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Research Article

Antioxidant activity of pineal methoxyindoles on hepatocyte plasmatic membrane

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Running title: Antioxidant activity of methoxyindoles on cell membrane.

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Abstract

Antioxidant effect of several pineal derived molecules has been well documented. Here, the protective effects of 5-methoxytryptophol (5-MTOH) and 5-methoxyindol-3-acetic acid (5-MIAA) on hepatic cell membrane lipid peroxidation and cell membrane rigidity induced by $FeCl_3$ plus ascorbic acid have been systemically investigated. The membrane fluidity was evaluated by fluorescence spectroscopy, malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations and carbonyl groups of protein were measured as the parameters of lipid and protein damage, respectively. Results showed that oxidative stress increased membrane rigidity, MDA and 4-HDA concentrations as well as carbonyl content in a concentration-dependent manner. 5-MTOH, but not 5-MIAA, significantly attenuated these oxidative indecies. In absence of oxidative stress, none of these methoxyindoleamines modified the content of MDA, 4-HDA or carbonylation. However 5-MIAA at its highest concentration slightly modified membrane fluidity. The results suggest that structural modification of C3 in the methoxyindoleamine, that is, the carboxyl group replaced by hydroxyl group in this site could improve the ability of 5-methoxyindoleamine derivatives to preserve membrane fluidity of cells which are under oxidative stress.

Keywords: 5-methoxytryptophol; 5-methoxyindol-3-acetic acid; membrane fluidity; lipid peroxidation; protein oxidation; antioxidant; free radical; melatonin; oxidative stress.

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1. NTRODUCTION

Melatonin, 5-methoxytryptophol (5-MTOH), and 5-methoxyindole-3-acetic acid (5-MIAA) are produced in the pineal gland from the essential amino acid tryptophan. The pathway of these methoxyindoles takes place in the pineal gland and also in other sites including retina (1), iris (2), lacrimal gland (3), bone marrow (4), gastrointestinal (5-7), ovary (8), and immune system (9). Melatonin, an unique methoxyindole, is a well known modulator of circadian rhythms and seasonal reproduction in photoperiodic reproductive mammals. Nevertheless, it is also classified as a potent free radical scavenger to protect

nervous system from oxidative stress. It plays a pivotal role in protection of hydroxyl radical (•OH)-induced carcinogenesis and neurodegeneration (10, 11), and preserves nucleic acids, lipids, proteins, and membrane fluidity disturbance caused by oxidative stress (12, 13). Antioxidant properties of melatonin are well documented in the *in vivo* and *in vitro* studies and several of its methoxyl metabolites including cyclic 3-hydroxylmelatonin, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine (AMK) also are potent oxidative stress inhibitors like their parental molecule, melatonin (14, 15). Thus, the antioxidant and free radical scavenging activity of additional two pineal gland derivatives, 5-MTOH and 5-MIAA, are investigated in the current study. These 5-methoxyindoleamines are the products of monoamine oxidase-B (MAO-B). Their antioxidative activities are examined for their protective effects on isolated hepatic cell membrane prooxidation. The membrane oxidative damage is induced by the combines $FeCl_3$ plus ascorbic acid (16-18). Exposure of membranes to this combination leads to lipid and membranal protein oxidation. The peroxidation of lipids further impairs the function of proteins and finally jeopardizes the physicochemical properties of membranes, including its fluidity (19,20).

Similar to melatonin which was isolated from bovine pineal gland by Lerner *et al*. in 1958 (21), several years later, the 5-MTOH and 5-MIAA were also found in pineal gland and their chemical structures were also characterized by researchers (22). Biosynthesis of 5-MTOH and 5-MIAA can be catalyzed by several enzymes including monoamine oxidase, alcohol dehydrogenase, and hydroxyindole-O-methyltransferase (HIOMT). Their precursors can be, serotonin or 5- methoxytryptamine, respectively (11,23) (Fig. 1).

