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Can melatonin delay oxidative damage of human erythrocytes during prolonged incubation?

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ABSTRACT

Purpose: Melatonin (MEL) is an effective antioxidant in numerous experimental models, both i*n vitro* and *in vivo*. However, it should be stressed that there are also papers reporting limited antioxidative activity of MEL or even giving evidence for its pro-oxidative properties. In the present paper we investigated the influence of MEL on the oxidative damage of human erythrocytes during prolonged incubation.

Material/Methods: Human erythrocytes suspended in phosphate-buffered saline (PBS), pH 7.4 were incubated at 37ºC either in absence or presence of melatonin at concentration range 0.02 mM–3 mM for up to 96 hrs. The influence of MEL on erythrocyte damage was assessed on the basis of the intensity of intracellular oxidation processes (the oxidation of HbO_2, GSH, fluorescent label $DCFH_2$) as well as damage to the plasma membrane (lipid peroxidation, the potassium leakage) and the kinetics of hemolysis.

Results: The prolonged incubation of erythrocytes induced a progressive destruction of erythrocytes. Melatonin prevented lipid peroxidation and hemolysis whereas the oxidation of HbO_2 and $DCFH_2$ was enhanced by melatonin at concentrations higher than 0.6 mM. In the case of erythrocytes incubated with 3 mM of MEL, the hemolysis rate constant (0.0498±0.0039 $H\%•h^{-1}$) was 50% lower than that of the control while the HbO2 oxidation rate constants were about 1.4 and 1.5 times higher for 1.5 and 3 mM of MEL, respectively. Melatonin had no influence on the oxidation of GSH and the potassium leakage.

Conclusions: Probably, MEL can stabilize the erythrocyte membrane due to interaction with lipids, thus prolonging the existence of cells. On the contrary, in the presence of MEL the accelerated oxidation of HbO2 and generally, increased oxidative stress was observed in erythrocytes. Pro- and antioxidative properties of melatonin depend on the type of cells, redox state, as well as experimental conditions.

Key words: melatonin, human erythrocytes, oxidative damage, prolonged incubation

INTRODUCTION

In mammalians melatonin (MEL) regulates circadian day and night rhythms, seasonal biorhythms, vascular tone, cancer inhibition, etc. [1,2]. There are many papers indicating that MEL is an effective antioxidant in numerous experimental models, both *in vitro* and *in vivo*. Its antioxidant activity relies on direct scavenging of reactive oxygen (ROS) and nitrogen (RNS) species as well as indirect control and/or modulation of the processes which disturb the balance between anti- and pro-oxidative species in biological systems [3-6]. This activity shows that MEL can play an important role in the reduction of oxidation stress under various conditions where free radicals are generated [7]. ROS are considered to be etiological factors of aging and of many metabolic diseases [8]. By decreasing the level of ROS, MEL can prevent oxidative damage to biologically important molecules [9-15]. However, it should be stressed that there are also papers reporting limited

antioxidative activity of MEL [16-18] or even giving evidence for its pro-oxidative properties [19-22]. In addition, it has been reported that the *in vitro* antioxidant and pro-oxidant activity of MEL depends on its concentration [23,24]. Taking the above literature into consideration, it seems that the antioxidative mechanisms of MEL activity are more complex than it has been thought so far. Therefore, in the present paper, we decided to investigate the influence of MEL on the oxidative damage of human erythrocytes during prolonged incubation in phosphate-buffered saline (PBS), pH 7.4 at 37ºC.

Mature human erythrocytes are an appropriate model for studying the oxidative damage during prolonged incubation of the cells in PBS at 37 ºC which probably triggers ROS mediated damage [25,26]. While erythrocytes are a source of ROS, they are also extremely sensitive to oxidative damage. Then, devoid of cellular organelles, they cannot replace their damaged components. Thus oxidative damage may induce permanent defects which finally lead to hemolysis *in vitro*. Taking into consideration data concerning the way of functioning of MEL depending on its concentration in various experimental systems, we examined the relationship between MEL concentration and the level of damage induced by oxidative stress generated during prolonged incubation of erythrocytes in PBS at 37ºC.

MATERIALS AND METHODS

Chemicals

Melatonin (MEL), 2′,7′-dichlorofluorescin diacetate ($DCFH_2$-DA), sodium tungstate, tiobarbituric acid (TBA), trichloroacetic acid (TCA), dithiothreitol (DTT), sodium dodecyl sulphate (SDS) were purchased from Sigma-Aldrich (Poznan, Poland).

All other chemicals and solvents used were of analytical grade.

Preparation of erythrocyte suspension

Erythrocytes were prepared from heparinized human blood. Blood samples were obtained from apparently healthy adult donors by venipuncture. Erythrocytes were separated from blood plasma and leukocytes by centrifugation and washed four times with phosphate-buffered saline (PBS), pH 7.4. Supernatant and buffy coat were carefully removed by aspiration after each wash. They were then suspended in PBS to obtain a hematocrit of 2%.

Incubation of erythrocytes

The 2% erythrocyte suspensions in PBS were incubated at 37ºC either in presence or absence of melatonin. The final concentrations of melatonin in the samples were: 0.02, 0.1, 0.6, 1.5, 3.0 mM. The samples were incubated for 96 h under aerobic conditions in the presence of antibiotics (penicillin and streptomycin). Melatonin was dissolved in PBS by stirring at 40ºC for 2 h in the dark.

