Characteristics of resveratrol and serotonin on antioxidant capacity and susceptibility to oxidation of red blood cells in stored human blood in a time-dependent manner

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Abstract

Objective: In stored red blood cells (RBCs), which are used in diseases (e.g., acute blood loss and leukaemia), storage lesions arise by oxidative stress and other factors over time. This study investigated the protective effects of resveratrol and serotonin on stored RBCs.

Methods: Blood from each donor (n = 10) was placed in different bags containing 70 mL of citrate phosphate dextrose (total volume: 500 mL) and divided into three groups (n = 30): control, 60 µg/mL resveratrol, and 60 µg/mL serotonin. Malondialdehyde (MDA) and glutathione (GSH) levels, activity of glutathione peroxidase (GSH-Px), catalase, and carbonic anhydrase (CA), and susceptibility to oxidation in RBCs, and pH in whole blood were measured at baseline and on days 7, 14, 21, and 28.

Results: MDA levels and susceptibility to oxidation were increased in all three groups time-dependently, but this increase was greater in the serotonin group than in the other groups. Activity of GSH-Px, CAT, and CA, as well as GSH levels, were decreased in the control and serotonin groups time-dependently, but were significantly preserved in the resveratrol group. The pH was decreased in all groups time-dependently.

Conclusion: Our study shows that resveratrol attenuates susceptibility to oxidation of RBCs and protects their antioxidant capacity, and partially preserves CA activity time-dependently.

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Introduction
Red blood cells (RBCs), which are used for transfusion, can be stored for up to 6 weeks at 2–6°C under suitable conditions. However, morphological, biochemical, and functional changes known as “storage lesions” can arise in RBCs at the initial stage of storage. Storage lesions negatively affect the lifespan and efficiency of RBCs in a time-dependent manner. The maximum storage period and effectiveness of RBCs depend on the content of their storage medium and protective solutions. Various preservative solutions have been developed for minimizing storage lesions and prolonging the lifespan of RBCs. The most common combinations involve addition of citrate phosphate dextrose (CPD), acid citrate dextrose, citrate phosphate dextrose adenine, and sorbitol glucose adenine mannitol. Although blood can be stored in these solutions, storage lesions may develop. Therefore, intensive studies have aimed to increase the storage times of RBCs. These studies reported that the addition of various antioxidants, such as vitamin E, vitamin C, β-carotene, melatonin, propofol, and tannic and caffeic acids, as protective solutions had a positive effect on the survival of RBCs.

We hypothesize that the antioxidant properties of resveratrol and serotonin (phenolic compounds) may benefit the susceptibility to oxidation and antioxidant capacity of RBCs in stored blood. This is because reactive oxygen species (ROS), such as hydroxyl radical (OH−), superoxide anion radical (1O2−), and hydrogen peroxide (H2O2), increase oxidative stress in a time-dependent manner. ROS induce oxidation in membrane lipids and proteins, and antioxidants in the phenolic structure can scavenge the factors that cause oxidative stress. Therefore, the lifespan of stored RBCs can be extended by reducing oxidative stress.

Resveratrol is a natural compound that acts on various intracellular messengers and is found in edible plant leaves and red wine, as well as in fruits, such as mulberry, grapes, and peanuts. Guˇlc¸in reported that resveratrol inhibited peroxidation of a linoleic acid emulsion by 89.1% and was more effective than standard antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, α-tocopherol, and Trolox. This author also demonstrated that resveratrol was effective for its 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2’-Azino-bis [3-ethyl benzo-thiazoline-6-sulfonic acid] (ABTS+), N, N-dimethyl-p-phenylenediamine radical cation (DMPD+), and O2− scavenging activity, H2O2 reduction power, and Fe2+ chelating activity. Guˇlc¸in also reported that resveratrol exhibited beneficial effects on cardiovascular diseases by promoting vasodilatation and inhibiting oxidative damage and platelet aggregation.

