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AN ASSAY FOR THE *IN VITRO* DEMONSTRATION OF SUPEROXIDE SCAVENGING CAPACITY OF MELATONIN

Kadir BATÇIOĞLU*
A. Alev KARAGÖZLER**
İ. Çetin ÖZTÜRK***
Aysun BAY***
M. Engin GÖZÜKARA***

Aim: *In vitro assay of superoxide scavenging capacity of melatonin.*
Materials and methods: *Scavenging capacity of melatonin for superoxide radicals produced by xanthine-xanthine oxidase system was investigated by using Fridovich I. method.*
Results and discussion: *Melatonin exhibited superoxide scavenging activity in a dose-dependent manner up to 45 pg/mL and activity was calculated to be 0.046 ±0.009 SOD equivalent units / pg of melatonin (n=9). A concentration of approximately 18 pg / mL melatonin served as one unit of superoxide dismutase enzyme.*

Key words: *Melatonin, superoxide scavenging activity, oxidative stress, antioxidant.*

Melatoninin Superoksit Yakalama Kapasitesinin In Vitro Incelenmesi

Amaç: *Melatoninin radikal süpürücü kapasitesinin in vitro koşullarda incelenmesi amaçlandı.*
Materyal ve metot: *Ksantin-ksantin oksidaz sisteminde oluşturulan superoksit radikallerinin melatonin tarafından süpürüldüğü sitokrom c kullanılarak Fridovich I. yöntemi ile araştırıldı.*
Bulgular ve sonuç: *Çalışma bulgularımız melatoninin in vitro koşullarda da superoksit radikallerine karşı anlamlı ölçüde temizleyici etkiyi gösterdiği yönündedir.*

Anahtar kelimeler:*Melatonin,superoksit,süpürücü aktivite, oksidatif stres,antioksidan*

*İnönü University, Institute of Health Science Department of Biochemistry, MALATYA
**Adnan Menderes University, Faculty of Arts and Sciences Department of Chemistry, AYDIN
***İnönü University, Faculty of Medicine Department of Biochemistry, MALATYA

A free radical is a specie containing an unpaired electron. The unpaired electron gives certain characteristic properties to the radical, such as paramagnetism and the chemical reactivity of free radicals is unusually high.[1] In all aerobic organisms reactive oxygen species (ROS) are formed as by-products of respiration and oxidative metabolism. The cell generally has protective mechanisms to control the concentration of intracellular ROS, some of which are necessary for physiological functions such as phagocytic mechanisms and in growth regulation or as a signal transduction messenger. When the delicate balance between the intracellular levels of oxidants and antioxidants is perturbed, ROS are produced in excess and cells reach the state of oxidative stress.[2] The defense mechanism of the cell against oxidative stress deploys enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx) and catalase (CAT); antioxidant vitamins such as A, E and C; antioxidative

Correspondence Address:
Kadir BATÇIOĞLU PhDs
İnönü University, Institute of Health Science Department of Biochemistry, MALATYA, TURKEY

molecules such as glutathione, melatonin and bilirubin; and elements such as selenium.

The pineal hormone melatonin (N-acetyl-5-methoxy tryptamin) has been found to be a highly effective antioxidant and a free radical scavenger, especially for hydroxyl and peroxyl radicals.[3] Melatonin also controls superoxide anion level *in vivo*.[4] Because of its lipophilic character, which allows it to cross the blood-brain barrier, its highly efficient free radical scavenging properties and its capacity of inducing antioxidative enzymes, melatonin has been proposed to be the endogenous protective agent against brain oxidative damage.[5] There are quite a number of reports on the *in vivo* antioxidant effects of melatonin.[6-8]

The aim of this work was to construct an *in vitro* assay in order to demonstrate the radical scavenging capacity of melatonin. The experiment was planned on the basis of melatonin's ability to inhibit some observable process. For this reason, the method developed by McCord and Fridowich[9] for the determination of SOD activity is modified by replacing SOD with melatonin.

MATERIAL AND METHODS

Materials: Xanthine, xanthine oxidase (XOD, from buttermilk), cytochrome *c* (cyt *c*, from bovine hearth), melatonin, $FeSO_4$, H_2O_2, KH_2PO_4, Na_2HPO_4, EDTA were purchased from Sigma Chemical Company (St. Louise, USA). A Shimadzu 1601 UV-VIS spectrophotometer with a Grand LTD6 thermostability unit adjusted to 37 ± 0.1°C was employed for the assays.

Method: The following solutions were prepared for the assay:

Solution A: 0.76 mg Xanthine in 10 mL of 0.001N NaOH solution was mixed with 24.8 mg cyt *c* in 100 mL of 50 mM phosphate buffer (pH=7.8) containing 0.1 mM EDTA.
Solution B: 0.2 U/ mL XOD in 0.1 mM EDTA solution.