Measurement of hemolysis

Hemolysis of erythrocytes was determined as described earlier [27]. The measurements of hemolysis were performed after 3, 8, 12, 24, 72 and 96 hours of erythrocyte incubation at 37ºC. The absorbance was measured at 523 nm (isobestic point for HbO_2 and MetHb).

The percentage of hemolysis was calculated according to the following equation:

$$\text{MetHb}[\%] = \frac{A_{630} - A_{700}}{(A_{630} - A_{700})_{ox}} \cdot 100\%$$

where:

A_1- absorbance of Hb at 523 nm in the supernatant

A_2- absorbance of Hb at 523 nm after total hemolysis of the remaining erythrocytes

Measurement of methemoglobin (MetHb)

The percentage of MetHb in erythrocytes was determined on the basis of measurement of absorbance at 630 nm after their hemolysis. The absorbance of solutions was measured before and after oxidation of oxyhemoglobin (HbO_2) with $K_3[Fe(CN)_6]$. The content of MetHb was calculated according to the following equation:

$$\text{MetHb}[\%] = \frac{\text{A630} - \text{A700}}{(\text{A630} - \text{A700})\text{utl}} \cdot 100\%$$

Measurement of intracellular GSH

GSH was determined by Ellman's method with some modifications [28,29]. In brief, erythrocyte suspension (hematocrit of 2%, volume of 0.75 mL) was centrifuged and the pellet of 0.15 mL was re-suspended in 1 mL 40 mM H_2SO_4. After 10 min incubation at 22ºC, 0.15 mL 300 mM Na_2WO_4 was added. The samples were shaken for 5 min and centrifuged. The clear supernatant (0.6 mL) was mixed with 0.75 mL of 1 M Tris-HCl buffer, pH 8 and 0.06 mL of DTNB. The GSH content was determined spectrophotometrically at 412 nm using the absorption coefficient 13.6 $mM^{-1}cm^{-1}$.

Measurement of lipid peroxidation

Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances TBARS [30]. Erythrocyte suspensions were mixed in a ratio of 1:1 with a solution containing 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA) and 0.25 M HCl. After 10 min incubation and centrifugation the supernatant was separated, heated in boiling water for 15 min, cooled and centrifuged. The absorbance of the supernatant was measured at 532 nm. Lipid peroxidation was expressed in absorbance units.

Potassium efflux

The erythrocyte suspensions were centrifuged at 650g for 10 min. The resulting supernatant was taken for assay of potassium content. The concentration of potassium was measured by flame atomic emission spectrometry using a SpectrAA-300 apparatus (Varian, Australia).

Preparation of erythrocyte membranes

Erythrocyte membranes were prepared according to the method of Dodge et al. [31] with some modifications. Hemolysis was carried out at 4ºC with 20 volumes of 20 mM Na-phosphate buffer (pH 7.4) containing 1 mM EDTA and 0.5 mM PMSF as an inhibitor of proteases. The membrane ghosts were washed successively with 20, 10 and 5 mM ice-cold Na-phosphate buffer (pH 7.4) until the ghosts were free of residual hemoglobin. The protein concentration in the membrane preparations was determined by a method of Lowry et al. [32].

Polyacrylamide gel electrophoresis (SDS-PAGE)

Erythrocyte membrane proteins were analyzed by SDS-PAGE as described earlier [33]. Erythrocyte membranes were solubilized by addition of a solution containing: 10% SDS, 0.05% bromophenol blue in 0.25 M Tris-HCl buffer (pH 6,8) with or without 0.5 M dithiothreitol (DTT). After incubation at 90ºC for 5 min, the samples containing 1mg/mL of protein were subjected to one dimensional SDS-PAGE. Electrophoresis were carried out with 4% and 7.5% gels for condensation and separation, respectively, in Tris-HCl buffer pH 8.8 containing 0.1% SDS. Electrophoresis was carried out in a Bio-Rad system with current of 20 mA. Protein bands were visualized by staining with Coomassie Brillant Blue R-250. The gels were digitalized and analyzed with the software GelScan (KTE, Poland). The relative quantities of proteins in selected bands were expressed as percentage of the total protein quantity.

Oxidation of $DCFH_2$

$DCFH_2$ -DA (2',7'-dichlorodihydrofluorescein diacetate) is widely used as a marker for studies of oxidative stress at the cellular level. Diacetate form of $DCFH_2$ is cell-permeable and hydrolyzed in the cell by intracellular esterases to $DCFH_2$ which, upon reaction with oxidizing species, forms its 2-electron oxidation product, the highly fluorescent compound 2'-7'-dichlorofluorescein (DCF). The oxidation of $DCFH_2$ is thought to occur as a result of the reaction of H_2O_2 with heme protein catalysts e.g.: peroxidase, cytochrome *c* or Fe^{2+} [34,35].

Suspensions of erythrocytes (2%) were incubated with 20 μM $DCFH_2$-DA for 30 min in the dark at 37ºC. The DCF fluorescence intensity was measured with a BD LSR II flow cytometer at 488 nm and 530 nm for excitation and emission, respectively.

Statistical analysis

Data were reported as the mean±standard deviation of at least triplicate determinations.

The data were analyzed with Fisher-Snedecor test (F test) with level of significance $p<0.05$.

RESULTS

Studying the influence of MEL on oxidative damage of erythrocyte during prolonged incubation, the cells were incubated as described above in the absence (control) or in the presence of MEL at the following concentrations: 0.02; 0.1; 0.6; 1.5; 3 mM. The influence of MEL on erythrocyte damage was assessed on the basis of the intensity of intracellular oxidation processes, damage to the plasma membrane and the kinetics of hemolysis.

