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Research paper

Interaction of melatonin with model membranes and possible implications in its photoprotective activity

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Abstract

It is well known that administration of antioxidants represents a successful strategy for preventing the occurrence and for reducing the severity of UV-mediated oxidative damage. Melatonin was recently shown to be an efficacious photoprotective agent. The aim of the present study was to better investigate the interaction of melatonin with model membranes and the possible implications in its photoprotective activity. The antioxidant activity of melatonin was tested in two 'in vitro' experimental models: (a) UV radiation-induced peroxidation in phosphatidylcholine multilamellar vesicles (MLVs); (b) scavenging activity against nitric oxide (NO). Furthermore, we investigated the melatonin/biomembrane interaction by differential scanning calorimetry (DSC) on dimyristoylphosphatidylcholine (DMPC) MLVs and unilamellar vesicles (LUVs). The findings of in vitro antioxidant tests suggest that the photoprotective effect of melatonin should be due, partially at least, to the drug scavenging activity against aqueous and lipophilic free radicals, including NO; besides, melatonin might provide its protective effect against UV radiation-induced damage also by acting as a UV-absorbing screen. The results of DSC experiments have evidenced a good capability of melatonin to interact with DMPC bilayers, causing a significant fluidifying effect; however, the transfer of melatonin in the LUVs is faster than that observed for MLVs, even if both values tend to the maximum values reachable. Our present data allow us to emphasize two points: (1) the fluidifying effect induced by melatonin on lipidic bilayers might act as a cooperative mechanism in its protective effect against peroxidative membrane damage; (2) melatonin appears able to cross biomembranes, so that it could protect intracellular components against peroxidative insult.

Keywords: Melatonin; Antioxidant; Liposomes; Differential scanning calorimetry; Photoprotection

1. Introduction

In the last decades, the knowledge of the effects of ultraviolet (UV) radiation on human health, especially in skin cancerogenesis, but also in immunosuppression, photoaging and eye damage, has enlarged strongly [1–5]. The human population is exposed to both UVA and UVB regions of the solar spectrum. UVA is absorbed by other cellular constituents and induces mainly oxidative damage indirectly, whereas UVB induces mainly dipyrimidine photoproducts in DNA by a direct photochemical mechanism.

The increasing solar UV radiation and changes in life style strengthen the necessity to seek preventive measures effective against deleterious effects caused by intense or prolonged UV light exposure. In particular, administration of antioxidants represents a successful strategy for preventing the occurrence and for reducing the severity of UV-mediated oxidative damage [6–12].

Melatonin has been widely reported to protect against damage caused by reactive oxygen species (ROS) in vivo [13–15]. This protective effect can occur as a result of at least two mechanisms: a direct antioxidant action and an upregulation of endogenous antioxidant defences. However data in literature demonstrate that melatonin is not a conventional chain-breaking antioxidant, but perhaps it functions as a retarder in metal ion-free autoxidations [13,16].

Several papers claim that melatonin may act as an efficacious photoprotective drug. In fact, topical application of melatonin was recently shown to almost completely suppress the development of UV-induced skin erythema [17,18]. Moreover, exogenously administered melatonin

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appeared able to protect the eye lens from the damaging effect of UV-A and UV-B exposure in adult rats [19,20] ; consistently with these findings, Longoni and coworkers [14] have hypothesized a functional role of melatonin as a free radical scavenger within retinal photoreceptors, by preventing oxidative damage due to UV light irradiation.

