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Actions of Resveratrol and Serotonin Added to Stored Bloods on Lipid Peroxidation and Oxidative DNA Damage

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ABSTRACT

Stored blood for use in diseases such as acute blood loss, injury and anemia can be maintained for about a month. It was reported that some negative changes called "storage lesions" occur in stored bloods time dependently. In order to delay these changes, preservatives are added to stored bloods. In this study, we aimed to determine the effects of resveratrol and serotonin on oxidative DNA and mitochondrial damage and lipid peroxidation in stored blood.

Blood was taken from 10 volunteers to different blood bags and each blood was separated into 3 groups (control, resveratrol and serotonin groups). Malondialdehyde (MDA), ubiquinone-10 (CoQ10) and 8-hydroxy-2-deoxyguanosine/10^6deoxyguanosine (8-OHdG/10^6dG) levels were determined at baseline and on the 7th, 14th, 21st and 28th day after addition of resveratrol and serotonin.

MDA, CoQ10 and 8-OHdG/10^6dG levels increased over time in the control and serotonin groups ($p<0.05$). However, resveratrol protected against the time-dependent increase in MDA and CoQ10 values ($p>0.05$). MDA and CoQ10 levels were significantly lower in the resveratrol-treated group on the 21st and 28th days compared with the other groups ($p<0.05$).

These results show that oxidation sensitivity increased time dependently in stored blood. However, the addition of resveratrol allowed the extension of the blood's shelf life by protecting against increased lipid peroxidation and oxidized CoQ10.

Key Words: DNA damage, malondialdehyde, oxidative stress, resveratrol, serotonin, stored blood

Introduction

Human blood is stored for a specific time for various uses (1). However, reactive oxygen species (ROS) formation, such as superoxide anion radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and H_2O_2, occurs during storage (2). In addition, erythrocytes of stored blood results in progressive deregulation of the redox poise time dependently, as it is accompanied increased GSSG levels and decreased GSH anabolism. In anaerobic conditions, erythrocytes of stored blood continue to consume glucose, resulting in acidosis and decreased PH. In addition, the decreasing ATP and 2,3 biphosphoglycerate levels of erythrocyte components in stored blood promotes apoptosis and eryptosis time-dependently. Thus, an increase in heme iron of the medium promotion the formation of $OH^{\cdot}$ and $O_2^{\cdot-}$ radicals with the reactions of Haber Weis and Fenton (3). Consequently, the formed free radicals cause lipid, protein and DNA oxidation, which are all structural membrane components, cellular elements, core proteins and nucleic acids in stored blood (2,4). Therefore, immediately some changes begin to occur in the stored blood cells starting from the initial time of blood storage (1). When there are at least three double bonds in lipid fatty acids, malondialdehyde (MDA) occur as end product in the result of lipid peroxidation (5). MDA quantities in stored blood provides information about free radical damage (6). Therefore, it is important to examine lipid peroxidation in stored blood to assess degree of oxidative damage. ROS does not only affect lipids. Reactive oxygen and nitrogen species can also induce DNA mutations. $O_2^{\cdot-}$, $OH^{\cdot}$ and peroxynitrite ($ONOO^-$) radicals are known to cause many types of DNA damage (7). Among base modifications, 8-hydoxy-2-deoxyguanosine (8-OHdG) also forms as a result of guanine residue oxidation. Therefore, 8-OHdG serves as a sensitive biomarker of oxidative DNA damage (8). Also, it is known that $OH^{\cdot}$ radicals are also responsible for the formation of 8-OHdG (9). Because mitochondria possess an electron carrying system and lack an RNA polymerase

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proofreading mechanism during mRNA synthesis, oxidative damage occurs more frequently in mitochondria compared with chromosomal DNA. ROS form as a result of mitochondrial respiration and causes single chain fractures and damaged bases such as 8-oxoguanine and thymine glycol (10).

One of the lipids that carries electrons in the electron transport chain (ETC) is ubiquinol-10 (CoQ10H). Its primary function is to transport electrons between nicotinamide dinucleotide and succinate dehydrogenase in the ETC (11). CoQ10 is present in two forms, oxidized and reduced (12). The reduced form is known as CoQ10H and protects against the oxidative damage of low density lipoprotein (LDL) (13). Because of CoQ10H antioxidant effect, oxidized CoQ10 levels are considered as an important biomarker of mitochondrial oxidative damage (14). Since leukocyte and platelet cells have mitochondria, it is important to protect mitochondrial membrane integrity. However, increasing ROS and oxidative stress may damage mitochondrial membranes depending on the storage time. If the oxidized form of CoQ10 in the electron transport chain structure is suppressed by antioxidants, this may provide a protection mechanism for the mitochondria.