Serotonin (5-hydroxytryptamine) is synthesized in Drosophila by tryptophan-phenylalanine hydroxylase activation, which hydroxylates phenylalanine and tryptophan. Serotonin is an important neurotransmitter that has several biological roles. ABTS+ radical scavenging activity and antioxidant properties of serotonin and some of its derivatives have been tested. Guˇlc¸in found that melatonin and serotonin exhibited effective DMPD+ radical
scavenging and cupric ion (Cu\(^{2+}\)) reduction activity.\(^{12}\) The molecular structures of resveratrol and serotonin are shown in Figure 1.

In this study, we investigated the protective effects of resveratrol and serotonin on lipid peroxidation and sensitivity to oxidation of RBCs in stored blood. We aimed to develop protective solutions and increase the viability of RBCs. The effects of resveratrol and serotonin on total carbonic anhydrase (CA), catalase (CAT), and glutathione peroxidase (GSH-Px) activity, as well as reduced glutathione (GSH) levels and pH, were also investigated.

**Materials and methods**

This study protocol was performed in accordance with the ethical rules in human experiments in the context of the World Medical Association Ethics Rules in the Helsinki Declaration. For this study, ethics committee approval was obtained from the Yuzuncu Yil University Non-Pharmaceutical Clinical Practices Local Ethics Committee (REC number: 06/13.08.2014). Before blood was taken, necessary information was provided to the volunteers of the study.

Resveratrol and serotonin were obtained from Sigma Aldrich Company (St Louis, MO, USA). Four-way paediatric bag systems connected to the main bag for blood samples were purchased from Kansuk (LOT:1104070; Kansuk, Istanbul, Turkey).

NADPH, molybdic acid, GSH, 1,1,3,3-tetraethoxy propane, methanol, ethanol, hydrogen peroxide, and other chemicals were obtained from Merck (Istanbul, Turkey).

**Obtaining blood samples from volunteers and preparing for measurements of parameters**

Blood from each healthy volunteer (n = 10) was collected in four different paediatric bag systems (Kansuk) containing 70 mL of CPD at the Yuzuncu Yil University Medical Faculty Blood Center. Blood was transferred to three paediatric bags attached to the main bag and categorized into three groups (n = 30). One group was the control group; 60 \(\mu\)g/mL resveratrol (resveratrol group) or 60 \(\mu\)g/mL serotonin (serotonin group) was added to blood for the other groups. Blood was stored at +4°C in a blood bank during the study.

Blood samples (12 mL, n = 30) were taken from stored blood in each group at 1-week intervals (weeks 0, 1, 2, 3, and 4). Measurement of pH was performed using 2 mL of whole blood. The remaining 10 mL of whole blood samples were centrifuged at 2500 \(\times\) g for 15 min and the plasma was discarded. Upper leucocytes and other impurities were carefully removed using a dropper. An equal volume of isotonic solution was added to the underlying erythrocyte (RBC) solution and was slowly overturned. The supernatant was discarded.

![Resveratrol and Serotonin](image-url)
after centrifugation at $2500 \times g$. This procedure was repeated three times and thorough washing of the RBCs was achieved. The susceptibility to oxidation of RBCs was measured in an erythrocyte suspension that was prepared in azide buffer solution. The remaining RBC packages were haemolyzed with distilled ice water and frozen at $-80^\circ C$ until measurement of GSH levels, and GSH-Px, CAT, and CA activity.

**Haemoglobin measurement in RBC packages**

Haemolysate (20 µL) was added to a 5-mL drabkin solution and incubated at room temperature for 10 min. The absorbance of the mixture was measured colourimetrically on a spectrophotometer (Shimadzu UV mini 1240; Korea, Japan) at 540 nm. Haemoglobin concentrations are expressed as g/dL.\(^{20}\)

