Beneficial Effects of Melatonin on Oxidative Damage Observed During Whole Blood Storage

Ilknur S. OZCELİK

The Scientific and Technological Research Council of Turkey, Ankara, TURKEY

ABSTRACT

It is well known that oxidative stress rises during the storing period of whole blood and there has been a great interest to improve the quality of stored blood for longer periods. In the present study, we determine the effects of melatonin as an antioxidant on the whole blood indices stored under blood bank conditions. Human blood was collected by venipuncture into citrate-dextrose-phosphate (CDP) bags from healthy volunteers. Immediately after collection, blood was subdivided into two parts and exposed to either melatonin or control saline solutions. Several biochemical parameters were measured on the day of collection and at weekly intervals up to 3 weeks. Mean corpuscular and platelet volumes tended to increase during storing process and with melatonin, these indices remained near to physiologic levels. As expected, acidity, oxidative damage and osmotic fragility increased in stored blood. Interestingly addition of melatonin reduced acidity, oxidative damage by lowering malondialdehyde levels and by increasing superoxide dismutase activity, and osmotic fragility during storage without adversely affecting other biochemical parameters. pH was 6.68 ±0.04 in day 14 of control group while it was 7.00 ± 0.03 in melatonin group. Malondialdehyde level was 1.223±0.05 in day 21 of control group while it was 0.723±0.04 in melatonin group (p< 0.05). Superoxide dismutase activity was significantly higher in melatonin group at days 14 and 21 (151.3±12.2 and 82.9±9.2) compared to saline (111.8±6.5 and 44.8±3.0) (p< 0.05). Through these results, we could confirm a central role for oxidative stress in the mechanism of the most evident blood storage lesions and melatonin seems to exhibit beneficial effects on these lesions.

Keywords: Whole blood storage, Oxidative stress, Melatonin

ÖZET

Kanın Depolanmasında Görülen Oksidatif Hasarda Melatoninin Koruyucu Etkileri

Kanın depolanması sırasında oksidatif stress arttığı iyi bilinmekle de depo kanın kalitesinin daha uzun sürelerde korunması için yoğun çalışmalar yapılmaktadır. Bu çalışmada, kan bankası şartlarında saklanan tam kan örneklerinde bir antioksidan olan melatoninun etkisi araştırılmıştır. Sağlıklı gönüllü bir grup insandan venöz kan örnekleri, antikoagulanlı torbalara alınmıştır. Bağıştan hemen sonra kan, kontrol grubu ve melatonin grubu olarak iki gruba ayrılmış ve bağış gününde ve sonraki üç hafta boyunca hafta çeytli biyokimyasal analizler yapılmıştır. Ortalama eritrosit ve trombosit hacim değerleri saklama boyunca artmıştır, ancak melatonin ile bu değerler normale yakın seyretemiştir. Beklendiği gibi, saklanan kanda asidite, ozmotik frajilite ve oksidatif hasar artmıştır. İlgincel olarak, melatonin ilavesi asiditeyi, ozmotik frajiliteyi ve oksidatif hasan (malondialdehit seviyelerini azaltıp superoksit dismutaz aktivitesini artırarak) anlamı olarak düştürtmüştür. Kontrol grubunda 14. gün pH değeri 6.68±0.04 iken, melatonin grubunda bu değer 7.00 ± 0.03 idi. Malondialdehit düzeyi kontrol grubunda 21. gün 1.223±0.05 iken, melatonin grubunda bu değer 0.723±0.04 idi (p< 0.05). Superoxid dismutaz aktivitesi de 14 ve 21. günlerde melatonin grubunda (151.3±12.2 and 82.9±9.2) kontrol grubuna göre anlamlı olarak yüksekti (111.8±6.5 and 44.8±3.0) (p< 0.05). Bu sonuçlar, oksidatif hasanın tam kanin saklanması ortaya çıkan hasarları neden olarak mekanizmalar arasında önemli bir yer tuttuğunu ve melatoninün bu hasarlar üzerinde faydalı etkileri olduğunu göstermektedir.