Intracellular oxidation processes during erythrocyte prolonged incubation in the presence of melatonin

The incubation of erythrocytes induced a marked and progressive oxidation of HbO_2, whose level decreased with time of incubation (*Fig. 1*). The obtained data were fitted to linear equations. The slopes of these curves corresponded to the rate constants of the oxidation of HbO_2. The rate constants were presented in *Tab. 1* and indicated that the kinetics of hemoglobin oxidation in erythrocytes incubated with 0.02 mM and 0.1 mM MEL was the same as in the control. A slight increase in the rate constant, though statistically insignificant, was observed in case of erythrocytes incubated with 0.6 mM MEL. However, a statistically significant increase in the oxidation rate in comparison to the control was observed in erythrocytes incubated with 1.5 and 3 mM of MEL. In these cases, the rate constants were about 1.4 and 1.5 times higher than the rate constant of the control. The presented results show that MEL, at sufficiently high concentrations, shows prooxidative properties with respect to hemoglobin.

It should be underlined that the absorption spectra of hemoglobin in erythrocytes incubated for 72 hours recorded in the range of 460-700 nm did not indicate the presence of any denatured forms of hemoglobin (*Fig. 2*). As a criterion of the possible presence of hemichromes, the ratio A_{505}/A_{563} was determined for totally oxidized hemoglobin [36]. The value of this ratio for native hemoglobin is about 2.3. The values 2.3-2.1 were observed for hemoglobin in erythrocytes incubated in the studied experimental systems. A slight decrease was observed only after 96 hours of incubation.

The intensity of the oxidation processes in incubated cells was shown by a very rapid decrease in the concentration of reduced glutathione (*Tab. 2*). After 24 hours of incubation only 34% of GSH remained in the reduced form and MEL did not affect this process. Hemoglobin oxidation and loss of GSH suggested that there was a high level of oxidation stress, which was measured by $DCFH_2$. $DCFH_2$ was oxidized to a

Table 1. **Rate constants of Hb oxidation within human erythrocytes incubated without and with melatonin (0.02 mM – 3.0 mM) at 37°C .**

Melatonin (mM)	Without melatonin	0.02	0.1	0.6	1.5	3.0
Rate constant (%oxyHb/h)	0.3721	0.3704	0.3759	0.4409*	0.5212*	0.5762*
±SD	0.0368	0.0750	0.0772	0.0414	0.0457	0.0344

** - statistically significant against control without melatonin at p<0.05 (Fisher-Snedecor test; F-test)*

Table 2. **The GSH level in erythrocytes incubated without or with melatonin (0.6 mM – 3.0 mM) at 37°C during 72 hrs; mean±SD.**

Time of incubation (h)	Without Melatonin	0.6 mM	1.5 mM	3.0 mM
3	1.312±0.090	1.338±0.104	1.412±0.182	1.467±0.151
8	1.181±0.110	1.169±0.087	1.119±0.115	1.131±0.110
12	1.128±0.110	1.097±0.081	1.076±0.063	1.036±0.110
24	0.543±0.085	0.525±0.051	0.536±0.046	0.498±0.057
48	0.300±0.049	0.284±0.024	0.261±0.015	0.261±0.018
72	0.176±0.014	0.154±0.014	0.141±0.007	0.147±0.017

highly fluorescent product (DCF). The intensity of DCF fluorescence reflects the level of intracellular ROS and other oxidizing species (*Fig. 3*). The level of DCF in control cells and those incubated with 0.02 and 0.1 mM MEL did not differ. A statistically significant increase in fluorescence in comparison to control was observed in erythrocytes incubated with 0.6 mM MEL after 72 hours of incubation. In the case of 1.5 mM MEL, the increase in fluorescence of DCF was observed either after 48 or 72 hours.

Modification of the plasma membrane of erythrocytes

Fig. 4 illustrates the intensity of lipid peroxidation in control erythrocytes and in those incubated with 0.02 mM and 0.1 mM of MEL. The level of lipid peroxidation was expressed in absorbance units corresponding to the substances reacting with TBA. It should be pointed out that at concentrations higher than 0.1 mM, MEL itself produced a positive reaction with TBA. As it can be seen in *Fig. 4*, the level of lipid peroxidation increased with incubation time but a marked rise in TBARS was observed only after 24 hours of incubation. Further incubation slightly influenced their increase. In the presence of 0.02 mM MEL after 24 hours of incubation there was a statistically significant decrease in lipid peroxidation. At longer incubation times and 0.1 mM of MEL the differences in TBARS levels between erythrocytes incubated with and without MEL remained within the experimental error but with a tendency to decrease in the presence of MEL.

Membrane proteins of erythrocytes incubated without MEL and in the presence of 0.1 mM and 0.6 mM of MEL were examined by the SDS-PAGE method in the presence and absence of DTT. The obtained patterns of separation are presented in *Fig. 5*. The incubation of erythrocytes leads to the aggregation of membrane proteins. After 48 hours of incubation, the participation of the aggregate fraction in the membrane protein was equal to 11.5%. The extension of the incubation time to 72 h led to an increase in the aggregate fraction to 13%. The presence of MEL during erythrocyte incubation did not influence the level of aggregates. It should be stressed that the aggregate fractions were not found in the preparations of membrane proteins before incubation of erythrocytes. The presence of aggregates is connected to a simultaneous disappearance of bands 4.2, 4.9 and actin as well as a decrease in band 3 protein by a few per cent. However, the amount of spectrin was not changed. The level of aggregates was decreased and reached 5% after reduction with DTT, which indicates that the aggregation was mainly caused by the formation of disulfide bonds. It is important to point out that under the same conditions bands 4.2 and actin are visible, whereas the level of band 3 protein does not change. Melatonin present during erythrocyte incubation does not significantly change the observed effects connected to the oxidative damage of erythrocytes during prolonged incubation.