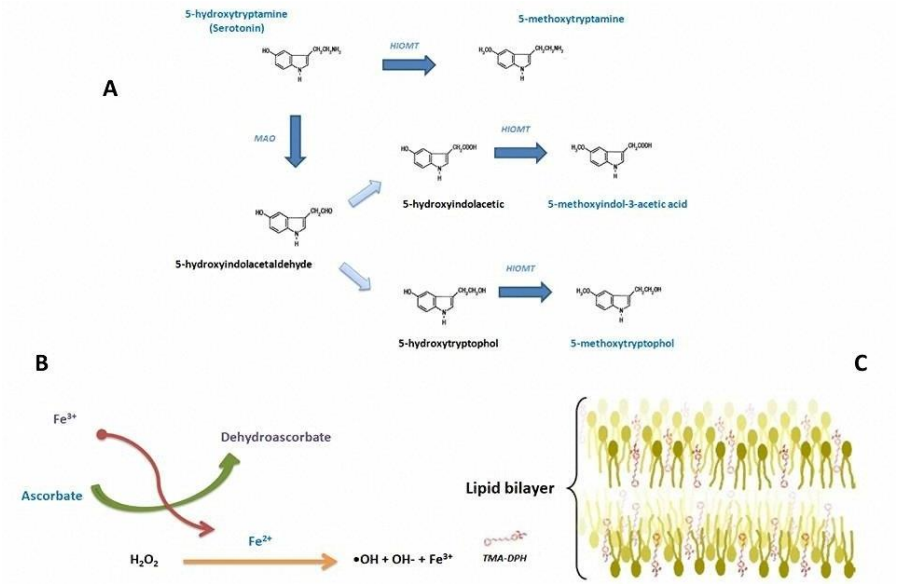

Fig. 1: Illustrations of biosynthetic pathways of the HIOMT and MIAA as well as Fenton reaction and TMA-DPH location in membrane.

A: Biosynthetic pathwys of methoxyindoleamines, 5-methoxyindol-3-acetic acid (5-MIAA) and 5-methoxytriptophol (5-MTOH). MAO: Monoamine oxidase; HIOMT: Hydroxyindole-O-methyltransferase. B: Role of iron and ascorbic acid in the Fenton reaction. H_2O_2: hydrogen peroxide; •OH: hydroxyl radical; Fe: iron. C: Location of TMA-DPH among the bilayer phospholipids. TMA-DPH, (1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatriene p-Toluenesulfonate).

In pineal gland, 5-MTOH (24-31) and 5-MIAA (32) show their highest concentration during daytime and low concentration at night, an opposite daily rhythm to melatonin. 5-MTOH seems to have less activity than melatonin in reproductive physiology tested in several animal species including humans, however; it is involved in the modulation of some aspects of puberty and gonadal function (33, 34). In contrast to melatonin, 5-MTOH plasma levels have shown significant differences with age and sex. For example, its level increases in boys and reduces in girls after 8 years old. It can be used as a biomarker of the different chronobiology in the pubertal development in boys and girls (31). In addition, melatonin and 5-MTOH, produced an equipotent and dose-dependent effects to reduce blood pressure in rats when both are administered through the jugular vein of the animals, however; this is not the case in 5-MIAA (35).

Cell membrane requires an optimal fluidity state for its proper functionality. The molecular components of membrane including lipids and proteins are susceptible to structural and functional changes because they are subjected to the continuous free radical attack which leads to cell damage and aging (36). On the other hand, free radical attack causes the oxidation of amino acid residue functional groups, and cross-linking and oxidation of the protein backbone, resulting in a modification of the structure, including the fragmentation of proteins (37).

L-ascorbic acid (vitamin C) is considered as antioxidant, but at high concentrations it also functions as prooxidant, especially in the presence of transition metals such as iron which acts as a reducing agent (Fig. 1) to facilitate the generation of free radicals by the Fenton reaction (11, 38-41). This combination is frequently used as *in vitro* free radical generator to study the deleterious effects of ferrous iron exposure on the hepatocyte cell membrane.

