Effects of Different Concentrations of Melatonin on the Time-course of Nitrite–induced Oxidation of Hemoglobin: *In vitro* Study[#]

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Abstract

Melatonin is a potent scavenger of reactive oxygen species or free radicals like superoxide and hydroxyl radicals. The oxidation of hemoglobin to methemoglobin (meth-Hb) by oxidizing compounds has been widely studied. The present work was designed to evaluate the ability of different concentrations of melatonin to inhibit nitrite–induced oxidation of hemoglobin. Blood samples were obtained from apparently healthy individuals from which erythrocyte hemolysate was prepared. Different concentrations of melatonin (10^{-9}-1.0 mg/ml) were incubated for 10 min with the hemolysate, then to the resultant mixture 1 ml of sodium nitrite (final concentration 0.6 mM) was added, and the formation of meth-Hb was measured by monitoring absorbance of light at 631 nm each min for 30 min. Control samples without melatonin were utilized for comparison. Nitrite caused rapid oxidation of hemoglobin to meth-Hb in control samples; in the presence of melatonin, the oxidation process was delayed in a dose–dependent manner. The effect of melatonin on the time course of nitrite-induced oxidation of Hb showed that melatonin has a protective effect initiated early after addition along with nitrite. Melatonin also affect the time required for the formation of meth-Hb, the time required to convert 50% of the available Hb to meth-Hb was 4 min in the absence of melatonin, and became 17, 22, 26, 30, 114 and 383 min with increasing melatonin concentrations (10^{-9}, 10^{-6}, 0.001, 0.01, 0.1, and 1.0 mg/ml respectively). In conclusion, melatonin in a concentration and time dependent manner can protect Hb from oxidation by nitrite; melatonin delays the onset of autocatalytic stage and the protective effect extended over long period of time.

***Key words*: melatonin, erythrocytes oxidation**

الخلاصة

ان عملية اكسدة الهيمغلوبين وتحوله الى ميتهيمغلوبين قد تمت دراستها بشكل واسع، وتهدف الدراسة الحالية الى تقييم مقدرة تراكيز مختلفة من مادة الميلاتونين على منع أو تأخير حدوث عملية الأكسدة بمادة نايترايت الصوديوم. تم الحصول على عينات دم من أشخاص أصحاء وتحضير محلول من الهيمغلوبين من الكريات المتحللة وحسب الطرق المعتمدة من قبل الآخرين. تم مزج محلول الهيمغلوبين مع تراكيز مختلفة من مادة الميلاتونين (١-١٠$^{-٩}$ ملغم/مل) لمدة ١٠ دقائق تم بعدها اضافة مللتر واحد من مادة نايترات الصوديوم كعامل مؤكسد. تمت متابعة عملية التأكسد من خلال قياس مستوى الميتهيمغلوبين المتكون كل دقيقة باستخدام مطياف الأشعة فوق البنفسجية. أظهرت النتائج ان للميلاتونين القدرة على تأخير تأكسد الهيمغلوبين بصورة تعتمد على التركيز وفترة الخلط. ويمكن الأستنتاج بان الميلاتونين بأمكانه حماية الهيمغلوبين من التأكسد بواسطة نايترات الصوديوم وبصورة تعتمد على التركيز وفترة المزج.

Introduction

Recently, many experimental data provided unequivocal evidence about the formation and role of free radicals in biological systems. [(1)] Such reactive species may bring about oxidative damage to virtually all cell compartments, eventually leading to various pathologies and aging. [(2)] These studies prompted research on physiological antioxidant systems and molecules, and stimulated the development of natural or synthetic compounds that prevent oxidative stress and damage mediated by an enhanced formation of free radicals. [(3)] After the discovery of its radical-scavenging properties, melatonin (N-cetyl-5-methoxytryptamine) has been considered as a putative biological antioxidant but it has been questioned to whether it may have a real antioxidant function under physiological conditions; [(4)] its molecular mechanisms of action remain to be clarified. Interactions of melatonin contributing to its antioxidant effects *in vivo* may be lost during *in vitro* experiments; when it behaves *in vitro* as an electron donor, many electrophilic compounds, such as the hydroxyl radical, Fe^{+3}, or carbon centered radicals may act as acceptors in one-electron transfer reactions, which convert the indolamine to the indolyl cation radical. [(5)] Reactivity of melatonin with oxygen centered radicals, such as peroxyl or alkoxyl radicals, as well as a moderate activity towards lipoperoxyl radicals, has also been demonstrated.