Under normal conditions, blood can be safely stored for about a month at $+4^0$C and longer period of stored blood can not be used (1). This situation causes an additional financial burden due to the disposal of the blood bags in which the stored blood is preserved. This can lead to budget, labor and time losses. Some studies have suggested that the loss of the antioxidant defense system and increased oxidative stress during and after storage shorten the shelf life time of blood cells (15,16). For this reason, it has been shown that adding some additives such as antioxidants to blood bags has a positive effect in order to increase the shelf life by protecting the antioxidant capacities of stored blood (8,17). Racek at al. (18) concluded that addition of antioxidants such as β-carotene, vitamin C, vitamin E and selenium to stored blood contributed positively to the total antioxidant capacity of erythrocytes.

The phenolic compound resveratrol is a natural compound that acts as an intracellular messenger. Due to its beneficial effects to the human body, it has become a focus of study all over the World (19). It was reported that approximately 30 μg/mL resveratrol inhibits linoleic acid emulsion oxidation by 89.1%, which is more effective than standard antioxidants such as butylene hydroxyanisole (BHA), butylene hydroxytoluene (BHT), α-Tocopherol and Trolox that are used as positive control (20). In addition, the study showed that resveratrol effectively scavenges free radicals and chelates ferrous ions (Fe^{2+}). In particular, resveratrol's positive effects on preventing platelets from oxidative damage and aggregation and inducing vasodilation in cardiovascular diseases have been reported (20). Studies on experimental animals have shown that resveratrol prevents carcinogenesis by inhibiting tumor formation and cell growth and differentiation through distinct mechanisms (21). In addition, in vivo and in vitro studies on human health related to Resveratrol have shown beneficial effects on circulatory system and vascular stiffness (22).

It has also been reported that serotonin, an important neurotransmitter with many biological functions, plays a role as antioxidant and radical scavenger at the molecular level in vitro (23). It was shown that melatonin and serotonin effectively scavenge N-N'-Dimethyl-phenylenediamine ($DMPD^{\cdot+}$) free radicals and reduce cupric ions (Cu^{2+}). Also, he stated that serotonin exhibits more effective radical elimination and copper reduction activity than melatonin due to the -OH group present in the phenolic structure (24).

We aimed to measure lipid peroxidation product MDA (25), mitochondrial damage indicator oxidized CoQ10 (26) and oxidative DNA damage indicator 8-OHdG/10^6dG ratios in stored blood and determined the effects of serotonin and resveratrol addition on lipid peroxidation, mitochondria and DNA damage that have a negative effects on the shelf life of stored blood.

Materials and Methods

Chemicals: Serotonin and reseveratrol were purchased from Sigma (SIGMA-ALDRICH Co., 3050 Spruce Street, St. Louis, USA, Product code: H9523, CAS Number: 153-98-0 for serotonin hydrochloride and Product code: R5010, CAS number: 501-36-0 for resveratrol). Quaternary pediatric pouch systems purchased from Kansuk (Kansuk®, Turkey).

Establishment of groups and measurement intervals: The study protocol was performed in accordance with the Helsinki Declaration as revised in 2000. The study protocol was approved by the local ethics committee (Decision number and date: 01/30.01.2014), and informed consent was obtained from each volunteer. Each blood was separately collected into quaternary pediatric pouch systems (Kansuk®, Turkey) containing citrate-phosphate-dextrose (CPD) from 10 healthy volunteers. The blood bags had a volume of 500 mL and contained 70 mL of CPD solution.

Each 500 mL of blood was transferred to 3 pediatric bags attached to the main bag. The first group was separated as a control group and no substances were added to these blood. A total of 60 μg/mL resveratrol

and 60 μg/mL serotonin was added to the second and third group, respectively. For blood storage time and measurement intervals, time intervals specified by previous studies were selected (15,17,26). For this purpose, MDA and oxidized CoQ10 were measured in the samples taken at the beginning, 7, 14, 21 and 28 days in all groups. Leucocyte DNA was isolated for 8-OHdG and dG analysis. After hydrolysis, 8-OHdG and 10^6dG levels were measured by HPLC using an electrochemical detector. The results were reported as 8-OHdG/10^6dG ratios.