Because of biological membranes are composed by lipids and proteins, the antioxidant properties of 5-MTOH and 5-MIAA against oxidative damage of lipids (lipid peroxidation) and proteins (carbonylation) are evaluated in isolated cell membranes of hepatocytes of rat. The Fenton reaction system of 0.1 mM $FeCl_3$ plus 0.1 mM ascorbic acid was used to generate free radicals and this method is widely accepted to induce oxidative stress (42,43). Levels of malondialdehyde (MDA), 4-hidroxyalkenals (4-HDA), and carbonylation of the proteins were measured as an index of oxidative damage to lipids and proteins, respectively; and the membrane fluidity, as a functional index of cell membrane damage, was also evaluated.

2. MATERIALS AND METHODS

2.1. Chemicals.

All chemicals and reagents used were of the purest available quality obtained from comercial sources. 5-MTOH, 5-MIAA, $FeCl_3$, ascorbic acid, ethylenediaminetetraacetic acid disodium (EDTA-Na_2), Tris(hydroxymethyl)aminomethane (TRIS), and 4-(2- hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), were purchased from Sigma-Aldrich (Madrid-Spain). 1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5 hexatriene-*p*-toluene-sulfonate (TMA-DPH) was obtained from Molecular Probes (Eugene, OR). Lipid peroxidation was measured with the LPO assay Bioxytech LPO-586 assay kit (Portland, OR). TMA-DPH was diluted in tetrahydrofuran (THF) and water. THF concentration in the incubation volume was 0.4%. 5-MTOH, $FeCl_3$, and ascorbic acid were diluted in Tris-HCl 50 mM (pH 7.4). 5-MIAA was diluted in methanol and incubation buffer (2% volume/volume). Other reagents were dissolved in incubation buffer. All solutions were prepared fresh just prior to use.

2.2. Animals and isolation of cell membranes.

The animal handling procedures were strictly accordance with the recommendations of the European Economic Community Committee (86/609/CEE) for the care and use of laboratory animals. 20 Sprague-Dawley male rats, weighing 210–225 g were purchased from Harlan-Ibérica S.A. (Barcelona-Spain) and acclimated (light:dark cycle: 12:12, room temperature 22±2°C) for two weeks before being sacrificed. Rats were anesthetized with thiopental (50 mg/kg) and subjected to intracardiac perfussion with cold saline solution (0.9% NaCl). The livers were quickly removed, washed in saline and homogenized 1:10 weight/volume in 140 mM KCl/20 mM HEPES buffer (pH 7.4) using a motor-driven Teflon homogenizer. Membranes were isolated following the protocol described by Millán-Plano *et al.* (43), briefly, the resulting suspension was centrifuged at 1,000×*g* for 10 min at 4°C to remove the nuclei and cellular debris. The supernatant was centrifuged at 50,000×*g* for 20 min at 4°C and the resulting pellet was resuspended in the buffer and centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant and buffy-coat were removed, homogenized and then recentrifuged at 50,000×*g* for 20 min at 4°C. The final pellet was washed and resuspended 1:1 w/v, and frozen at −80°C until assay.

2.3.Oxidative Model and Peroxidation of membranes.

The induction of oxidative stress to cell membranes was performed by using a free radical generator system based on the models proposed by Wong *et al.* (44) and Sahu & Washington (40), that is, the combination of Fe^{+3} plus ascorbic acid in which Fe^{+3} is reduced to Fe^{+2} by ascorbic acid to generate •OH via Fenton reaction. The concentrations of $FeCl_3$ and ascorbic acid are 0.1 mM, respectively. At the end, the reaction was stopped by adding 2 mM EDTA into the mixture.