The changes in the erythrocyte membrane led to an increase in the efflux of potassium ions. The leak of potassium increased with incubation time up to 48 hours, reaching a balanced level of about 1.2-1.4 mM in medium. The presence of MEL did not change the course of this process (data not shown).

Melatonin affects hemolysis

Erythrocytes incubated for up to 96 hours were subjected to gradual hemolysis. The fraction of the hemolysis in all the experimental systems grew in a linear manner with incubation time (*Fig. 6*). The slopes of these curves are defined as the rate constants of hemolysis. The inset in *Fig. 6* illustrates the relationship between the rate constants of hemolysis and the concentration of MEL. In the range of applied incubation time, hemolysis took place relatively slowly. In control samples, *i.e.*

Figure 1. **Kinetics of hemoglobin oxidation during 96-hour-incubation of human erythrocytes at 37ºC without or with MEL in concentration range 0.02 mM – 3 mM. Each point represents the mean of 3-5 separate experiments ± SD.**

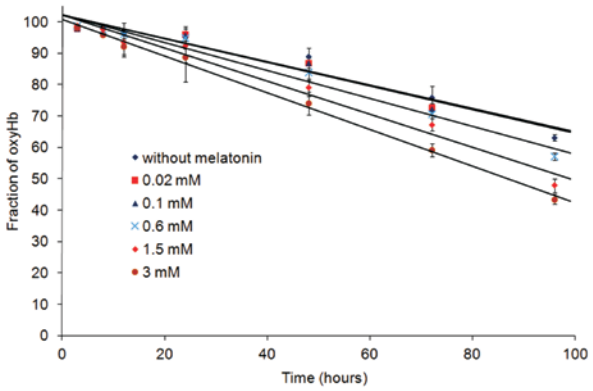


Figure 2. **UV-Vis spectra of hemoglobin released from erythrocytes incubated with MEL at 0.6 mM for 72 hrs at 37ºC before and after total oxidation with $K_3[Fe(CN)_6]$.**

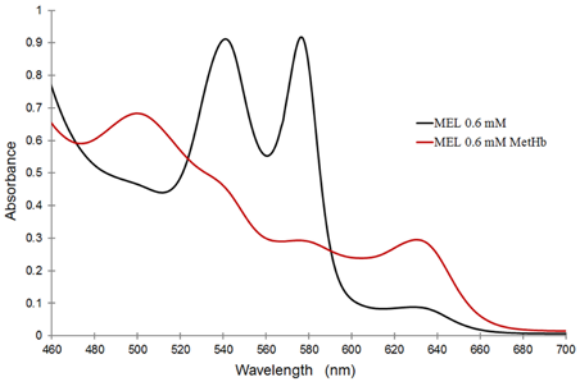


Figure 3. **$DCFH_2$ oxidation in control erythrocytes (without MEL) and erythrocytes incubated with MEL at 0.02 mM - 1.5 mM. The level of DCF was assessed after 48 h and 72 h of the incubation at 37ºC. The background value of DCF fluorescence in fresh erythrocytes was 80±6. Each bar represents the mean of 3-5 separate experiments ± SD, *(asterisk) - statistically significant against control without melatonin (test F).**

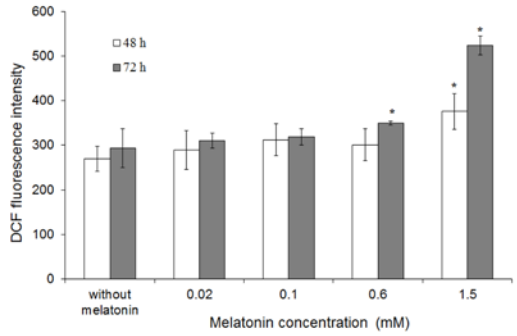
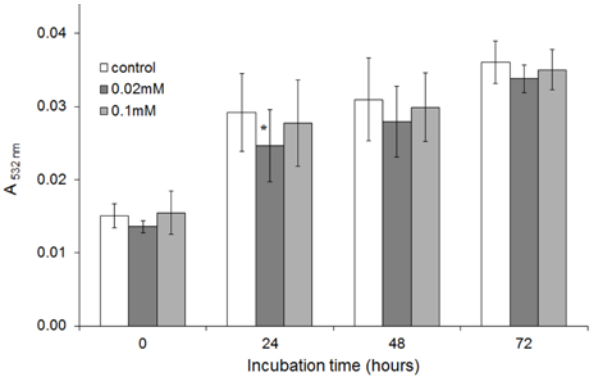


Figure 4. **Lipid peroxidation in control erythrocytes (without MEL) and erythrocytes incubated with MEL in concentrations 0.02 mM and 0.1 mM. Incubation was carried out at 37ºC. Each bar represents the mean of 3-5 separate experiments ± SD. *(asterisk) - statistically significant against control without melatonin (test F).**



those without MEL, after 96 hours the level of hemolysis was about 8.6% and the rate constant was equal to 0.0831±0.0024 H%•h^{-1}. In samples incubated with MEL the level of hemolysis and rate constants were different depending on MEL concentration. MEL at concentrations of 0.02 mM and 0.1 mM did not affect the hemolysis rate. In the presence of 0.6, 1.5 and 3 mM MEL erythrocyte hemolysis took place slower than in the control. In these experimental systems, the rate constants of hemolysis decreased with increased MEL concentration. In the case of erythrocytes incubated with 3 mM MEL, the hemolysis rate constant (0.0498±0.0039 H%•h^{-1}) was 50% lower than that of the control. Thus, 0.6, 1.5 and 3 mM MEL inhibited erythrocyte hemolysis under conditions used in this work.