**Measurement of MDA by high-performance liquid chromatography (HPLC)**

Briefly, 250 µL thiobarbituric acid (42 mM), 750 µL $H_3PO_4$ (0.44 M), and 450 µL distilled water were added to the 50-µL RBC suspension in a test tube and the mixture was incubated for 60 min in a boiling water bath (total volume: 1.5 mL). After cooling in ice water, the same volume of alkaline methanol (50 mL methanol + 4.5 mL of 1 M NaOH) was added to the test tube and centrifuged at $3000 \times g$ for 3 min. The supernatant (200 µL) was transferred to a vial and injected into an HPLC apparatus (Agilent, Boblingen, Germany). An RP-18 column (150 ×4.6 mm) and 5-µm particle size were used. The mobile phase consisted of a mixture of 400 mL of 50-mM phosphate buffer (pH 6.8) and 600 mL of methanol (total volume 1 L). The flow rate of the mobile phase and the injection volume of samples were set to 0.8 mL/min and 20 µL, respectively. Measurements were made against standard samples of different concentrations at an excitation wavelength of 527 nm and an emission wavelength of 551 nm.\(^{21}\) MDA levels are expressed as nmol/gHb.

**Determination of susceptibility to oxidation of RBCs**

The susceptibility to oxidation of RBCs in vitro was determined using the method of Stocks et al.\(^{21}\) The RBC suspension was prepared with azide buffer. A volume of 5 mL of $H_2O_2$ (0.03% concentration prepared in buffered saline solution) was then added to the same RBC suspension, and the mixture was incubated for 2 h at 37°C for oxidation. After 2 h, MDA levels were measured on the HPLC apparatus,\(^{22}\) and the haemoglobin concentration of the RBC suspension was determined. MDA values are expressed as nmol/gHb.

**Measurement of GSH-Px activity in RBCs**

Erythrocyte GSH-Px activity was determined using the method described by Paglia and Valentín.\(^{23}\) In this method, GSH reductase transforms oxidized GSH to reduced GSH in the presence of NADPH, accompanying the conversion of NADPH to NADP\(^+\). For this purpose, a 970-µL reaction mixture (mixture of Tris, $Na_2$EDTA, GSH, NADPH, sodium azide, and GSH reductase) was added to 20 µL of sample and incubated for 5 min at 37°C. After incubation, 10 µL of $H_2O_2$ solution was added to initiate the reaction, and decreasing absorbance values were measured at 340 nm at 30-s intervals for 3 min. GSH-Px activity is expressed as IU/gHb.

**Measurement of CAT activity in RBCs**

CAT activity in RBCs was measured according to the colourimetric method of Goth.\(^{24}\) A volume of 1 mL of 65 µmol/mL
H$_2$O$_2$ (prepared in phosphate buffer 0.06 M, pH of 7) was added to the 0.2-mL RBC package. After 1 min, 1 mL of ammonium molybdate solution (32.4 mmol/L) was added to the test tube to terminate the reaction. Absorbance of the yellow complex formed by molybdate and H$_2$O$_2$ was measured spectrophotometrically at 405 nm on a spectrophotometer (Shimadzu UV mini 1240). CAT activity is reported as kU/gHb.

**Measurement of reduced GSH in RBCs**

GSH values in RBCs were measured spectrophotometrically according to the method of Fairbanks and Klee.$^{25}$ The non-protein sulphydryl groups in RBCs are normally in the form of reduced GSH. DTNB, a disulphide chromogen, is reduced to a yellow compound by the sulphydryl groups of the GSH in RBCs. Absorbance of the reduced chromogen shows maximum absorbance at 412 nm. The concentration of this complex is directly proportional to the GSH concentration. Although the DTNB method measures all free sulphydryl groups (protein-bound or protein unbound), the sulphydryl groups are thought to reflect the GSH value in RBCs because GSH levels are high in RBCs. GSH values are expressed as mg/gHb.