Measurement of Plasma MDA by High Pressure Liquid Chromatography (HPLC): MDA levels were measured according to the method described by Khoschsorur et al (27). A total of 0.44 mol/L H_3PO_4 and 42 mmol/L thiobarbituric acid (TBA) was added to 50 μL plasma and incubated for 60 minutes in boiling water. After rapidly cooling with ice water, an equal volume of alkaline methanol was added to the samples. After centrifugation at 3000 *xg* for 3 min, the supernatant was collected. RP-C18 (5μm, 4.6x150 mm, Eclipse VDB-C18 Agilent) columns were used. For elution, the mobile phase volume was prepared as a 40:60 ratio with methanol and 50 mmol/L phosphate buffer. The flow rate was adjusted to 0.8 mL/minute a total of 20 μL supernatant was loaded onto the device and measured at 527 nm excitation and 551 nm emission wavelength by HPLC using a fluorescent detector (FLD). MDA-TBA product peaks were calibrated with 1,1,3,3-Tetraethoxypropane standard solution. The obtained results are expressed as μM.

Analysis of Oxidized CoQ10: Oxidized CoQ10 levels were measured according to the method previously described by Littarru et al (28). Briefly, reduced CoQ10 was forced to oxidize in plasma samples treated with para-benzoquinone following extraction with 1-propanol directly injected into the HPLC device. For HPLC analysis, a reverse-phase ODS supercoil C-18 (15x0.46 cm i.d. 3 μm) column was used. For analysis, 50 μL 1,4-benzoquinone (2 mg/mL) was vortexed and added to 200 μL plasma. After 10 minutes incubation at room temperature, 1 mL 1-propanol was added and during 10 min the mixture was vortexed. The mixture was centrifuged at 600 *xg* for 2 min. A total of 200 μL was collected from the supernatant, put into a vial and loaded onto the HPLC device. For spectral analysis, the detector was adjusted to 275 nm, and the ethanol-methanol (65-35% v/v) mobile phase flow rate was adjusted to 1 mL/min. The system was stabilized after balancing the column and oxidized CoQ10 was measured by the electrochemical detector adjusted to 0.35 V. The results are expressed as μM.

DNA Isolation and Hydrolysis: DNA was isolated from whole blood leukocytes using a DNA isolation kit (Vivantis GF-1 blood DNA extraction kit, Vivantis Technologies SDN. BHD, Malaysia) with the spin column method. A total of 200 μL blood sample was vortexed in a microcentrifuge tube and 200 μL lysis buffer was added. Then, 20 μL proteinase K was added and incubated at 65^0C for 10 minutes. A total of 200 μL pure ethanol was added and centrifuged at 5000 *xg* for 1 min. Then, 500 μL wash buffer-1 was added to the spin column and centrifuged at 5000 *xg* for 1 min. The membrane and protein residues accumulated in lower tube and were discarded. A total of 500 μL wash buffer-2 was added to the spin column and centrifuged at 5000 *xg* for 1 min. This was repeated and centrifuged for 3 minutes. Lastly, 100 μL DNA elution buffer was added to the spin column and centrifuged at 5000 *xg* for 1 min and the DNA was collected. The DNA samples were stored at -20^0C. DNA aliquots were thawed for 8-OHdG analysis and hydrolysed as previously described by Kaur and Halliwell (29). Hydrolysed DNA samples were dissolved in HPLC elution buffer (final volume, 1 mL). 8-OHdG levels were measued by HPLC using EC and UV detectors. A total of 20 μL final hydrolysate was measured by HPLC using ECD. Reverse-phase-C-18 (RP-C18) analytical columns (250 mm × 4.6 mm × 4.0 μm, Phenomenex, CA) were used. The mobile phase was 0.05 M potassium phosphate buffer (pH 5.5) and acetonitrile mixture (97:3, v/v) and the flow rate was adjusted to 1 mL/min. The levels of 8-OHdG were measured by HPLC with the ECD adjusted to 600 mV. To measure 8-OHdG and dG levels, 8-OHdG ve dG standards from Sigma Aldrich Company (Sigma diagnostics, St. Louis, MO, USA) were purchased. The 8-OHdG values for each 10^6dG were expressed as 8-OHdG/10^6dG ratios (30).

