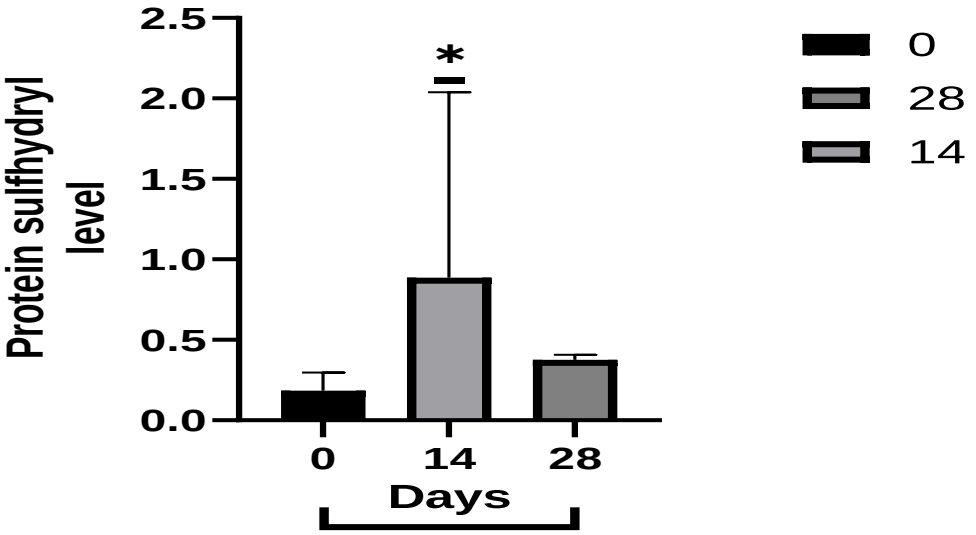

Fig. 10: The protein sulfhydryl level in blood stored for 28 days without an antioxidant. Values are mean ±SE; *P<0.05 when compared with day 0.

The effect of cinnamic acid on protein sulfhydryl level in blood kept for 14 days is shown in Figure 11. There was a substantive increase in the protein sulfhydryl level when cinnamic acid at 50µg and 100µg was added (P<0.0001).

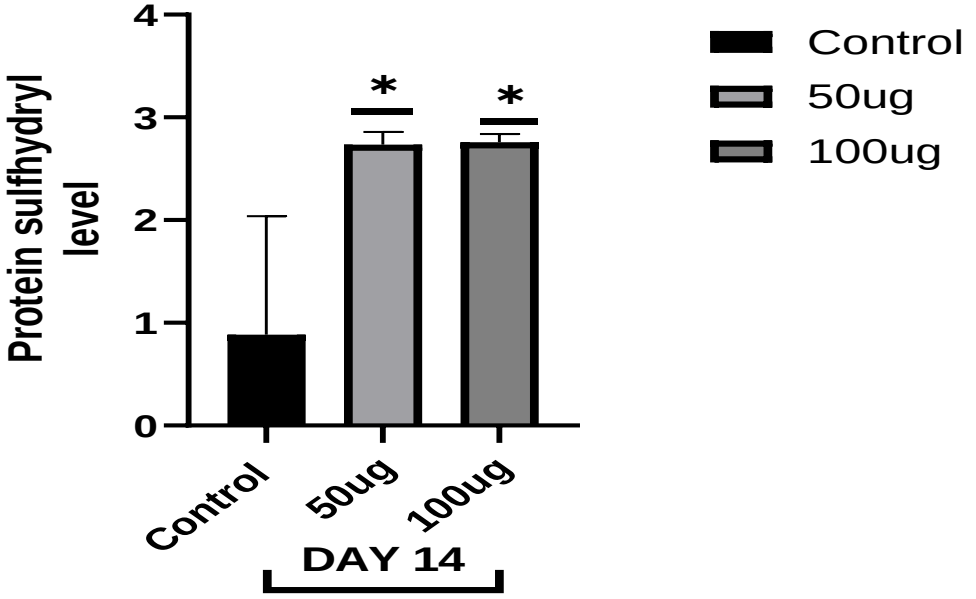

Fig. 11: The effect of cinnamic acid on protein sulfhydryl level at 50µg and 100µg on day 14 when compared to the control. Values are mean ±SE. *P<0.0001 when compared to control.