**Determination of total CA activity in RBCs**

Total CA activity in RBCs was measured using the method described Rickly et al.$^{26}$ and modified by Wilbur and Anderson.$^{27}$ Purified water (0.55 mL), bromothymol blue (0.1 mL, 0.04%), and haemolysate (50 μL enzyme solution) were added to tubes containing 1 mL of veronal buffer (0.025 M, pH of 8.2). The elapsed time for the conversion from the blue colour of the indicator to the yellowish-green (pH 6.8) colour was determined by addition of 2.5 mL of CO$_2$ solution (total volume: 4.2 mL) as the substrate ($t_c$). As a reference measurement, we used the elapsed time for the colour change using distilled water instead of haemolysate ($t_0$). CA activity (CO$_2$–hydratase activity) was calculated using the ($t_0$ – $t_c$)/$t_c$ equation$^{28,29}$ and is expressed as U/gHb.

**Measurement of pH**

Values of pH were measured with a commercially purchased blood gas kit (ABL90 FLEX PLUS, Radiometer, Denmark) on an ABL 90 apparatus (Radiometer Medical Materials Industry and Trade Ltd. Inc., Istanbul, Turkey).

**Statistical analyses**

Descriptive statistics for continuous variables are expressed as the mean and standard deviation. Following analysis of variance, Duncan’s multiple comparison test was used for inter-group comparison within the same week and for intra-group comparison between weeks. Statistical significance was considered at 5% ($p < 0.05$). The SPSS 15 statistical package program (Chicago, IL, USA) was used for calculations.

**Results**

Volunteers involved in the study (n = 10) were people who had lived in Van city centre in Turkey for at least 2 years. Four of the volunteers were Kurdish and six of them were of Turkish origin. Volunteers were healthy men between the ages of 21 and 25.

MDA levels in RBCs in all of the groups increased in a time-dependent manner ($p < 0.05$). However, this increase was greater in the serotonin group from week 3 onwards (on days 14, 21, and 28) compared with the other groups ($p < 0.05$) (Figure 2). We also tested the susceptibility of RBCs to oxidation in vitro. Two hours after in vitro oxidation with H$_2$O$_2$ (0.03%),
MDA levels were increased in all groups in a time-dependent manner during 5 weeks. However, after in vitro oxidation, MDA levels in the resveratrol group were significantly lower than those in the other groups on days 14, 21, and 28 ($p < 0.05$). After in vitro oxidation with H$_2$O$_2$, MDA values on days 21 and 28 in the serotonin group were significantly higher than those in the control and resveratrol groups ($p < 0.05$) (Figure 2).

We also compared changes in GSH-Px and CAT activity and levels of GSH, which function as antioxidants in RBCs, over time. GSH-Px and CAT activity, as well as GSH levels, were decreased in the control and serotonin groups, but were significantly preserved in the resveratrol group in a time-dependent manner ($p < 0.001$). GSH-Px and CAT activity, as well as GSH levels, in the resveratrol group were also significantly higher than those in the other groups on days 21 and 28 ($p < 0.001$) (Figure 3). However, GSH-Px and CAT activity, as well as GSH levels, did not significantly change in the serotonin group, and were similar to those of the control group.

We further examined the change in CA activity, which provides pH and ionic balance in RBCs over time. CA enzyme activity in all of the groups was decreased in
intra-group comparisons in a time-dependent manner \((p < 0.001)\). However, CA activity of the resveratrol group was significantly higher than that in the other groups on days 21 and 28 \((p < 0.05)\) (Figure 4).

We investigated changes in pH and pCO\(_2\) in stored whole blood in association with CA time-dependently. We found that pH was decreased in all groups time-dependently \((p < 0.05)\). However, pH values on day 28 in the resveratrol group were partially preserved according to the control group, although this was not significant (Figure 4). Additionally, pCO\(_2\) values rapidly increased during the first 3 weeks, and then began to fall \((p < 0.05)\). However, pCO\(_2\) values in the resveratrol group were lower than those in the other groups on day 28 \((p < 0.05)\) (Figure 4).

**Discussion**

We investigated the effects of resveratrol and serotonin on antioxidant defence status and oxidative alterations that distort the physiological functions of RBCs. We also examined their protective effects on CA enzyme activity, which plays important roles, such as pH and ion balance in the body.