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Although the exact relationship between such activity and the concentrations required to perform it is not clarified, its ability to scavenge a broad spectrum of radicals could allow melatonin to behave as an antioxidant in various and possibly complex ways. [6] This study was designed to investigate the antioxidant activity of melatonin in different concentrations using an *in vitro* model of nitrite-induced hemoglobin oxidation.

Material and method

Blood samples were obtained from apparently healthy individuals, and were centrifuged at 2500 rpm and 4°C for 10 min to remove plasma and the buffy coat of white cells. The erythrocytes obtained were washed thrice with phosphate-buffered saline and lased by suspending in 20 volumes of 20mM phosphate buffer pH 7.4 to yield the required hemolysate concentration of 1:20. Different concentrations of melatonin were incubated for 10 min with the hemolysate starting with stock solution (melatonin 1mg/ml) from which serial dilutions were made to give concentrations of 0.1, 0.01, 0.001, 10^{-6} and 10^{-9} mg/ml melatonin solution. Then to these incubated mixtures 1ml of sodium nitrite (final concentration 0.6 mM) were added and the formation of methemoglobin was measured by monitoring absorbance at 631 nm each min for 30 min using a spectrophotometer. [7] In the second part of the study, melatonin was added either before or at various time intervals (5 min and 10 min) after the addition of sodium nitrite to the hemolysate solution, and the formation of methhemoglobin was measured by monitoring the absorbance of light at 631 nm, and the results were compared with control samples without melatonin; all experiments were performed in triplicate and repeated many times.

Results

Nitrite causes a rapid oxidation of hemoglobin to methemoglobin, as shown in control curve (figure 1). In the presence of melatonin, the oxidation process was delayed in a dose-dependent manner. Figure1 describes the effect of different melatonin concentrations on the time- course of nitrite oxidation of hemoglobin; without melatonin, the time-course of oxidation shows a characteristic pattern of slow initial transformation followed by a rapid autocatalytic process; in presence of melatonin there is slow increase in absorbance related to reduced levels of methemoglobin formation in all test samples. Figure 2 showed that addition of melatonin to the incubation mixture, at different time intervals (after 5 and 10 min) during the autocatalytic phase, did not affect its ability to decrease meth-Hb formation. The time required to convert 50% of the available hemoglobin to met hemoglobin was (4 min) in the absence of melatonin, whereas with 1 mg/ml melatonin solution the time was increased to 383 min (6.4 hr) (table 1).