The effect of cinnamic acid on protein sulfhydryl level in blood storage for 28 days is shown in Figure 12. Cinnamic acid at 50µg and 100µg reduced protein sulfhydryl level. Although this was not significant.

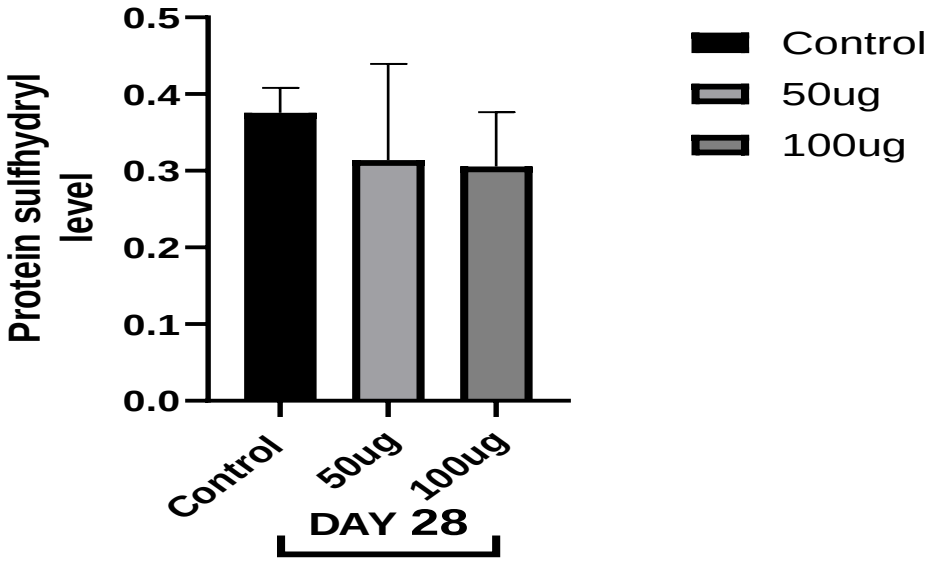

Fig. 12: The effect of cinnamic acid on protein sulfhydryl level at 50µg and 100µg on day 28 when compared to the control. Values are mean ±SE; P>0.05 when compared with the control.

AOPP Formation

The formation of AOPP in blood preserved for 28 days without an antioxidant is shown in Figure 13. There was a significant decrease in AOPP formation in blood stored over 28 days when compared to control (P<0.0001)

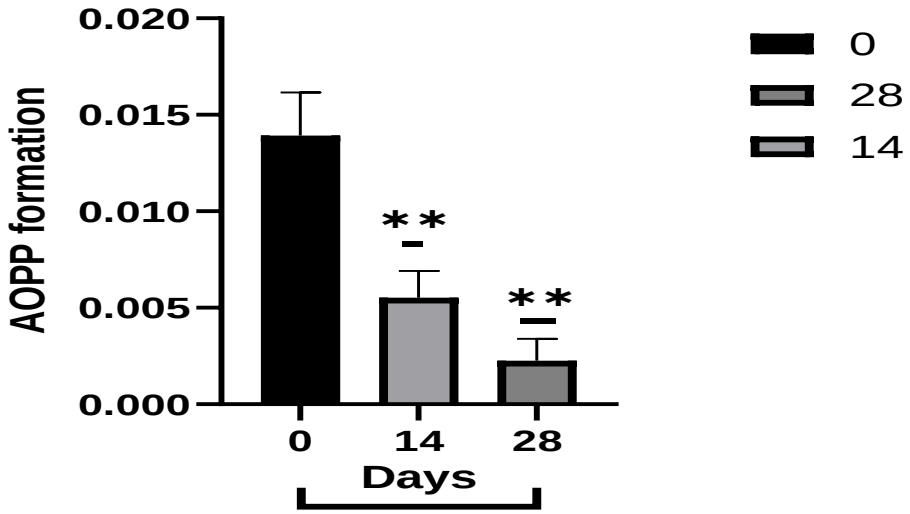

Fig. 13: The AOPP formation in blood stored for 28 days. Values are mean ±SE; **P<0.0001 when compared with day 0.