Anahtar Kelimeler: Tam kan saklama, Oksidatif stress, Melatonin
INTRODUCTION

Whole blood was the only commonly available blood product until component production systems came into general use.\textsuperscript{1,2} However, whole blood is still used in some developing countries and has been used repeatedly by the military of developed countries in Somalia, Bosnia, Kosovo and Afghanistan to replace red blood cells (RBCs) when the supply chain could not keep up with local need.\textsuperscript{3-5} At present, the most widely used protocol for the storage of whole blood is the collection of blood into anticoagulant solutions (typically citrate-dextrose-phosphate: CDP) and storage at 4 ± 2°C. The studies suggest that the quality (in terms of safety and efficiency) of blood decreases during the storage period.\textsuperscript{6,7} The most dramatic changes that occur during storage include acidosis,\textsuperscript{8,9} loss of function of cation pumps and consequent loss of intracellular potassium\textsuperscript{10,11} and increased oxidative stress.\textsuperscript{11-13} An increase in oxidative stress during storage results in lipid peroxidation in which reactive oxygen species (ROS) attack the membrane and lead to loss of membrane integrity and cell death.\textsuperscript{14} To counter this potential damage, cells have antioxidant defense mechanisms of enzymatic (superoxide dismutase (SOD), catalase)\textsuperscript{15} and nonenzymatic (tocopherols, ascorbic acid) types.\textsuperscript{16} Oxidative stress occurs when there is either an overproduction of ROS or a decrease in antioxidant defenses, provoking an imbalance between antioxidant and pro-oxidant species in favor of the latter.\textsuperscript{17} This situation has been established as an underlying factor in many pathological conditions.\textsuperscript{17} Malondialdehyde (MDA) is the break-down product of the lipid peroxidation and thus serves as a reliable marker of oxidative stress.\textsuperscript{18,19} In parallel with studies on storage lesions through oxidative stress,\textsuperscript{20-23} blood preservation studies have also been conducted to understand the potential benefits arising from the addition of antioxidants into the blood.\textsuperscript{24-27} While a number of compounds have already been demonstrated to directly defend against oxidative stress in RBC membranes,\textsuperscript{28,29} melatonin as a potent antioxidant has not been used so far to defend against oxidative stress in whole blood.\textsuperscript{29} The object of the present study was therefore to investigate the effects of melatonin on several vital parameters, lipid peroxidation and anti-oxidant status in whole blood stored for up to 3 weeks.

MATERIALS AND METHODS

The procedures that follow were in accordance with the ethical standards of the local committee responsible for human experimentation and performed upon informed consent in accordance with the declaration of Helsinki. Units of whole blood were collected from healthy donor volunteers in citrate-dextrose-phosphate (CDP) anticoagulant at the blood donation center of a local hospital in Ankara and stored under standard blood bank conditions. All donors met standard blood donor criteria. 300 ml venous blood was obtained from each of 6 healthy adult male volunteers with ages ranging from 32 to 38 years of different ABO blood types and then stored at 4°C in 2 pediatric CPD-bags (2x150 ml) for each (totally 12 bags). Blood obtained from the same persons were used as both control and experiment group (n=6 for each group). 0.5 mg melatonin dissolved in 2 ml saline was added into each of 6 different bags just after blood collection. The same amount of saline was added to control bags. 10 ml samples were taken from bags and the number of RBC, WBC and PLTs, indexes of RBCs and platelets (MCV, MCH, MCHC, MPV, PCT), hematocrit, hemoglobin, potassium (K\textsuperscript{+}), lactate dehydrogenase (LDH), glucose, pH, osmotic fragility, prothrombin time (PT), and activated partial thromboplastine time (aPTT) were measured on all samples initially and at 7, 14 and 21 days of storage. Osmotic fragility was performed as to measure RBC resistance to hemolysis when exposed to a series of increasingly dilute saline solutions. All analyses were performed directly on the whole-blood samples and samples were removed aseptically for the analyses every week. Visual inspection of all units demonstrated no indications of bacterial contamination. The study was terminated at 21st day because CDP is known to serve blood for 3 weeks.

For the measurement of malondialdehyde levels and superoxide dismutase activity, 4 ml blood from each bag for each period was separated into plasma and erythrocytes by centrifugation at 1500 g for 10 min, at 4°C. The erythrocyte samples were washed three times with cold PBS and then hemolyzed by adding a fourfold volume of distilled water. Hemolyzed erythrocytes were stored at –80°C for measurements, and the following parameters were studied in these hemolyzates.