DISCUSSION

The results presented in this paper reveal that the effects of MEL on human erythrocytes under the conditions of accelerated damage generated during prolonged incubation of erythrocytes in PBS at 37 ºC remain in the range from prooxidative to antioxidative. These effects are related to MEL concentration and also to the kind of examined parameters characterizing the damage of the cells. As it was mentioned in the introduction, the generally known theories concerning oxidative damage of erythrocyte during prolonged incubation [26,37,38] suggest that the basis of this process is oxidative damage to the plasma membrane components, which finally leads to hemolysis.

Figure 5. **SDS-PAGE of membrane proteins of erythrocytes incubated with MEL at 37°C during 72 hrs. Reductive conditions, dithiothreitol (DTT - 0.25 mM) was used as reducing agent: (1) without MEL (control erythrocyte); (2) with 0.1 mM of MEL; (3) with 0.6 mM of MEL. Non-reductive conditions: (4) without MEL (control erythrocyte); (5) with 0.1 mM of MEL; (6) with 0.6 mM of MEL.**

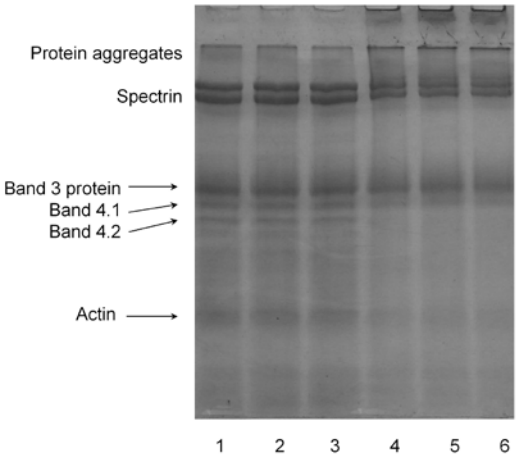
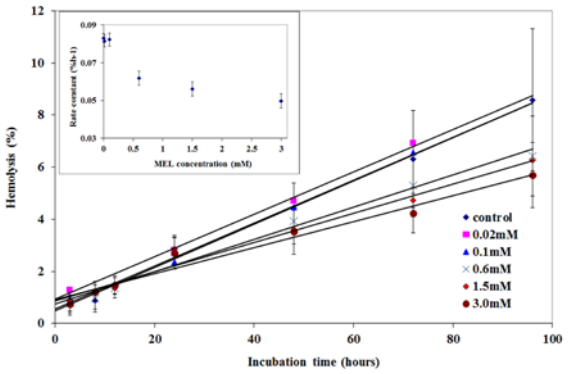


Figure 6. **The influence of MEL on the kinetics of the erythrocyte hemolysis. Erythrocytes were incubated at 37°C without (control) or with MEL at concentration range 0.02 - 3 mM. Each point represents the mean of 3-5 separate experiments ± SD. The inset represents the relationship between the rate constants of hemolysis and concentration of MEL.**



Oxidative cellular damage is initiated by ROS. The source of ROS in erythrocytes is hemoglobin, which undergoes autoxidation to methemoglobin producing O_2^- [39]. Both products of autoxidation are dangerous for cells. MetHb is the first step in the formation of the harmful hemichromes. Superoxide is easily transformed into the potent oxidant H_2O_2. Under physiological conditions, significant oxidative damage is prevented by a very efficient system consisting of small antioxidant compounds such as GSH, vitamin E, vitamin C, and enzymes such as GSH-peroxidase (GSH-Px), catalase, superoxide dismutase (SOD), and MetHb-reductase. ROS can be detoxicated and MetHb is reduced to ferro-Hb. An important role in erythrocyte antioxidant defense is played by reduced co-enzymes. NADH produced by the glycolytic pathway is the main reductant of MetHb to ferro-Hb. NADPH produced by the hexose monophosphate shunt is a co-substrate for glutathione reductase and plays an important role in maintaining the catalytic activity of catalase [26].

Under the conditions used in this work, i.e. PBS without glucose, erythrocytes were deprived of the exogenous energy source. Thus, with the prolongation of the incubation time, the metabolic processes of erythrocytes, i.e. the production of reducing equivalents: NADH and NADPH decreased gradually. As a result, the efficiency of the erythrocyte defense system declined. The lack of a properly functioning defense system resulted in increased hemoglobin oxidation (*Fig. 1*). At the same time, the increase in the fluorescence of $DCFH_2$ indicated increased oxidative stress in the cells (*Fig. 3*).

On the basis of literature data, it is known that even though $DCFH_2$ oxidation is a non-specific reaction, it is mainly caused by H_2O_2 [34,40], which in case of improperly functioning catalase is a source of further ROS generation. Under these conditions erythrocytes underwent an accelerated damage, which in this work was shown by loss of reduced glutathione (*Tab. 2*), increased lipid peroxidation (*Fig. 4*), membrane protein aggregation (*Fig. 5*) and a leak of potassium from cells. These modifications finally led to erythrocyte hemolysis.