In order to understand further the in vivo potential photoprotective effect of melatonin, we have tested its antioxidant activity in two in vitro experimental models, which may be rationally predictive for a successful employment of a drug as skin photoprotective agent: (a) UV radiation-induced peroxidation in liposomal membranes; (b) scavenging activity against nitric oxide (NO). On the basis of the results obtained in these tests, further studies have appeared necessary to better clarify the mechanisms subserving the photoprotection elicited 'in vivo' by melatonin. With this aim, we investigated the melatonin/biomembrane interaction by differential scanning calorimetry (DSC) on dimyristoylphosphatidylcholine (DMPC) multilamellar vesicles (MLVs) and unilamellar vesicles (LUVs). This biomimetic experimental model was chosen for two reasons. First, given that the antioxidant effect of a drug may be modulated by its capability to affect biomembrane fluidity, these experiments can help to understand the molecular basis of the pharmacological behavior of melatonin. Secondly, by studying the kinetic features of drug/phospholipid bilayer interaction, one can obtain information about drug capability to cross biological membranes and reach the intracellular compartment.

2. Materials and methods

2.1. Materials

Synthetic L-α-dimyristoylphosphatidylcholine was obtained from Fluka Chemical Co. (Buchs, Switzerland); solutions of the lipid were chromatographically pure as assessed by two-dimensional thin-layer chromatography. Melatonin, phosphatidylcholine, sodium nitroprussiate, sulphanilamide, naphthylethylene-diamine, Tris–HCl, ethanol and phosphoric acid were purchased from Sigma-Aldrich srl (Milan, Italy).

2.2. UV radiation-induced peroxidation in liposomal membranes (UV-IP test)

The protective effect of melatonin against UVC-induced peroxidation was evaluated on phosphatidylcholine (PC) MLVs by monitoring malondialdehyde (a final product of fatty acid degradation; MDA) production [21]. Briefly, 100 mg of PC, dissolved in chloroform, were transferred to a small stoppered tube. The lipid was thoroughly dried under nitrogen. It was then dissolved in warm ethanol (80 mg), and 25 mM Tris–HCl, pH 7.4 (200 mg) was added to yield a (100:80:200 w/w/w) lipid/ethanol/water mixture. This mixture was heated to 60°C for a few minutes and then allowed to cool to room temperature (20°C) yielding a proliposome mixture. The proliposome mixture was finally converted to a liposome suspension by the dropwise addition of 25 mM Tris–HCl, pH 7.4, to a final volume of 10 ml. The suspension was vortex-mixed throughout this last stage. Liposome dispersion (1 ml in a glass flask with a 3 cm^2 exposure surface area) was maintained at room temperature and exposed for 1.5 h to UV-radiation from a 15 W Philips germicidal lamp (254 nm) at a distance of 10 cm; the dose rate of UV-radiation was 105 erg/mm^2/s. Different concentrations of melatonin were added to the system; an equal volume (50 μl) of the vehicle alone (ethanol) was added to control tubes. MDA concentration in the mixture was measured by using a colorimetric assay kit (Calbiochem-Novabiochem Corp., La Jolla, CA, USA). All experiments were carried out in triplicate and repeated at least three times. Results were expressed as percentual decrease with respect to controls and mean inhibitory concentrations (IC_{50}) were calculated by using the Litchfield and Wilcoxon test.

Furthermore, a water/ethanol (95/5, v/v) solution of melatonin (25 μM) was exposed to UV radiation under the same experimental conditions described above and the spectra were recorded at different times (0–345 min) from the beginning of the incubation.

2.3. Scavenging activity against nitric oxide (NO test)

Nitric oxide interacts with oxygen to produce stable products, nitrite and nitrate. Scavengers of nitric oxide compete with oxygen, leading to a reduced production of nitrite. The concentration of nitrite in aqueous solution can be assayed spectrophotometrically by using the Greiss reagent, with which nitrite reacts to give a stable product absorbing at 542 nm [22,23] . Briefly, sodium nitroprusside solution was prepared immediately before the experiment, dissolving 10 mM sodium nitroprusside in 20 mM phosphate buffer, pH 7.4, previously bubbled with argon. Melatonin was dissolved in 20 mM phosphate buffer, pH 7.4, to obtain the opportune concentrations. Greiss reagent solution was obtained by preparing a solution A, containing 2% (% w/v) sulfanilamide and 4% (% w/v) H_3PO_4, and a solution B, containing 0.2% (% w/v) naphtylethylenediamide. Immediately before the assay, 50 ml of solution A were added to 50 ml of solution B.