Malondialdehyde (MDA) levels were measured with the thiobarbituric acid (TBA) reaction by the method...
This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to TBA with MDA at 535 nm. For this purpose, 2.5 ml of 100 g/l trichloroacetic acid solution was added to 0.5 ml hemolysate in each centrifuge tube and placed in a boiling water bath for 15 min. The mixture was cooled and centrifuged at 1000 g for 10 min. Next, 2 ml of the supernatant was added to 1 ml of 6.7 g/l TBA solution in a test tube, and placed in a boiling water bath for 15 min. The solution was then cooled and its absorbance was measured with a spectrophotometer (Helios, Epsilon, USA). MDA levels were expressed as nmol/g hemoglobin in erythrocyte hemolysates.

Superoxide dismutase (SOD) activity was assayed using the nitroblue tetrazolium (NBT) method of Sun.31 The stock solution contained 10 mg of Cu,Zn-SOD from bovine liver dissolved in 10 ml of isotonic saline and was diluted to 600 microgram/l with distilled water before it was used in the assay. The SOD assay reagent consisted of a combination of the following reagents: 80 ml of 0.3 mmol/l xanthine solution, 40 ml of 0.6 mmol/l ethylenediaminetetraacetic acid (EDTA) solution, 40 ml of 150 micromol/l NBT solution, 24 ml of 400 mmol/l Na₂CO₃ solution, and 12 ml of bovine serum albumin. The samples were subjected to ethanol-chloroform (62.5/37.5%) extraction prior to the assay of enzyme activity. Briefly, 400 microliter of ice-cold ethanol/chloroform mixture was mixed thoroughly with 250 microliter of sample. After vortexing for 30 s and centrifugation at 3000g at 4°C for 5 min, the upper aqueous layer was collected. The collected hemolysate was diluted by a factor of 100, and 0.5 ml of the diluted solution was used for the assay by adding to 2.5 ml of SOD assay reagent. NBT was reduced to blue formazan by O₂⁻, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. The calculated SOD activity was expressed as U/g hemoglobin in erythrocyte hemolysates.

Evaluation was made by alteration curves of measured parameters and the Wilcoxon test was performed for statistical analysis. P values less than 0.05 were assessed as significant.

<table>
<thead>
<tr>
<th>TABLE 1. Counts and indexes of blood cells for melatonin study (Mean ± SEM); *p&lt;0.05 when compared with control value of same day.</th>
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<tbody>
<tr>
<td><strong>INITIAL LEVELS</strong></td>
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<tr>
<td><strong>RED BLOOD CELLS &amp; INDEXES</strong></td>
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<tr>
<td>Cell count (millions/mm³)</td>
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<td>Hemoglobin (g/dl)</td>
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<tr>
<td>Hematocrit (%)</td>
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<td>MCV (fl)</td>
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<td>MCHC (%)</td>
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<td>RDW</td>
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<td><strong>PLATELETS &amp; CLOTTING FUNCTIONS</strong></td>
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<tr>
<td>Cell count (thousands/mm³)</td>
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<td>MPV (fl)</td>
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<td>aPTT (sec)</td>
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<td><strong>WHITE BLOOD CELLS</strong></td>
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<td>Cell count (thousands/mm³)</td>
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RESULTS

Red and white blood cell counts fluctuated during the storage period but did not change significantly over time and counts in melatonin group were not different compared to saline groups. However, mean corpuscular and platelet volumes (MCV and MPV) tended to increase during storing process, and with melatonin, these indices remained near to physiologic levels (Table 1). Mean corpuscular volume was 91.18 ± 1.57 and 93.42 ± 1.53 fl in days 14 and 21 of control group, while it was 90.10 ± 1.37 and 91.90 ± 1.28 fl respectively in melatonin group (p< 0.05). Mean platelet volume was 8.20 ± 0.40; 8.70 ± 0.56; 8.73 ± 0.48 fl in days 7, 14 and 21 of control group, while it was 7.60 ± 0.40; 7.27 ± 0.23; 8.05 ± 0.43 fl respectively in melatonin group (p< 0.05). There was no significant difference in mean PT and aPTT between the two groups (Table 1). Osmotic fragility test showed that prolonged storage caused linear increases in hemolysis curve percentages proportionally to osmotic stress increase (at increasingly dilute NaCl solutions). Highest values of osmotic fragility (increase likelihood of hemolysis at lower osmotic stress) were obtained for day 21 samples, in comparison with day 0 controls, which showed the lowest osmotic fragility (Table 2).