Statistics: Descriptive statistics were defined as mean and standard deviation. In terms of these properties, two factor (one of the factor being repeated measurements) variance of analysis (two-way ANOVA) was applied to compare different groups and times (periods). Duncan multiple-comparison test was used to determine the different groups and measurement periods after the variance analysis. Satistical analysis was performed using the SPSS 15 statistics package program (SPSS Inc., IL, USA). $p<0.05$ was considered statistically significant.

Results

Demographic data such as age and gender of the volunteers are shown in Table 1. The volunteers had an age of aging of 21 and an average weight of 64.2 kg. Volunteers were selected from humans who did

Table 1. Demographic data of volunteers

Volunteers	Gender	Age	Weight (kg)	Height (cm)	Smoking Status	Chronic Discomfort	Drug Use
1	Male	21	66	169	None	None	None
2	Male	20	71	173	None	None	None
3	Male	21	62	170	None	None	None
4	Male	22	68	175	None	None	None
5	Male	22	65	168	None	None	None
6	Male	20	63	167	None	None	None
7	Male	21	75	172	None	None	None
8	Female	22	61	165	None	None	None
9	Female	20	54	162	None	None	None
10	Female	21	57	164	None	None	None

Table 2. Comparison of the effect of resveratrol and serotonin on MDA levels in the plasma of stored whole blood

MDA (μM)			
	Control	Resveratrol	Serotonin
Baseline	1.316 ± 0.346	1.288 ± 0.341	1.412 ± 0.309
7th day	1.713 ± 0.322	1.677 ± 0.345	4.567 ± 0.424*
14th day	1.861 ± 0.337	1.729 ± 0.332	12.435 ± 0.953*
21st day	2.941 ± 0.340	1.851 ± 0.322**	16.866 ± 0.934*
28th day	3.255 ± 0.399	2.081 ± 0.362**	18.748 ± 0.938*

"*"According to the control and resveratrol groups ($p<0.05$)

"**" According to the control and serotonin groups ($p<0.05$)

not have any disease and did not use cigarettes or any drugs.

The effects of resveratrol and serotonin on MDA, CoQ10 levels and 8-OHdG/10^6dG ratios are shown in Table 2, 3 and 4, respectively. Furthermore, comparisons within groups are shown in Figure 1. Resveratrol considerably protected against changes in MDA and oxidized CoQ10 levels especially on the 21st and 28th days ($p<0.05$). MDA levels in the control and resveratrol groups were lower than those in the serotonin group on the 14th, 21st and 28th days (Table 2, $p<0.05$). In addition, MDA levels in the resveratrol group were lower than those in the control and serotonin groups on the 21st and 28th days (Table 2, $p<0.05$).

CoQ10 values in the resveratrol group were lower than the other groups on the 14th, 21st and 28th days (Table 3, $p<0.05$). However, CoQ10 levels in the serotonin group were higher than those in the other groups on the 14th, 21st and 28th days ($p<0.05$). The 8-OHdG/10^6dG ratios in the serotonin group were higher than the other groups on the 21st and 28th days (Table 4) ($p<0.05$). However, the 8-OHDG/10^6 dG ratios of the resveratrol group was significantly lower than the control and serotonin group, especially at 28th day ($p<0.05$).

In addition, the MDA, CoQ10 levels and 8-OHdG/10^6dG ratios in the serotonin group were higher than the starting day values on the 14th, 21st and 28th days and on the 21st and 28th days ($p<0.05$) in the control group, respectively (Figure 1). In contrast, there was no significant difference in MDA, CoQ10 levels ($p<0.05$) in the resveratrol group, compared with the starting day values, but the 8-OHdG/10^6dG ratios were statistically higher on the 21st and 28th days ($p<0.05$).