At the beginning of the experiment, 0.5 ml of drug solution (at various concentrations) was diluted with 0.5 ml of sodium nitroprusside solution and incubated at 25°C for 150 min. At the end of the incubation, 1 ml of Greiss reagent was added to each sample and the absorbance was read at 542 nm. Nitrite concentration was calculated by referring to the absorbance of standard solutions of potassium nitrite. All experiments were carried out in triplicate and repeated at least three times. Results were expressed as percentual nitrite production with respect to control values (drug concentration: 0 μM).

2.4. Differential scanning calorimetry (DSC)

Experiments were carried out according to the previously described method [24]. Dimyristoylphosphatidylcholine (DMPC) MLVs were prepared in the presence and absence of melatonin by the following procedure: chloroform–methanol (1:1, v/v) stock solutions of DMPC and melatonin (1:1, v/v) were mixed to obtain the chosen mole fractions (X_D). The solvents were removed under a nitrogen flow in a rotoevaporator, and the resulting film was freeze-dried under vacuum to remove the residual solvents. Liposomes were obtained by adding to the film 50 mM Tris buffer (pH 7.4), then heating at a temperature above that of the gel–liquid crystalline phase transition (37°C) and vortexing three times for 1 min. The samples were shaken for 1 h in a water bath at 37°C to homogenize the liposomes.

Aliquots of 120 μl of lipid aqueous dispersion (5 mg of lipid), pure or containing melatonin, were transferred to a 150 μl DSC aluminium, hermetically sealed pan (Mettler Toledo Group, Greifensee, Switzerland) and submitted to DSC analysis. The temperature of the maximum of the transition endotherm (T_m) and the enthalpy (ΔH) were determined by using a Mettler TA 4000 system equipped with a DSC-30 cell and a TC-11 processor (Mettler Toledo Group). The scan rate employed was 2°C/min in a temperature range of 2–37°C. The sensitivity was 1.72 mW, and the reference pan was filled with Tris buffer solution. ΔH values were evaluated from the peak areas using the integration program of the TA processor, permitting the choice of different baselines and ranges of integration. For curves showing an ill-defined baseline, a fixed arm planimeter was also employed. The areas calculated with these different methods lie within the experimental error (±5%).

After calorimetric scans, all samples were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorous assay [25].

Furthermore, DSC analysis allows us to obtain evidence of membrane penetration by comparing the thermotropic effects exerted by a compound put in contact with empty MLVs and/or LUVs and then following the reaching of the maximum perturbative effect on the two layered membranes [26]; if the perturbative effect (T_m shift) of the two systems tends to the same value, which is comparable with that observed when the compounds have been completely dispersed in the bilayer during the liposomal preparation, this should represent a signal of the happened membrane penetration. Thus, to study the capacity of melatonin to permeate the model membrane, a kinetic experiment was performed by putting a suspension of empty DMPC LUVs or MLVs in contact with a fixed amount of powdered melatonin (0.18 molar fraction) placed in the bottom of the DSC crucible [24–26]. The empty LUVs were prepared by submitting empty MLVs to extrusion through polycarbonate membranes of 100 nm (Avestin Inc., Ottawa, Canada) in an extruder system (LiposoFast™ Basic, Avestin Inc.) [27]. The crucible was gently shaken for 10 s and submitted to the following heating, isothermal and cooling calorimetric cycles: (1) a first scan between 2 and 37°C, to detect the interaction between the compound and model membrane; (2) an isothermal period of 1 h at 37°C to permit melatonin to permeate (if able) the lipid layer(s) in a disordered state at a temperature above the lipid transitional temperature; (3) a cooling scan between 37 and 2°C to restart the heating program. This procedure was run at least five times.