With regard to the osmotic fragility ratios, average values of the melatonin-added group were significantly lower than that of the control bloods. Osmotic fragility ratio was 36.69 ± 2.00; 53.33 ± 2.81 and 81.49 ± 2.99 in days 7, 14 and 21 of control group, while it was 24.77 ± 2.68; 38.50 ± 2.66 and 61.90 ± 2.79 respectively in melatonin group (p< 0.05).

Changes in pH during storage period are shown in Table 3, in which a general decrease during blood storage may be observed. Melatonin significantly prevented this decrease. pH was 6.68 ±0.04 in day 14 of control group while it was 7.00 ± 0.03 in melatonin group. Similarly, the glucose concentrations decreased and potassium and LDH increased in both groups (Table 3). However melatonin presented no significant effect on these parameters.

The results obtained in the present study indicate that storage of whole blood results in higher levels of malondialdehyde but the melatonin is very effective in arresting the dramatic increase in the malondialdehyde levels (Table 4). Malondialdehyde level was 1.223±0.05 in day 21 of control group while it was 0.723±0.04 in melatonin group (p< 0.05). Superoxide dismutase (SOD) activity during blood preser-

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<th>TABLE 2. Osmotic fragility; expressed as ratio to fragility in distilled water (Mean ± SEM); *p&lt;0.05 when compared with control value of same day.</th>
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<td><strong>NaCl concentration Level</strong></td>
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<tr>
<td>6%</td>
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<td>5.5%</td>
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<tr>
<td>5%</td>
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<tr>
<td>4.5%</td>
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<td>4%</td>
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<tr>
<th>TABLE 3. Evaluation of hemolysis rate (K+ and LDH), glucose consumption and decrease of pH (Mean ± SEM); *p&lt;0.05 when compared with control value of same day.</th>
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<tbody>
<tr>
<td><strong>INITIAL LEVELS</strong></td>
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<td></td>
</tr>
<tr>
<td>K+ (mEq/l)</td>
</tr>
<tr>
<td>LDH (IU)</td>
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<tr>
<td>Glucose (mg/dl)</td>
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<tr>
<td>pH</td>
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</table>
vation is also shown in Table 4, in which a variable decrease may be observed during three weeks in both groups, however SOD activity was significantly higher in melatonin group at days 14 and 21 (151.3±12.2 and 82.8±9.2) compared to saline (111.8±6.5 and 44.8±3.0) (p<0.05). There was no statistically significant difference in the changes in the other parameters between groups (p>0.05).

**DISCUSSION**

In the present study, we provide supporting evidence for the relation between whole blood storage duration, and alterations in RBC metabolism and oxidative stress. It is well known that during blood preservation, blood hydrogen ion concentration and potassium levels increase. As expected, the pH decreased and potassium levels increased over time during storage in our study. Our findings for the pH and potassium are thus in agreement with previous reports. These effects of storage appear at present to be unavoidable. Potassium increase is mainly due to adenosine triphosphate (ATP) depletion, since potassium homeostasis depends on ATP-dependent maintenance of proper functioning of sodium-potassium cationic pumps. Storage of whole blood with melatonin for 21 days has led to marked reductions in acidity in this study while it did not affect the potassium levels. Melatonin seems to exhibit beneficial effects on pH in stored blood.

Results of the present study indicate that storage of whole blood for 21 days induced lipid peroxidation in RBCs. MDA is particularly released as a result of toxic effects of active oxygen radicals which destroy unsaturated fatty acids in the cell membrane. In RBCs, endogenous free radical scavengers seem to fail to prevent oxidative injury. Malondialdehyde levels showed a progressive increase both in control and melatonin supplemented groups, although control units showed constantly higher levels than the supplemented counterparts. In Table 4, we report that lipid peroxidation (malondialdehyde levels) was consistently lower in whole blood stored with melatonin compared to saline. On the basis of the aforementioned evidences, the observed lower levels of malondialdehyde in melatonin group may be attributed to RBC protection from oxidative stress. Although MDA levels were clearly decreased by melatonin, its mechanism is not clear. Melatonin may eliminate free oxygen radicals or directly increase the antioxidant enzyme activity and prevent the inhibition of these enzymes. Since antioxidant properties of melatonin are well documented, decrease in MDA levels in melatonin group is probably due its antioxidant effect. A number of drugs or chemicals have been used to prevent lipid peroxidation. This is the first study in which melatonin was used for preventing lipid peroxidation injury in whole blood and it seems to be a good preservative regarding oxidant generation.