Storage lesions are defined as changes that result in irreversible damage and decrease post-transfusion survival of blood or RBCs.\(^{30}\) The most obvious changes that affect RBCs during storage are an increase in lipid peroxidation levels and a decrease in the activity of enzymes that play a role in the antioxidant defence system, such as superoxide dismutase (SOD), CAT, and GSH-Px. Additionally, there are haemorheological changes, such as an increase in RBC sphere membrane deformation and mean Hb concentrations, and osmotic fragility.\(^{30,31}\)

Most irreversible events occur as a result of long-term oxidative stress under non-physiological storage conditions. One of the most important reasons for an increase in oxidative stress is decreased antioxidant capacity of RBCs or stored blood.\(^{32}\) ROS, which result from increasing oxidative stress in stored RBCs, lead to impaired membrane integrity and cell death by attacking lipids and proteins in membranes.\(^{30}\) Therefore, cells possess enzymatic or non-enzymatic antioxidant defence mechanisms against this situation.\(^{31,32}\) However, researchers have suggested that these negative changes occur even if stored RBCs or blood are maintained under suitable conditions. Various studies have been

Figure 4. Protective effects of resveratrol and serotonin on total carbonic anhydrase activity in red blood cells, as well as pH and pCO\(_2\) in whole blood
A: Carbonic anhydrase activity \((U/gHb)\); B: pH; and C: pCO\(_2\). *\(p < 0.01\) versus baseline; **\(p < 0.001\) versus controls; #\(p < 0.001\) versus resveratrol and controls.
performed on stored blood to decrease the observed negative changes in a time-dependent manner and to increase the lifespan of RBCs.\textsuperscript{5,6,32,33} Dumaswala et al.\textsuperscript{34} showed that an increasing concentration of GSH in stored blood protected RBCs against free radical damage. Additionally, numerous studies have shown that lipid peroxidation is attenuated by the addition of metal chelators and antioxidant substances to stored blood or RBCs.\textsuperscript{32,35,36} Before blood is taken, donors are provided various antioxidant preparations in their diet. Racek et al.\textsuperscript{37} showed that stored blood was more resistant to free radical damage if various antioxidant tablets were provided to volunteers before blood collection.

Melatonin is one of the strongest antioxidants.\textsuperscript{38} Allegra et al.\textsuperscript{38} demonstrated that erythrocyte membrane proteins and lipids increased the susceptibility to oxidation with a cytotoxic effect of MDA \textit{in vitro}. However, addition of melatonin to stored blood resulted in a protective effect of RBCs against these adverse events. Sekeroglu et al.\textsuperscript{5} found that addition of melatonin to stored blood significantly preserved the levels of erythrocyte MDA, glutathione, GSH-Px, and SOD in a time-dependent manner. In previous studies, MDA levels in erythrocytes increased in a time-dependent manner.\textsuperscript{5,9,39} Our study showed that MDA levels increased in RBCs in a time-dependent manner, consistent with these previous studies. We also tested the susceptibility of RBCs to oxidation \textit{in vitro}. The susceptibility to oxidation of RBCs was increased under conditions of oxidative stress by addition of H\textsubscript{2}O\textsubscript{2} over time. This result supports the hypothesis that some changes occur in RBC membranes because of oxidative damage during the waiting period and that RBCs become less stable. In our study, resveratrol added to stored blood significantly preserved the susceptibility to oxidation of RBCs compared with the control group. However, serotonin showed an adverse effect.

In addition to the endogenous antioxidant systems that play a protective role against oxidative stress in RBCs, there are other factors, such as SOD, GSH-Px, CAT and GSH.\textsuperscript{30,31} GSH-Px acts as the primary antioxidant defence system and performs decomposition of H\textsubscript{2}O\textsubscript{2} at high H\textsubscript{2}O\textsubscript{2} concentrations.\textsuperscript{40} CAT completes the detoxification reaction of H\textsubscript{2}O\textsubscript{2} initiated by SOD to H\textsubscript{2}O and O\textsubscript{2}.\textsuperscript{41} CAT activity is initially low and increases with storage time.\textsuperscript{5,32} This situation may be due to activity of GSH-Px that scavenges H\textsubscript{2}O\textsubscript{2} at low H\textsubscript{2}O\textsubscript{2} concentrations. Previous studies have shown that an increasing storage time decreases GSH-Px activity.\textsuperscript{5,31} Our study suggested that GSH-Px activity in RBCs was attenuated with increasing storage time, in accordance with previous studies.\textsuperscript{5,31} However, addition of resveratrol and serotonin to stored blood increased GSH-Px activity in RBCs during the first 2 weeks and significantly protected RBC in the following weeks compared with the control group.