3. Results and discussion

A brief methodological comment is needed before discussing the results obtained in the UV-IP test. Biologically relevant solar radiation is represented mainly by UVB and, to some extent, UVA wavelengths, while UVC radiation from sunlight does not reach the surface of the earth. However, each region of the UV spectrum can induce strong degradation of fatty acids (the main components of biological membranes). High energy UVC radiation is widely employed in 'in vitro' cell-free systems (particularly liposomal systems), where production of MDA is monitored as index of lipid peroxidation; in fact in these experimental systems higher MDA levels may be expected following UVC exposure than after UVB or UVA irradiation.

In our study, melatonin appeared to be able to protect PC within liposomal bilayers from UVC-induced peroxidation. In fact, exposure of PC liposomes to UV radiation for 1.5 h elicited a large increase in MDA production. As shown in Fig. 1, the addition of melatonin reduced the amount of formed MDA in a dose-dependent manner; the IC_{50} value calculated (expressed as mean ± SD of three experiments) was 0.523 ± 0.064 mM. Since there is considerable evidence relating radical oxygen species with UV light-induced phospholipid degradation, the photoprotective effect of melatonin might be due to its capability to scavenge UV-induced free radicals and, thus, to inhibit propagation of lipid peroxidative chain reaction.

As to the NO test, a linear time-dependent production of nitrite was obtained for at least 3 h from 5 mM sodium nitroprusside. In the presence of melatonin, the concentration of nitrite detected after 150 min of incubation decreased depending on the antioxidant concentration (0.1–1.5 mM; Fig. 1). These data demonstrate the antioxidant activity of melatonin against NO radicals or (since the test employed in our study is not specific for nitric oxide detection, but measures the final product, nitrite, of the reaction of nitric oxide with oxygen through intermediates such as NO_2, N_2O_4 and N_2O_3) nitrogen oxides in general. Consistently with these results, Noda and coworkers [28] showed that melatonin and its precursors exhibit good NO scavenging activity in an experimental model using 1-hydroxy-2-oxo-3-(*N*-methyl-3-amino-propyl)-3-methyl-1-triazene as NO generator.

On the basis of these findings, one can speculate that the 'in vivo' observed photoprotective effect of melatonin