The effect of cinnamic acid on AOPP formation in blood stored for 14 days is shown in Figure 14. There was a significant formation of AOPP when cinnamic acid was added at both concentrations used (P<0.0001).

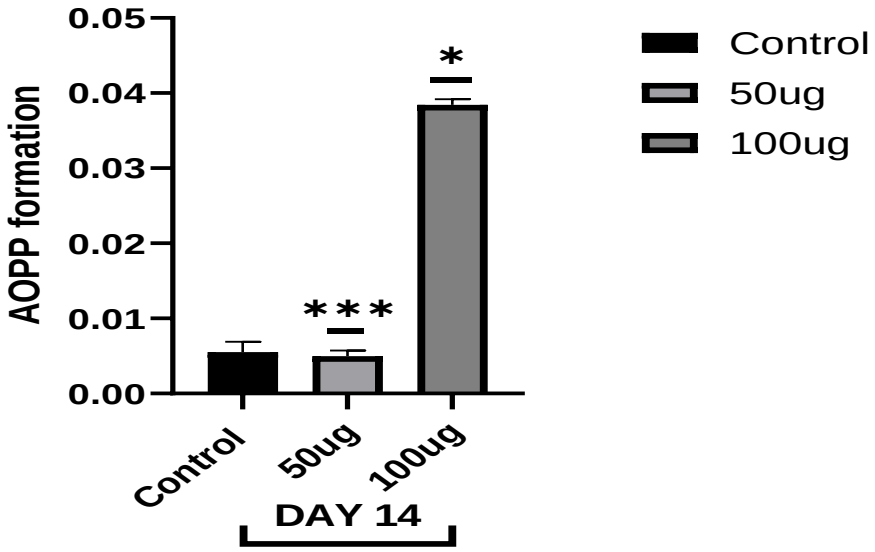

Fig. 14: The effect of cinnamic acid on AOPP formation at 50µg and 100µg on day 14 when compared to the control. Values are mean ±SE. ***P<0.0001 and *P<0.05 when compared to control.

The effect of cinnamic acid on AOPP formation in blood stored for 28 days is shown in Figure 15. There was an elevation in AOPP formation when cinnamic acid was added (P>0.05).