CAT and NADPH protect RBCs against acute and high exogenous H\textsubscript{2}O\textsubscript{2} levels, while GSH protects RBCs against endogenously produced low H\textsubscript{2}O\textsubscript{2} levels.\textsuperscript{42} In a previous study conducted by our group, CAT activity and GSH levels increased during the first 2 weeks and decreased in the following weeks.\textsuperscript{5,32} Our current study showed that CAT levels in the resveratrol and serotonin groups increased during the first 3 weeks and were then attenuated in the following weeks, consistent with previous studies.\textsuperscript{5,31,32} However, when we examined total CAT levels at the end of day 28, CAT levels in the control and serotonin groups were significantly lower compared with the resveratrol group. Additionally, similar changes in GSH and CAT levels showed a significant association between
these two parameters. The higher levels of CAT and GSH in the resveratrol group on days 21 and 28 may be due to the antioxidant properties of resveratrol.

CAs (carbonate hydrolase, EC 4.2.1.1) represent a large family of Zn-containing enzymes.43 A total of 16 different CA isoenzymes have been identified in mammals.44,45 In various tissues, different CA isoenzymes participate in many important biological processes, such as respiration, gas balance, calcification, photosynthesis, bone resorption, lipid synthesis, urea synthesis, glucose synthesis, body fluid exchange, tumour cell growth, electrolyte release, CO2 and ion transport, and acid-base balance by regulating pH in the eye and inner ear.46,47 CAs contribute to pH balance in the human body and other tissues by catalysing two steps: providing H+ and HCO3− with hydration of CO2 and converting HCO3− to CO2 in acidic medium.48 CA activity is important because a drop in pH has a negative effect on stored RBCs.32 There have been few studies on stored blood or RBCs concerning how CA activity changes over time. In a previous study carried out by our group, CA activity decreased in a time-dependent manner.32 In the current study, CA activity was attenuated in a time-dependent manner, similar to our previous study.32 The decrease in CA enzyme activity over time may be due to a loss of function of proteins depending on the elapsed time, as in other enzymes. However, resveratrol may protect stored blood and RBCs against time-related acidosis, as indicated by the partially preserved CA activity, and pH and pCO2 values on days 21 and 28 in the resveratrol group. In our previous study, resveratrol (30 μg/mL) significantly preserved the change in pH in stored RBCs.29 We believe that the reason why resveratrol did not exhibit a protective effect on the change in pH in this study may be because resveratrol was used at a higher dose (60 μg/mL).

Conclusions
Our study shows that the antioxidant capacity of RBCs is attenuated because of their increasing susceptibility to oxidation and decrease in GSH levels, and GSH-Px and CAT activity, in a time-dependent manner. Furthermore, the decrease in GSH-Px and CAT activity and GSH levels in RBCs is negatively associated with CA enzyme activity and antioxidant capacity with susceptibility to oxidation over time. However, addition of resveratrol to stored blood protects their antioxidant capacity and partially preserves CA enzyme activity and pH and pCO2 levels by decreasing the susceptibility to oxidation of RBCs. For this reason, low concentrations of resveratrol that are added to stored blood may positively contribute to the shelf life of stored RBCs. However, the dose of resveratrol that should be added to stored blood or RBCs must be adjusted accordingly. More detailed studies are required to determine the mechanism by which serotonin increases the susceptibility to oxidation.

Declaration of conflicting interest
The Authors declare that there is no conflict of interest.

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