Discussion

The studies have shown that increased lipid peroxidation and loss of antioxidant defense system depend on the over time in stored blood (16,17). ROS causes lipid, protein and DNA oxidation, which are structural elements in cells (31,32). Upon lipid and protein oxidation in the cell membrane, the cells become susceptible for penetration and their integrity disrupted. Accordingly, stored blood can not be used after a certain time period. It has recently become possible to extend the shelf life and increase the

Table 3. Comparison of the effect of resveratrol and serotonin on CoQ10 levels in the plasma of stored whole blood

	CoQ10 (μM)		
	Control	Resveratrol	Serotonin
Baseline	1.719 ± 0.339	1.513 ± 0.285	1.417 ± 0.413
7th day	2.141 ± 0.215	1.860 ± 0.169	1.734 ± 0.305
14th day	2.058 ± 0.196	1.703 ± 0.189**	3.280 ± 0.349*
21st day	2.290 ± 0.228	1.467 ± 0.203**	4.227 ± 0.348*
28th day	2.641 ± 0.229	1.925 ± 0.158**	6.998 ± 0.336*

"*"According to control and resveratrol groups ($p<0.05$)

"**" According to the control and serotonin groups ($p<0.05$)

Table 4. Comparison of the effect of resveratrol and serotonin on 8-OHdG/10^6dG ratios in stored whole blood

	8-OHdG/10^6dG		
	Control	Resveratrol	Serotonin
Baseline	0.703 ± 0.044	0.676 ± 0.055	0.592 ± 0.055
7th day	0.795 ± 0.082	0.757 ± 0.029	0.697 ± 0.090
14th day	0.878 ± 0.089	0.825 ± 0.026	0.926 ± 0.095
21st day	0.902 ± 0.087	0.889 ± 0.020	1.117 ± 0.078**
28th day	1.146 ± 0.080	0.966 ± 0.045*	1.541 ± 0.070**

"*"According to control and serotonin groups ($p<0.05$)

"**"According to the control and resveratrol groups ($p<0.05$)

antioxidant capacity of stored blood by adding certain substances (17). Knight et al. showed that substances such as vitamin E and vitamin C positively contribute to the antioxidant defense capacity of red blood cells (RBC), allowing for reduced lipid peroxidation (33). Also, Dumaswala et al. showed that older stored erythrocytes exhibit decreased defense capacitiy against oxidative damage, and this can be counteracted by adding fresh stock erythrocytes exposed to in vitro oxidative stress with ferric ammonium sulfate and ascorbic acid (Fe/Asc) oxidant (34). In our previous study, we also, showed that the addition of melatonin to stored blood strengthens the total antioxidant defence capacity of RBCs (16).

In stored blood in pouches containing the CPDA-1 (citrate-phospate-dextrose-adenine) anticoagulant, there was a decrease in SOD, catalase, glutathione-S-transferase and glutathione reductase activity and an increase in glutathione peroxidase activity (35). Furthermore, Şekeroğlu et al. and Gültekin et al. showed that lipid peroxidation and MDA levels increase as the storage period increases, but addition of melatonin decreased MDA levels and lipid peroxidation and preserved the blood's antioxidant capacity (16,17). In this study, we also found that the MDA levels in the resveratrol group were significantly preserved, but there was an interesting increase in serotonin group compared with the other groups (Figure 1). In this case, we can say that resveratrol has an inhibitory effect on lipid peroxidation in stored blood, on the contrary serotonin has an oxidative effect.

The primary role of CoQ10 is to carry electrons between nicotinamide adenine dinucleotide and succinate dehydrogenase (11). Because of its antioxidant effects, CoQ10 is sometimes used as a dietary supplement (36,37). Consequently, oxidized CoQ10 is an important indicator of mitochondrial damage (38). In the literature review we conducted, we could not find any study of how changes in CoQ10 levels from the mitochondrial damage markers, depending on the time course in stored blood, have previously been observed (Figure 1). Since whole blood used for transfusion contains cells with mitochondria (such as leukocytes), it is important to prevent mitochondrial membrane damage. In our study, CoQ10 levels of the resveratrol group were maintained in a significant proportion according to the control and serotonin group in the stored blood. However, there was a significant increase in serotonin group compared to control and resveratrol groups. These results show that mitochondrion is also affected by oxidative damage in the stored blood, whereas resveratrol prevents this effect, but on the contrary serotonin further increases this effect.