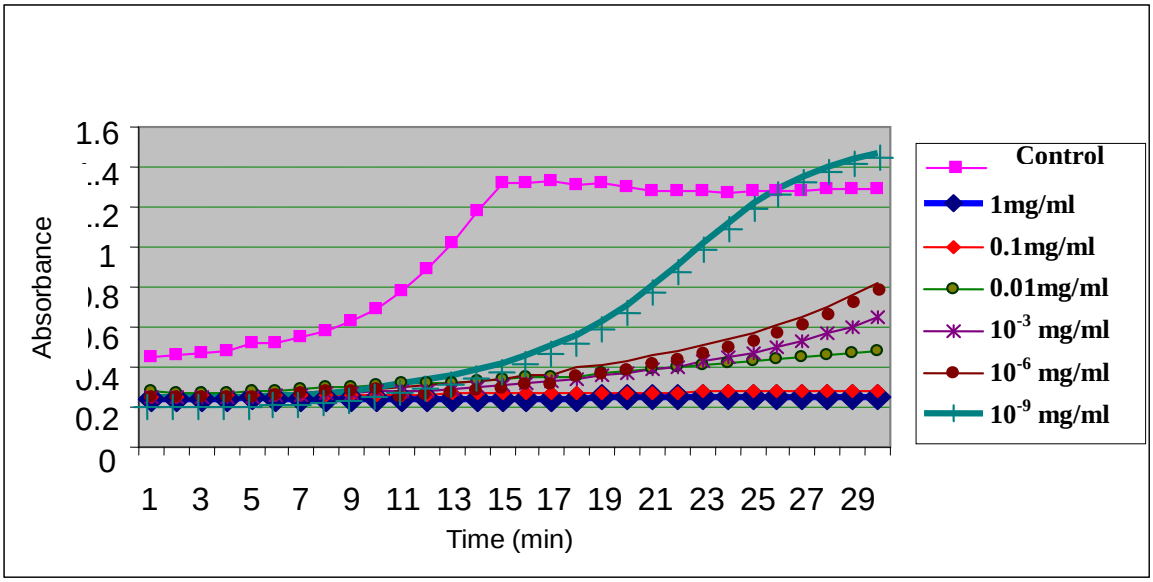

Figure 1. Effect of different melatonin concentrations on the time-course of nitrite–induced oxidation of hemoglobin.

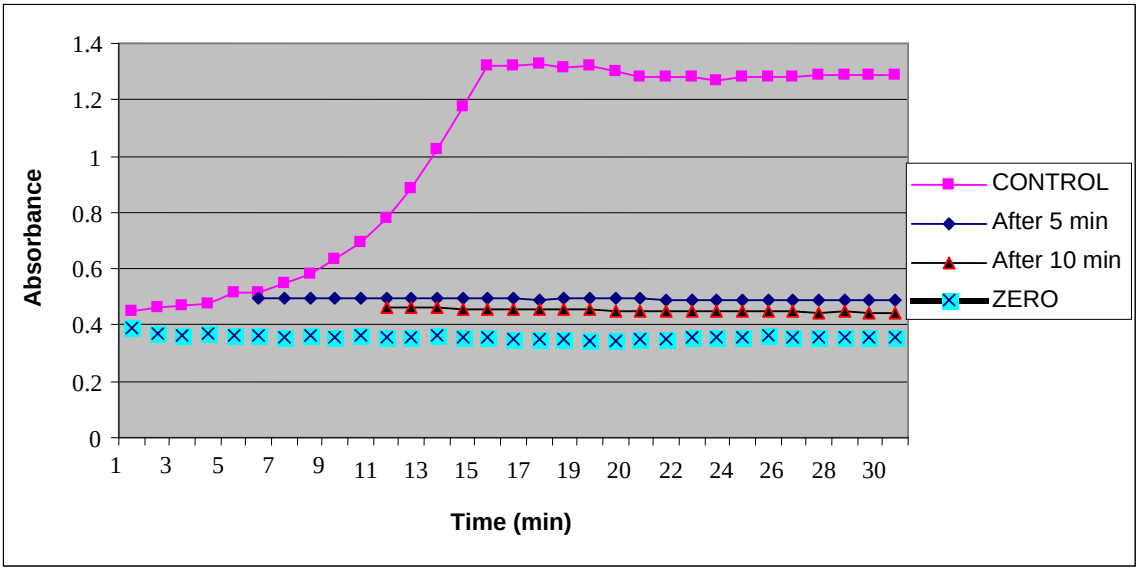

Figure 2. Effect of melatonin on the time course of methhemoglobin formation at various time intervals from nitrite addition.

Table(1) : Time to form 50% Meth-Hb in presence of different concentrations of melatonin.