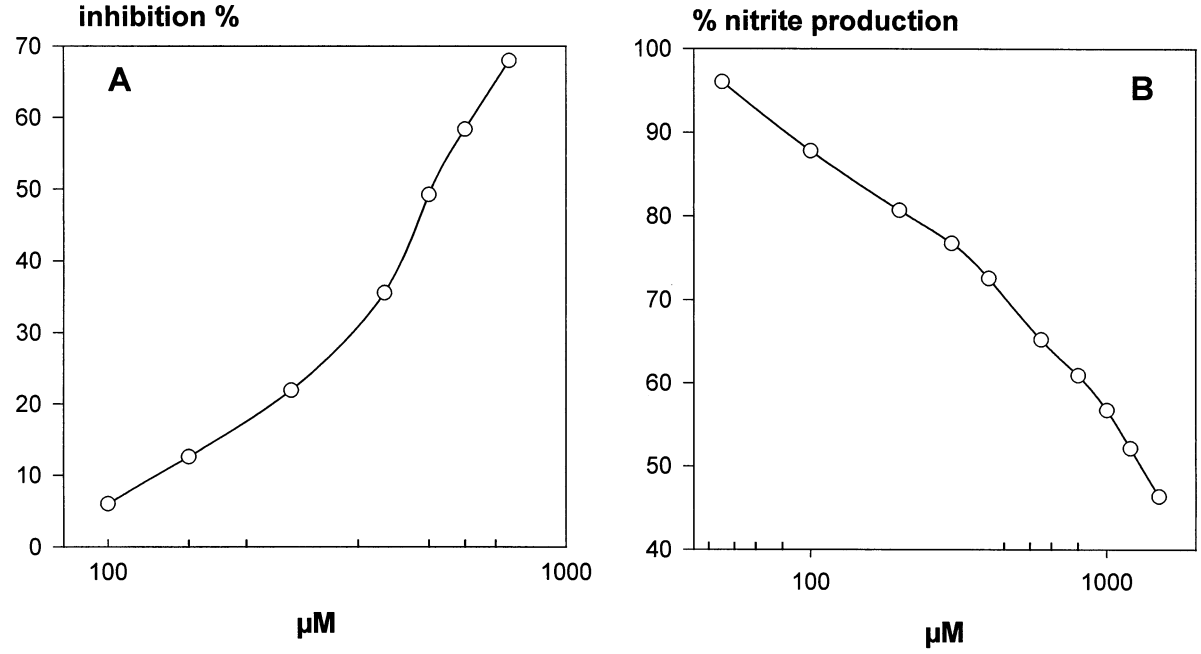

Fig. 1. Inhibition of UVC light-induced peroxidation on phosphatidylcholine vesicles (A) and percentage of nitrite production with respect to control (B) in presence of different concentrations of melatonin (○). Experiments were carried out as described in Section 2.

should be due, partially at least, to the drug scavenging activity against aqueous and lipophilic ROS, including NO (whose overproduction is stimulated by UV radiation especially in human skin keratinocytes) [29,30].

Aiming at better clarifying its photoprotective action, we exposed a 25 μM solution of melatonin to UVC irradiation and recorded the spectra at different times from the beginning of the incubation. Exposure of melatonin to UVC radiation caused marked changes in its absorption spectra and an excellent isosbestic point was obtained, indicative of new product formation (Fig. 2). These spectral modifications lead us to hypothesize that melatonin might provide its protective effect against UVC radiation-induced damage also by acting as a UV-absorbing screen. Interestingly, Nickel and Wohlrab [31] have recently demonstrated that melatonin protects human keratinocytes from UVB irradiation by light absorption.

However, in both 'in vitro' tests employed in our study, high doses of melatonin (in the 0.1–1.5 mM range) were needed to achieve a satisfactory antioxidant effect. This observation is in agreement with data obtained in other 'in vitro' experimental systems, showing that melatonin has only limited direct antioxidant activity [13–16]. Several papers point out the capability of melatonin to interact with lipidic membrane models [32]. Thus, in order to clarify if other receptor-independent mechanisms different from antioxidant/free radical scavenging activity might contribute to the protective effect (particularly the photoprotective one) of melatonin observed in 'in vivo' studies, we investigated, by DSC, the potential capability of melatonin to penetrate phospholipid bilayers and modify their fluidity characteristics.

The first series of experiments was carried out on DMPC multilamellar vesicles. Table 1 reports the effect of melatonin, at increasing drug molar fractions, on the thermodynamic parameters (T_m and ΔH) of DMPC MLVs; in this table, the shift of T_m values are axpressed also as $(\Delta T/T_m^0) \times 10^3$, where $\Delta T = T_m - T_m^0$, where T_m^0 is the transition temperature of pure DMPC MLVs and T_m is the transition temperature obtained at increasing drug molar fractions. The results of these experiments, analyzed by the Student's *t*-test to determine the significance

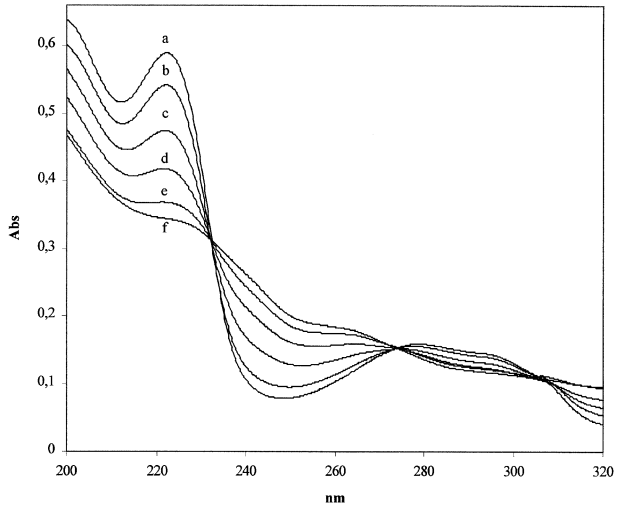

Fig. 2. Spectral changes of a water/ethanol (95/5, v/v) solution of 25 μM melatonin, exposed to UV irradiation. The spectra were registered at different times (a = 0 min; b = 15 min; c = 60 min; d = 130 min; e = 225 min; f = 345 min) from the beginning of the incubation.