In the presence of MEL at concentrations of 1.5 and 3.0 mM we observed the accelerated oxidation of ferro-Hb in comparison to control (without MEL) and generally increased oxidative stress in the cells. The acceleration of hemoglobin oxidation in the presence of the above MEL concentrations indicated its reaction with hemoglobin. It is generally known that hemoglobin reacts with various redox active compounds [41]. Some of them react directly, while some others via reactive intermediates. Gilad and Zisapel [42] showed that MEL bound to hemoglobin but not directly to the heme group. Results obtained by these authors suggested that MEL was bound to the HbO_2 state. It may be proposed that binding MEL to HbO_2 functions as an allosteric effector changing the protein structure and tending to accelerate heme Fe(II) oxidation. At such MEL concentrations as 0.6 mM and lower, probably the binding positions are not completely saturated and thus accelerated HbO_2 oxidation is not observed.

Tan et al. [43] hypothesized that MEL recycled $NAD^\bullet$ to NADH and as a consequence, NADH promoted more H_2O_2 formation via reaction:

$$NADH + O_2^{\bullet -} \rightarrow NAD^\bullet + H_2O_2$$

H_2O_2 produced in the above reaction accelerated the HbO_2 oxidation but only in cell-free systems. In such cases

there were no enzymatic systems which could protect hemoglobin against oxidation.

Under the conditions used in our work we observed the accelerated level of H_2O_2 for MEL concentrations above 0.6 mM (*Fig. 3*). Thus, we can assume that MEL presence in the erythrocyte suspensions incubated in the glucose-free medium (very low levels of NADH, NADPH and ATP in erythrocytes) may lead to the H_2O_2 overproduction. However, either the efficiency of this reaction in erythrocytes is much lower compared to cell-free systems or the mechanism of MEL action is different.

Hemoglobin oxidation leads to the loss of its capability of oxygen binding. The relationship between HbO_2 oxidation and MEL concentration suggests the possibility of choosing such MEL concentrations at which it does not influence MetHb levels, but at the same time it positively influences other parameters of erythrocytes. Under the conditions applied in this work, it seems that the optimal MEL concentration would be 0.6 mM. In that case, there was not any accelerated increase in MetHb levels. However, defense against hemolysis was visible.

Another indicator of the intracellular oxidation processes is also the amount of GSH present in cells. GSH concentration rapidly declined in the cells during the first 24 hours of incubation. MEL was not able to prevent the depletion of intracellular GSH, and the loss of GSH was about 70% (*Tab. 2*).

GSH is an important intracellular antioxidant which protects cells against oxidative damage. GSH may be oxidized to GS-SG as a result of direct scavenging of ROS or during elimination of H_2O_2 and lipid peroxides during GSH-Px activity, which used GSH as a cofactor or indirectly through the participation in repairing processes as a reductor of oxidized protein thiol groups. A low GSH level suggests that GSH is the first line antioxidant defense to combat oxidative damage to proteins and lipids. This conclusion was confirmed in other studies in which erythrocytes were subjected to radiation [27,44] or chemically induced ROS [30].

Thus, in the present work, MEL did not influence the level of GSH within the incubation time. If erythrocytes cannot defend themselves adequately against free radicals, they are subjected to accelerated oxidative damage caused by endogenously generated ROS.

In the presence of MEL, a further destruction of incubated erythrocytes appeared at various intensity depending on its concentration. In fact, a visible influence of MEL on erythrocytes was observed at a concentration of 0.6 mM and higher. Concentrations lower than 0.6 mM did not influence the hemolysis rate (*Fig. 6*) or the level of oxidative stress. However, in the case of lipid peroxidation a slight effect of MEL at a concentration of 0.02 mM suggests its antioxidative action. The fact that it is impossible to determine lipid peroxidation at higher concentrations of MEL does not allow us to make clear inferences. It seems that the peroxidation inhibition at low MEL concentrations contributes to its stabilizing effect on the lipid bilayer, which could lead to hemolysis inhibition.

It should be stressed that despite the decrease in the erythrocyte hemolysis rate in the presence of 0.6 mM of MEL, its influence on the membrane proteins was not observed. The membrane proteins of incubated erythrocytes were subjected to aggregation which took place mainly by disulfide bridges. The presence of MEL during the erythrocyte incubation did not influence the level of aggregates, which showed that MEL did not protect thiol protein groups.

Nevertheless, as far as the influence of MEL on the lipids and proteins of the erythrocyte membrane under oxidation stress is concerned, there are contradictory data in the literature. Although Tesoriere et al. [45] did not observe any influence of MEL on lipid peroxidation induced in erythrocytes by cum-OOH, they observed partial protection of the membrane proteins by MEL against oxidative modifications. Furthermore, Sadowska-Woda et al. [46] observed that 2 mM of MEL had no effect on beta-cyfluthrin induced lipid peroxidation and hemolysis of human erythrocytes.

On the other hand, Dikmenoglu et al. [47] showed that MEL at a relatively high concentration prevented MDA formation in erythrocytes exposed to H_2O_2. At the same time, MEL led to increased erythrocyte deformation, which suggests increased oxidative damage to membrane proteins. These findings indicate that MEL has an antioxidant effect on lipids and a pro-oxidant effect on proteins. The pro-oxidant effect of MEL resulting from inducing increased intracellular ROS formation was observed in U937 cells [20]. In Jurkat cells MEL caused the promotion of fast-induced cell death [19].