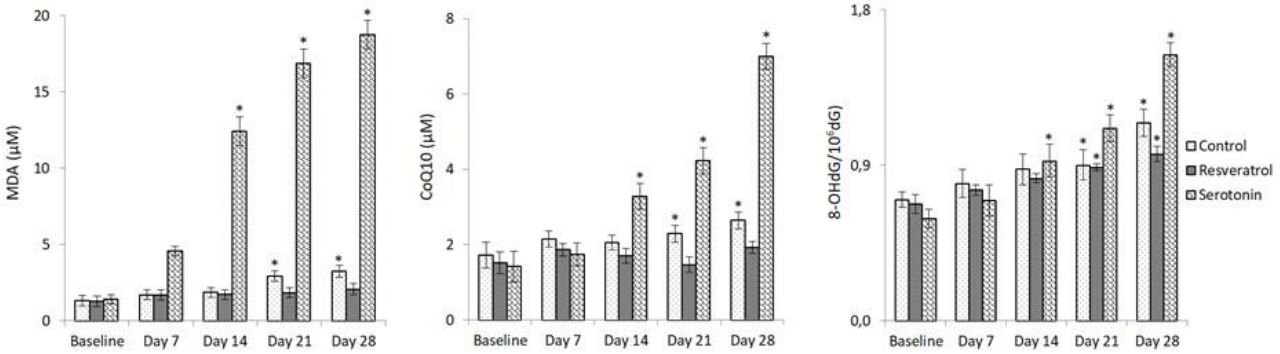

Fig. 1. The comparison of resveratrol and serotonin on levels of MDA, CoQ10 and 8-OHdG/10^6 dG within the group in stored blood, time-dependently. *p<0.05: According to baseline within the group.

ROS also affects DNA, as it causes oxidative modification of nucleic acids. 8-OHdG is an important biomarker of oxidative DNA damage (39,40). To the best of our knowledge, no previous study has evaluated 8-OHdG levels as indicators of DNA damage in stored blood. Because of a possible increase in 8-OHDG levels in stored blood can cause gene mutations and false protein coding after blood transfusion, it is important to prevent injury in the DNA bases. Increasing oxidative stress and decreasing antioxidant capacity due to storage time (3,9,15,17,26), especially OH· radicals, can lead to DNA oxidation by damaging nuclear proteins and DNA (9). Because of a possible increase in 8-OHDG levels in stored blood can cause gene mutations and false protein coding after blood transfusion, it is important to prevent injury in the DNA bases (41). In our study, we found that 8-OHdG/10^6dG ratios increase in stored blood over time. We also found that the resveratrol added to the stored blood only partially inhibited the increase in the ratio of 8-OHdG/10^6dG (Figure 1). In contrast, serotonin significantly increased these ratios compared with control and resveratrol groups. In this study, altough the mechanism of the increase in 8-OHdG levels is fully unclear time dependently, it should not be ignored the fact that increased oxidative stress and free radicals such as OH· radical can be cause to oxidation of bases in DNA by damaging to the membrane and proteins of the cell nucleus.

No other studies have examined the protective effect of resveratrol and serotonin on oxidative stress in stored blood. The current study has shown that resveratrol supplementation in storage blood will contribute significantly to prevent oxidative damage, but serotonin does not have such an effect but has an adverse oxidative damage enhancing effect.

In our literature research, Litarru et al. suggested that superoxide radicals and increased protein oxidation in the lungs after smoking are triggered by serotonin metabolism by MAO (42). In addition, Battal et al. reported that high doses of the reuptake inhibitor of serotonin sertraline increases oxidative stress (43). When considered that one of the most important components of whole blood is thrombocytes, it can be said that serotonin metabolism causes the oxidative stress by MAO enzyme activity linked to their mitochondrial membrane (44) in stored blood. If serotonin is used as a preservative in blood preparations separated from its components (for example, only stored mitochondria-free erythrocytes), this negative effects may be absent.

Our study showed that oxidative stress increased in stored bloods over time, leading to inreased MDA and, mitochondrial CoQ10 levels and 8-OHdG/10^6dG ratios in DNA. The addition of resveratrol to stored blood protected against lipid oxidation and mitochondrial CoQ10 oxidation. However, the addition of serotonin to stored blood showed prooxidant activity and caused lipid oxidation and mitochondrial and oxidative DNA damage. Therefore, we can easily say that resveratrol added to the stored blood protects the antioxidant capacity and can make a significant contribution to the shelf life of the stored blood.

Conflict of interest: The authors declare no conflict of interest.

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