Table 1
Effect of melatonin, at different molar fractions, on the thermodynamic parameters of aqueous dispersion of DMPC MLVs (data are expressed as mean ± SD of at least five calorimetric scans)

Molar fraction	T_m (°C)	$(\Delta T/T°_m)$ ($\times 10^3$)	ΔH (J/g)
0.000	23.6 ± 0.11	0.00	33.78 ± 1.30
0.015	23.3 ± 0.15	1.01	32.72 ± 1.45
0.030	23.0 ± 0.18	2.02	34.71 ± 1.22
0.045	22.7 ± 0.12	3.03	31.43 ± 1.38
0.060	22.4 ± 0.13	4.04	28.06 ± 1.41
0.090	22.1 ± 0.16	5.05	30.11 ± 1.52
0.120	21.4 ± 0.12	7.41	29.32 ± 1.56
0.180	20.4 ± 0.17	10.78	28.27 ± 1.47

($P < 0.05$), have evidenced a good capability of melatonin to interact with phospholipid bilayers. In fact, a statistically significant shift of T_m values associated to the gel to liquid-crystal phase transition toward lower temperatures was observed at increasing drug molar fractions ($T_m = 23.6 \pm 0.11$°C and 20.4 ± 0.17°C, respectively for 0.0 and 0.18 molar fraction). These changes are very likely due to the introduction of lipophilic molecules into the ordered structure of the lipidic bilayer.

As to permeation experiments, results of which are shown in Fig. 3, the transfer of melatonin in the LUVs is faster than that observed for MLVs, even if both values tend to the maximum values reachable (represented by the value obtained for the organic preparation of the MLV vesicles in the presence of melatonin). The fact that the permeation process took longer to reach equilibrium (maximal interaction) with the MLVs than with the LUVs reflects a slower transfer kinetic of melatonin through a multilayer system compared to a single bilayer.

Taken together, our present data allow us to emphasize two points: (1) melatonin is able to induce a fluidifying effect on lipidic bilayers; such alteration of biomembrane fluidity might act as a cooperative mechanism in the protective effect of melatonin against the peroxidative membrane damage; (2) as shown by permeation experiments, melatonin can cross biomembranes, so that it could protect intracellular components against peroxidative insult.

Concerning the first issue, the existence of a relationship between the rate of lipid peroxidation and membrane composition and fluidity is well known. For example, lipid bilayers in which membrane probes are relatively less mobile are, very likely, more prone to peroxidation than are fluid bilayers [33]. Moreover, several drugs, known to interact with biomembranes and affect their fluidity, proved to be able to prevent oxidant-induced damage; however, their protective effect was shown to be mediated by a direct interaction with biomembranes [34] as also by other mechanisms different from free-radical scavenging activity, metal chelation or xanthine oxidase inhibition. Finally, a higher transbilayer mobility and/or a stronger disorder of membrane lipids should make interaction of antioxidant molecules with lipid radicals more efficient [35,36]. In the light of data obtained in DSC experiments and in antioxidant tests, one could speculate that melatonin might operate not only with a specific radical scavenging mechanism (related to the structural features of the compound), but