CONCLUSIONS

The results of our work show that MEL can present pro- and antioxidative properties in the same cells, which depend on the components of the cells interacting with MEL. Out of the concentrations of MEL used in this work, 0.6 mM is optimal, as then MEL does not present any prooxidative properties (no Hb oxidation, no ROS increase). Additionally, MEL at 0.6 mM protects erythrocytes against hemolysis. Probably, MEL stabilizes the erythrocyte membrane due to interaction with lipids, thus prolonging the existence of cells. In spite of the fact that erythrocytes were starved in our experiments and the levels of NADH, ATP and NADPH must have been low, we had no convincing evidence that MEL recycled NAD radicals efficiently to NADH in human erythrocytes, since GSH and hemoglobin were oxidized and the increase of H_2O_2 was rather low even at very high concentrations of MEL.

Taking into consideration our results and studies concerning the influence of MEL on other cells, one could propose that its pro- and antioxidative properties depend on the type of cells, redox state, as well as experimental conditions.

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REFERENCES

1. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev. 1991 May;12(2):151-80.

2. Tan DX, Manchester LC, Hardeland R, Lopez-Burillo S, Mayo JC, Sainz RM, Reiter RJ. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. J Pineal Res. 2003 Jan;34(1):75-8.

3. Reiter RJ, Tan DX, Manchester LC, Qi W. Biochemical reactivity of melatonin with reactive oxygen and nitrogen species: a review of the evidence. Cell Biochem Biophys. 2001;34(2):237-56.

4. Shimauti EL, Silva DG, de Almeida EA, Zamaro PJ, Belini Junior E, Bonini-Domingos CR. Serum melatonin level and oxidative stress in sickle cell anemia. Blood Cells Mol Dis. 2010 Dec 15;45(4):297-301.

5. Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res. 2007 Jan;42(1):28-42.

6. Mahal HS, Sharma HS, Mukherjee T. Antioxidant properties of melatonin: a pulse radiolysis study. Free Radic Biol Med. 1999 Mar;26(5-6):557-65.

7. Galano A, Tan DX, Reiter RJ. Melatonin as a natural ally against oxidative stress: a physicochemical examination. J Pineal Res. 2011 Aug;51(1):1-16.

8. Harman D. Free radical theory of aging. Mutat Res. 1992 Sep;275(3-6):257-66.

9. Ionov M, Burchell V, Klajnert B, Bryszewska M, Abramov AY. Mechanism of neuroprotection of melatonin against beta-amyloid neurotoxicity. Neuroscience. 2011 Apr 28;180:229-37.

10. Karasek M, Reiter RJ. Melatonin and aging. Neuro Endocrinol Lett. 2002 Apr;23 Suppl 1:14-6.

11. Poeggeler B. Melatonin, aging, and age-related diseases: perspectives for prevention, intervention, and therapy. Endocrine. 2005 Jul;27(2):201-12.

12. Bonnefont-Rousselot D, Collin F. Melatonin: action as antioxidant and potential applications in human disease and aging. Toxicology. 2010 Nov 28;278(1):55-67.

13. Tain YL, Kao YH, Hsieh CS, Chen CC, Sheen JM, Lin IC, Huang LT. Melatonin blocks oxidative stress-induced increased asymmetric dimethylarginine. Free Radic Biol Med. 2010 Sep 15;49(6):1088-98.

14. Reiter RJ, Paredes SD, Korkmaz A, Manchester LC, Tan DX. Melatonin in relation to the „strong" and „weak" versions of the free radical theory of aging. Adv Med Sci. 2008;53(2):119-29.

15. Sener A, Ozsavci D, Bingol-Ozakpinar O, Cevik O, Yanikkaya-Demirel G, Yardimci T. Oxidized-LDL and Fe3+/ascorbic acid-induced oxidative modifications and phosphatidylserine exposure in human platelets are reduced by melatonin. Folia Biol (Praha). 2009;55(2):45-52.

16. Marshall KA, Reiter RJ, Poeggeler B, Aruoma OI, Halliwell B. Evaluation of the antioxidant activity of melatonin in vitro. Free Radic Biol Med. 1996;21(3):307-15.

17. Wölfler A, Abuja PM, Schauenstein K, Liebmann PM. N-acetylserotonin is a better extra- and intracellular antioxidant than melatonin. FEBS Lett. 1999 Apr 23;449(2-3):206-10.

18. Antunes F, Barclay LR, Ingold KU, King M, Norris JQ, Scaiano JC, Xi F. On the antioxidant activity of melatonin. Free Radic Biol Med. 1999 Jan;26(1-2):117-28.

19. Wölfler A, Caluba HC, Abuja PM, Dohr G, Schauenstein K, Liebmann PM. Prooxidant activity of melatonin promotes fas-induced cell death in human leukemic Jurkat cells. FEBS Lett. 2001 Aug 3;502(3):127-31.

20. Albertini MC, Radogna F, Accorsi A, Uguccioni F, Paternoster L, Cerella C, De Nicola M, D'Alessio M, Bergamaschi A, Magrini A, Ghibelli L. Intracellular pro-oxidant activity of melatonin deprives U937 cells of reduced glutathione without affecting glutathione peroxidase activity. Ann N Y Acad Sci. 2006 Dec;1091:10-6.

21. Cristofanon S, Uguccioni F, Cerella C, Radogna F, Dicato M, Ghibelli L, Diederich M. Intracellular prooxidant activity of melatonin induces a survival pathway involving NF-kappaB activation. Ann N Y Acad Sci. 2009 Aug;1171:472-8.

22. Ximenes VF, Pessoa AS, Padovan CZ, Abrantes DC, Gomes FH, Maticoli MA, de Menezes ML. Oxidation of melatonin by AAPH-derived peroxyl radicals: evidence of a pro-oxidant effect of melatonin. Biochim Biophys Acta. 2009 Aug;1790(8):787-92.