Melatonin concentration mg/ml	% formation of Meth-Hb	Time to form 50% Meth-Hb (min)
Control	100	4.0
10^{-9} mg/ml	96.1	17.0
10-6 mg/ml	86.9	22.0
0.001 mg/ml	50.5	26.0
0.01 mg/ml	37.9	30.0
0.1 mg/ml	29.9	114.3
1.0 mg/ml	16.6	383.0

Discussion

The oxidation of Hb to Meth-Hb by nitrite has been widely studied, [7-9] formation of Meth-Hb occurs in two stages; there is a slow initial stage followed by a rapid autocatalytic stage, which carries the reaction to completion. [10] The present study has shown that melatonin can protect hemoglobin from oxidation by sodium nitrite in hemolysate, and there are two suggested theories for the mechanism through which melatonin produces this protective role; erythrocytes are utilized as a traditional target for studying oxidative damage, when exposed to high oxygen tensions and in presence of high iron contents (transition metal promoting the formation of oxygen free radicals) oxidative damage occur due to both endogenous and exogenous insults. Sodium nitrite as a prooxidant induces a primary extensive methemoglobin formation as a result of generation of several free radical species like super oxide anion, peroxynitrite, and nitric dioxide, which are generated during the course of nitrite–induced oxidation of hemoglobin. [11] After the discovery of radical-scavenging properties of melatonin, it has been considered a putative biological antioxidant, but it has been questioned whether it may have a real antioxidant function under physiological *in vivo* conditions. [6] The molecular mechanisms of actions of melatonin remain to be better clarified; it is capable to prevent the onset of the autocatalytic stage since superoxide is implicated in the autocatalytic stage , and the fact that melatonin is a potent scavenger of superoxide anion, [5] the results of the present study suggests that the protective action of melatonin might be due to its scavenger effect and not due to reduction of methemoglobin to hemoglobin, since it fails to reverse the oxidation of hemoglobin; additionally, direct interaction between nitrite and melatonin as a reason for protection can be ruled out because the concentrations of melatonin which protect erythrocytes is very low. [11] Kinetic evidence indicates that melatonin delays oxidative denaturation of Hb through it's reaction with Hb-derived oxoferryl radicals, and this may

explain the reported antioxidant effects; Tesoriere *et al* (2001) studied the reaction of melatonin with hemoglobin-derived oxoferryl radicals and the inhibition the oxidant effects of hydroxyl peroxide-induced hemoglobin denaturation in red blood cells, they found that the basic requirement for oxidative denaturation of Hb by hydroperoxides is the transient formation of the perferryl-Hb; [12] perferryl-Hb, which includes a hypervalent-iron oxoferryl heme group and a radical species, localized in the globin is a strong oxidant towards the globin moiety, which leads to Hb denaturation with the formation of hemichrome and heme release. [13] The perferryl species, generated from met-Hb and H_2O_2, [14] comprises a radical localized on the globin, possibly an aromatic amino acid radical , and an oxoferryl heme group. [12] After exhaustion of H_2O_2, decay of the perferryl to the oxoferryl form occurs, and then the latter is slowly converted to met-Hb by a so-called autoreduction reaction; [14] this process involves intramolecular electron transfer and modification of the globin moiety. [13] Such oxidative modifications of globin on exposure to H_2O_2 may be avoided by the presence of certain antioxidant compounds such as melatonin, ascorbate or Trolox at the time of reaction, suggesting that rapid deactivation of the protein radical in the perferryl species is crucial for protection; [15] the mentioned mechanisms may prove that melatonin acts through its reducing activity towards perferryl-Hb, and this may include the reduction of the oxoferryl moiety or the unpaired electron electrophile center at the globin moiety by melatonin, or both. [11] Although in our study we did not investigate the reactivity of melatonin as a reducing agent, we can not exclude this effect and it needs further investigations. In conclusion, melatonin protects hemoglobin against nitrite-induced oxidation and delay the formation of meth-Hb in concentration dependent pattern.

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