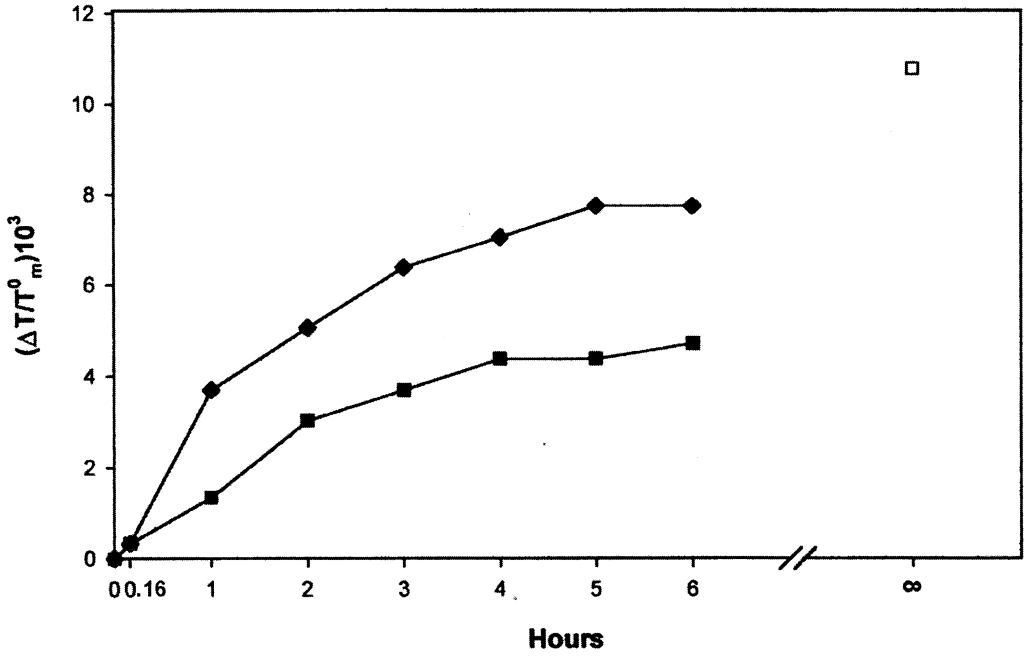

Fig. 3. Transitional temperature variations, expressed as $(\Delta T/T^0_m) \times 10^3$, of DMPC LUVs (full circles) and MLVs (full squares) in the presence of 0.18 molar ratio of melatonin for increasing incubation time (scan 0 = pure lipid; scan 1 = 10 min; scans 2–7 = successive 1 h-isotherm period at 37°C). The value of scan *T* (empty square) refers to samples prepared for direct interaction between melatonin and MLVs, by organic solvent dissolution and MLV preparation; these values are to be considered as the maximum interaction between compounds and vesicles.

also through a fluidifying effect on biomembranes. In accordance with this hypothesis, a correlation was suggested between the capability of melatonin to significantly prevent lipid peroxidation and to decrease microviscosity of platelet membranes [37].

As to the second point, besides to confirm that melatonin is able to cross biological barriers [38], the results of the permeation experiments allow us to speculate that melatonin may have access to intracellular sites critical for oxidative damage; thus it can protect not only cell membranes, but also intracellular components against peroxidative attack. In agreement with this hypothesis, melatonin delays hemoglobin denaturation and hemin release in erythrocytes under cumen hydroperoxide-induced oxidative stress, suggesting that the drug is able to permeate across red blood cell membrane [39]. Furthermore, melatonin is able to reduce hydrogen peroxide-induced DNA damage in U-937 cells [40].

In conclusion, these data provide a rationale for the protection that melatonin affords against UV radiation-induced damage. It is evident that several mechanisms (direct antioxidant effect, alteration of biomembrane fluidity, UV radiation absorption) appear to play a role in such photoprotection; moreover, the capability of melatonin to permeate across the lipidic bilayer may also contribute to its protective effect. Finally, melatonin is claimed to be free of toxicity and side effects, although a moderate phototoxicity has been recently predicted by an 'in vivo' test carried out in guinea pigs [41]. Further studies are needed to investigate the safety of melatonin treatment in humans.

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