Determination of the rate of cyt c (Fe^{3+}) reduction in the presence of melatonin: 100 μL of iron (II) sulphate solution was added to 750 μL of solution A. 25 μL of ethanol solution of melatonin (to give a final concentration of 10 pg / mL) was added to this solution. Then, 100 μL of H_2O_2 solution (2.00 mM in phosphate buffer) was added to the reaction mixture. After incubation for 30s, 25 μL of solution B was added and absorbance change at 550 nm was followed for 1 min. The assay was repeated with increased amounts of melatonin. Each assay was repeated three times and the arithmetic means were calculated. A blank was run by substituting 0.5% ethanol solution with melatonin solution.

RESULTS AND DISCUSSION

The strategy to test melatonin's radical scavenging capacity was established by combining a reaction that generates superoxide radical from H_2O_2 with a reaction that generates indolyl radical from melatonin. Xanthine-xanthine oxidase system was utilized to generate the superoxide flux (reaction 1).

$$Xanthine + O_2 + H_2O_2 \xrightarrow{XOD} O_2 + H_2O + Uric\ acid \quad (1)$$

The amount of O_2 produced was measured by its capacity to reduce the oxidized cyt *c*, which is the electron acceptor and the chromophore for the reaction that may be followed spectrophotometrically at 550 nm (reaction 2).

$$O_2 + cyt\ c\ (Fe^{3+}) \longrightarrow O_2 + cyt\ c\ (Fe^{2+}) \quad (2)$$

In the method developed by McCord and Fridovich the aforementioned reactions were used to measure SOD activity. When SOD was added to the reaction medium the rate of cyt *c* (Fe^{3+}) reduction was progressively inhibited. One unit of SOD activity was defined as the amount of enzyme necessary to decrease the (reference) rate to 50% of maximum inhibition. SOD is the metalloenzyme which catalyses the dismutation of O2 into O_2 and H_2O_2.

Table 1. Percentage of inhibition observed in cyt *c* reduction with increased melatonin concentration (Reaction medium: 50 mM phosphate buffer, pH 7.8, containing 0.1 mM EDTA, 0.037 mM Xanthine, 186 μg / mL cyt *c*, 5 mU/mL XOD, 0.02 mM $FeSO_4$).

[Melatonin] (pg / mL)	% Inhibition	Log [Melatonin]
0	---	---
10	14.6	1.397
15	35.9	1.431
20	55.8	1.462
25	64.0	1.491
30	68.6	1.518
35	78.2	1.544
40	81.9	1.568
45	87.9	1.591

p= 0,000; r= 0.971; Regression equation : % Inhibition =-474.7 +357.0 (Log [Melatonin])

It was suggested that when SOD is replaced with melatonin, indolyl radical produced from melatonin will scavenge the superoxide radical and the expected result was the inhibition of the reduction of oxidized cyt *c*.

Hydroxyl radical is generated by Fenton reaction (reaction 3) and indolyl radical is generated by the reaction of hydroxyl radical and melatonin (reaction 4).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH + OH \quad (3)$$

$$OH + Melatonin \longrightarrow Indolyl\ radical \quad (4)$$

The inhibition observed in cyt *c* (Fe^{3+}) reduction by increased amount of melatonin is listed in Table 1. Correlation analysis is applied to the data and p=0.000 and r=0.971 is found. Regression equation is calculated to be % Inhibition=-474.7 + 357.0 (Log [Melatonin]).

In this study, free radical scavenging activity of melatonin for superoxide radical was clearly demonstrated. Melatonin concentrations were between 10 pg/mL to 45 pg/mL; first the average daytime serum melatonin concentration and the latter average nocturnal serum melatonin concentration in human serum [10]. Scavenging activity exhibited a dose-dependent manner up to 45 pg/mL and activity was calculated to be 0.046 ± 0.009 SOD equivalent units/pg of melatonin (n=9) which means a concentration of approximately 18 pg/mL melatonin serves as 1 unit of SOD enzyme.

In order to estimate the magnitude of potency of melatonin as a superoxide radical scavenger, it may be compared with the therapeutic agent, gliclazide, which is also known to be a general free radical scavenger. In a study the effect of gliclazide on free radicals were examined *in vitro* and it was shown that gliclazide scavenged superoxide radical in a dose-dependent manner with a calculated superoxide scavenging activity of 0.18 ± 0.8 SOD equivalent units/mg of gliclazide, which is far less than that of melatonin.[11]

It is not clear whether the *in vitro* observations with melatonin as a free radical scavenger are related directly to the *in vivo* behavior. Since 1958, when Lerner *et al* discovered the pineal hormone melatonin, a broad spectrum of metabolic and physiological effects of melatonin have been described in a wide variety of species.[12] Melatonin constitutes the primary non-enzymatic antioxidant agent against hydroxyl radicals, because there is no physiological enzymatic system for the detoxification of this highly reactive and toxic radical. Superoxide radical is primarily scavenged by SOD enzyme in the cell. But there may be cases in which SOD is inhibited or is not produced enough to work efficiently, or the amount of superoxide radical produced in the cell is more than the amount that is scavenged by SOD. In recent years, it is suggested that melatonin is protective against free radical damage at physiological concentrations and would thereby contribute to the total antioxidative defense system of the organisms.[13,14] This *in vitro* study implies that

endogenous or exogenous melatonin may compensate for SOD and it is highly effective for the detoxification of superoxide radical.

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