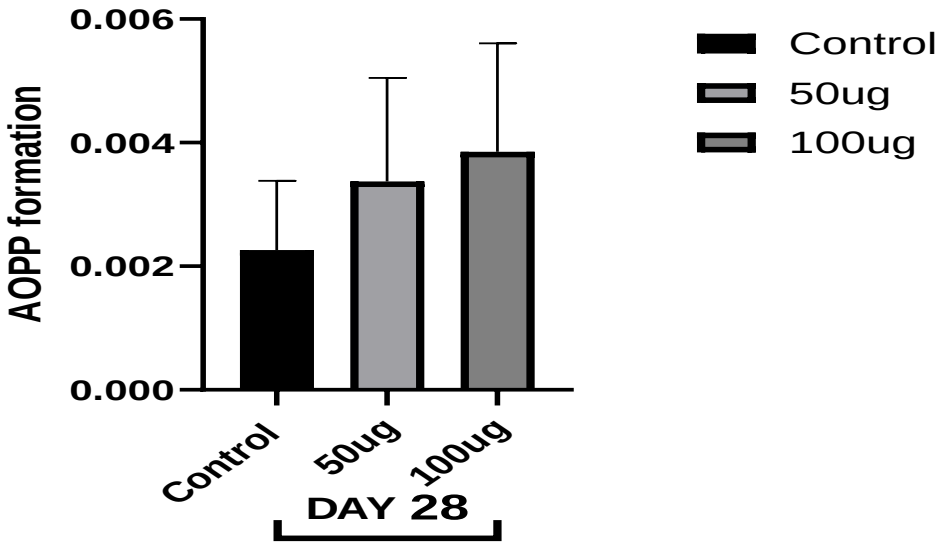

Fig. 15: The effect of cinnamic acid on AOPP formation at 50µg and 100µg on day 28 when compared to the control. Values are mean ±SE; P>0.05 when compared to the control.

Discussion

Studies have shown that increased storage lesions of the red blood cell units could result from a decline in the antioxidant defense system, which can increase the susceptibility of stored blood to oxidative stress (*Şekeroğlu et al., 2012;* Czubak *et al*., 2017). Oxidative stress is known to be a major contributor to storage lesions during blood storage (Barzegar *et al*., 2022). The study attempts to evaluate the antioxidant capacity of cinnamic acid on oxidative stress induced in blood stored for 28 days.

SOD is an antioxidant enzyme that catalyzes the dismutation of superoxide anion to the toxic product hydrogen peroxide (Halliwell, 2001). Data showed a slight decrease in the SOD activity of the blood stored without antioxidants for 28 days, as compared to blood stored at day 0, indicating oxidative stress. Intervention with cinnamic acid caused a slight increase in SOD activity compared to the control, a process that could suppress the oxidative stress initially observed. The decrease in catalase activity in the blood can be harmful to antioxidant enzymes activity (Carl *et al.,* 2016). The increase in catalase activity as a result of the addition of cinnamic acid suggests a potential reduction in oxidative stress (Chaudhary *et al*., 2023) which could restore the antioxidant capacity of the blood cells.

Oxidative stress promotes lipid peroxidation with an increase in malondialdehyde level (MDA). MDA is one of the byproducts of lipid peroxidation and an oxidative stress indicator. It cross-links the phospholipids and proteins of the erythrocyte membrane and consequently impairs its functions and decreases its survival (Cheraghi *et al*., 2019). Aslan *et al.* (1997) reported that plasma MDA levels of stored blood increased with time. In the present investigation, the level of TBARS significantly increased in stored blood on day 14 and day 28 indicating lipid peroxidation. Cinnamic acid at 50µg and 100µg significantly decreased TBARS level till day 14, which can be related to the effect of cinnamic acid decreasing lipid peroxidation.

There was an increase in the level of protein sufhydryls throughout the 28 days of blood storage. Addition of cinnamic acid at both concentrations could not reduce the protein sulfhydryl level. Cinnamic acid was not efficient at these concentrations and a higher concentration could have been more effective in protection against sulfhydryl formation.

Protein sulfhydryl (PSH) groups are mainly present in the cysteine group of proteins and at smaller concentrations in glutathione. They are predisposed to oxidation to disulfides, a reversible reaction that can undermine the antioxidant capacity of plasma and protein function (Adeyanju *et al*., 2021) High and significant PSH levels were noticed, especially on day 14, in the blood stored for 28 days, which may be due to its oxidation. However, there was a decrease in the AOPP formation during the time of storage, which is in contrast with the expected increase in AOPP formation during blood storage due to protein oxidation as reported by Antonelou *et al*. (2010). The inability of cinnamic acid in this study, to sufficiently prevent oxidative protein damage, even at higher concentrations could be a result of prooxidation reported for phenolic compounds, where higher concentrations could not scavenge free radicals (Nowak *et al*., 2022).

Conclusion

The results obtained in this study indicate that cinnamic acid was capable of protecting against oxidative stress and lipid peroxidation in blood stored for 28 days. However, its inability to suppress protein formation in the current study suggests the inefficiency of cinnamic in this regard and may require the use of high concentration for optimization and possible combination with other antioxidants to achieve protection.

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