23. Abuja PM, Liebmann P, Hayn M, Schauenstein K, Esterbauer H. Antioxidant role of melatonin in lipid peroxidation of human LDL. FEBS Lett. 1997 Aug 18;413(2):289-93.

24. Duell PB, Wheaton DL, Shultz A, Nguyen H. Inhibition of LDL oxidation by melatonin requires supraphysiologic concentrations. Clin Chem. 1998 Sep;44(9):1931-6.

25. Bosman GJ, Lasonder E, Groenen-Döpp YA, Willekens FL, Werre JM, Novotný VM. Comparative proteomics of erythrocyte aging in vivo and in vitro. J Proteomics. 2010 Jan 3;73(3):396-402.

26. Cimen MY. Free radical metabolism in human erythrocytes. Clin Chim Acta. 2008 Apr;390(1-2):1-11.

27. Krokosz A, Szweda-Lewandowska Z. Induction of transient radioresistance in human erythrocytes. Radiat Phys Chem. 2006 Sep;75(9):967-76.

28. Ellman GL, Courtney KD, Andres V Jr, Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961 Jul;7:88-95.

29. Maj S. Badania krwinek czerwonych i hemoglobiny [Studies of red blood cells and hemoglobin] In: Pawelski S, editor. Diagnostyka laboratoryjna w hematologii [Laboratory Diagnostic in Hematology]. 3rd ed. rev. and updated. Warszawa: PZWL; 1990. p. 52-110. Polish.

30. Sandhu JS, Ware K, Grisham MB. Peroxyl radical – mediated hemolysis: role of lipid, protein and sulfhydryl oxidation. Free Radic Res Commun. 1992;16(2):111-22.

31. Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristic of hemoglobin free ghosts of human erythrocytes. Arch Biochem Biophys. 1963 Jan;100:119-30.

32. Lowry OH, Rosebrough NJ, Farr AL, Randall JR. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265-75.

33. Szweda-Lewandowska Z, Krokosz A, Gonciarz M, Zajeczkowska W, Puchała M. Damage to human erythrocytes by radiation-generated HO* radicals: molecular changes in erythrocyte membranes. Free Radic Res. 2003 Oct;37(10):1137-43.

34. Bukowska B, Rychlik B, Krokosz A, Michałowicz J. Phenoxyherbicides induce production of free radicals in human erythrocytes: oxidation of dichlorodihydrofluorescine and dihydrorhodamine 123 by 2,4-D-Na and MCPA-Na. Food Chem Toxicol. 2008 Jan;46(1):359-67.

35. Loetchutinata C, Kothana S, Dechsupaa S, Meesungnoena J, Jay-Gerinb JP,S Mankhetkorn. Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2′,7′-dichlorofluorescein diacetate assay. Rad Phys Chem. 2005 Feb;72(2-3):323-31.

36. Puchała M, Szweda-Lewandowska Z, Leyko W. Gamma irradiation of aqueous solutions of human hemoglobin in atmospheres of air and argon. Radiat Res. 1979 Jun;78(3):379-89.

37. Arese P, Turrini F, Schwarzer E. Band 3/complement-mediated recognition and removal of normally senescent and pathological human erythrocytes. Cell Physiol Biochem. 2005;16(4-6):133-46.

38. Kanias T, Acker JP. Biopreservation of red blood cells--the struggle with hemoglobin oxidation. FEBS J. 2010 Jan;277(2):343-56

39. Misra HP, Fridovich I. The generation of superoxide radical during the autoxidation of hemoglobin. J Biol Chem. 1972 Nov 10;247(21):6960-2.

40. Wrona M, Patel KB, Wardman P. The roles of thiol-derived radicals in the use of 2',7'-dichlorodihydrofluorescein as a probe for oxidative stress. Free Radic Biol Med. 2008 Jan 1;44(1):56-62.

41. Winterbourn CC. Free-radical production and oxidative reactions of hemoglobin. Environ Health Perspect. 1985 Dec;64:321-30.

42. Gilad E, Zisapel N. High-affinity binding of melatonin to hemoglobin. Biochem Mol Med. 1995 Dec;56(2):115-20.

43. Tan DX, Manchester LC, Sainz RM, Mayo JC, Leon J, Hardeland R, Poeggeler B, Reiter RJ. Interactions between melatonin and nicotinamide nucleotide: NADH preservation in cells and in cell-free systems by melatonin. J Pineal Res. 2005 Sep;39(2):185-94.

44. Krokosz A, Koziczak R, Gonciarz M, Szweda-Lewandowska Z. Study of the effect of dose-rate on radiation-induced damage to human erythrocytes. Radiat Phys Chem. 2006 Jan;75(1):98-105.

45. Tesoriere L, D'Arpa D, Conti S, Giaccone V, Pintaudi AM, Livrea MA. Melatonin protects human red blood cells from oxidative hemolysis: new insights into the radical-scavenging activity. J Pineal Res. 1999 Sep;27(2):95-105.

46. Sadowska-Woda I, Wójcik N, Karowicz-Bilińska A, Bieszczad-Bedrejczuk E. Effect of selected antioxidants in beta-cyfluthrin-induced oxidative stress in human erythrocytes in vitro. Toxicol In Vitro. 2010 Apr;24(3):879-84.

47. Dikmenoglu N, Ileri E, Seringec N, Ercil D. Melatonin prevents lipid peroxidation in human erythrocytes but augments deterioration of deformability after in vitro oxidative stress. Clin Hemorheol Microcirc. 2008;40(3):235-42.