Since oxidative stress can result from increased ROS production, and/or from decreased ROS scavenging capability, SOD activity was measured. We observed a significant decrease in the activity of enzyme in control group by the 3rd week. This may suggest that oxidative stress reduced the activity of the enzyme. As enzyme lost activity during this period, it hints that those unstable forms of enzymes that depend on fine physiological environmental requirements early become inactive. But, most of the forms do keep their functional properties until the 3rd week, suggesting that they represent more stable enzyme forms in spite of the times passed. There are several reports indicating either decrease or increase in the antioxidant enzyme activities in oxidative injuries. A decrease in antioxidant enzyme activity was explained as being due to the interaction of enzymes with oxygen free radicals and peroxidation products which affect their active sites. Interestingly, SOD activity in the melatonin group is statistically higher than in the control group. An explanation for this effect of melatonin might be that it prevents the inhibition of the enzyme by toxic products, since melatonin was

**TABLE 4. Evaluation of Oxidant (MDA) and Antioxidant (SOD) Levels (Mean ± SEM); *p<0.05 when compared with control value of same day.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (mmol/gHb)</th>
<th>SOD (U/gHb)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>Control</td>
<td>0.579±0.03</td>
<td>0.618±0.03</td>
</tr>
<tr>
<td>Melatonin</td>
<td>0.538±0.02</td>
<td>0.563±0.03</td>
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reported to preserve the activity of SOD. Higher activity of SOD in melatonin supplemented blood might also be due to a reduced consumption of SOD, promoted by decreased oxidative stress levels in supplemented units (also confirmed by lower accumulation of malondialdehyde (Table 4). These results are indicative that melatonin supplementation is effective in protecting RBCs from oxidative stress. The beneficial effects of melatonin supplementation are evident at the SOD level, but also when focusing on lower accumulation of lipid oxidation product - malondialdehyde. Replenishing the RBC antioxidant battery through melatonin seems to help preserving RBC indices, thus confirming a central role for oxidative stress in the accumulation of the most evident RBC storage lesions.

We observed that RBCs progressively suffered from increased corpuscular volume and osmotic fragility during the storage period, as they failed to withstand higher osmotic stresses as they aged ex vivo (Table 2). These changes might be triggered both by metabolic alterations (pH decrease, potassium accumulation) and, like other studies suggest, oxidative stress. The observed increase in corpuscular volume and osmotic fragility may suggest that RBC membranes had substantial alterations due to oxidative stress and lipid peroxidation (Table 4). We could confirm therefore that lipid peroxidation leads to storage membrane lesions which then increase osmotic fragility.

Whole blood drawn into plastic bags was also studied for variations of coagulation parameters. Storage of whole blood seems to have relatively little effect on the levels and function of various coagulation components. The data show that CDP-anticoagulated whole blood stored at 4°C for 3 weeks did not change PLT number and plasma coagulation protein activities. The observation that the PLT number and function were stable over time for the full three weeks reinforces the earlier observations that PLTs and plasma coagulation factors were not greatly damaged by cold storage.

A very large body of evidence indicates that melatonin is a major scavenger of oxygen-based reactive molecules. A number of studies have shown that melatonin is significantly better than the classic antioxidants in resisting free-radical–based molecular destruction. In these studies, melatonin was more effective than vitamin E, β-carotene, and vitamin C. This effect is indirect, owing to its ability to scavenge free radicals and protect the protein from damage. Our study offers encouraging data supporting storage of whole blood with melatonin for at least 21 days. We conclude that whole blood seems to suffer from oxidative stress within the 21 days of storage, while the addition of melatonin is effective in arresting the dramatic oxidative changes that takes place during storage of whole blood storage. As the clinical implications for the transfusion recipient are unknown, this study should be supported with clinical investigations including different doses and a greater n value to get more reliable outcome.

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REFERENCES


Correspondence
Dr. İlknur ŞENVER ÖZÇELİK
TÜBİTAK MAG
Tunus Caddesi No: 80
Kavaklıdere, ANKARA / TURKEY
Tel: (+90.312) 468 53 00 / 1130
e-mail: ilknur.ozcelik@tubitak.gov.tr