First, the kinetics with time reagarding the membrane fluidity, MDA, 4-HDA, and protein carbonylation with/without Fe^{+3} plus ascorbic were established. The hepatocyte membrane was incubated in Tris-HCl 50 mM (pH=7.4) buffer at a constant temperature of 37°C. The samples were collected and measured at the time periods of 0, 10, 20, 30, 60 and 120 min, respectively. The suitable incubation time was selected at 120 min based on the study of the kinetics. Then, the subsequent set of experiments were conducted to incubate 0.5 mg/mL membrane protein in Tris-HCl 50 mM (pH=7.4) buffer in a water bath with shaking at 37°C for 120 min with or without 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, respectively and in the absence or presence of the 5-MTOH or 5-MIAA (0.1, 0.3, 1.0, 3.0 and 5.0 mM, respectively). Membrane fluidity, MDA+4-HDA, and carbonyl groups were measured immediately after thes incubations.

2.4.Analytical membrane fluidity, MDA+4-HDA and carbonyl group content.

Fluidity of the hepatic cell membranes was monitored according to the method of Yu *et al*. (20), based on incorporation of a marker, TMA-DPH, into the bilayer as a fluorescent probe (Fig.1). 0.5 mg protein/mL of hepatic cell membranes were re-suspended in Tris 50 mM (3 mL final volume) with TMA-DPH (66.7 nM). After mixing vigorously, the membrane suspensions were incubated at 37°C for 30 min. Fluorescence measurements were carried out in a Perkin-Elmer LS-55 Luminescence Spectrometer, equipped with a circulatory water bath to maintain a temperature of 22±0.1°C. Excitation and emission wavelengths of 360 nm and 430 nm were used, respectively. The emission intensity

of vertically polarized light was detected by an analyzer oriented parallel (I_{VV}) or perpendicular (I_{VH}) to the excitation plane. A correction factor for the optical system, G, was used. Polarization (P) was calculated according to the equation (a):

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}$$

Since an inverse relationship exists between membrane fluidity and polarization (13,19,20,45,46); membrane fluidity was expressed as 1/P and it was calculated from triplicate determinations. Protein concentrations were determined employing the method of Bradford (47) using bovine serum albumin as standard.

Measurements of MDA+4-HDA concentrations were used as an index of lipid peroxidation of the hepatic cell membranes (48,49). Levels of these products were measured using the Bioxytech LPO-586 kit following the instructions of the manufacturer. In this assay, MDA+4-HDA react with 1-methyl-2-phenylindole yielding a chromophore with a peak of maximum absorbance at 586 nm; 1,1,3,3 Tetramethoxypropane was used as standard. Results are expressed as nmol MDA+4-HDA per mg of membrane protein.

Carbonyl group content, as index of oxidative protein damage, were measured using the reaction with 2,4-dinitrophenylhydrazine (DNPH), yielding a stable complex according to the method described by Levine et al. with slight modifications (50-52). Briefly, the membranes of hepatocytes were added into Tris-HCl 50 mM buffer with DNPH 10 mM, and the mixture was incubated at 37 °C for 1 h. Aliquots of 50% ice-cold trichloroacetic acid was added to the mixture. The samples were placed on ice for 10 min, and centrifugated at 3,000 × g for 10 min, the pellets obtained were washed three times with ethanol/ethyl acetate (1:1, volume/volume). The final pellets were dissolved in 6 M guanidine and incubated at 37°C for 15 min. After centrifugation at 12,000 × g for 10 min, the absorbance of the supernatants was measured spectrophotometrically at 375 nm in the ultraviolet spectrum. Guanidine solution was used as a blank. Protein carbonyl groups were estimated by using the molar absorption coefficient of DNPH (ε=22.000 M^{-1} cm^{-1}), and its concentration was expressed as nmol carbonyl groups/mg protein.

2.5. Statistical analysis

Results were expressed as arithmetic means ± standard error of at least five independent experiments. Statistical analysis was performed via ANOVA and comparisons of the means of two groups were performed using a Student's t-test. Differences were accepted as being statistically significant when $p < 0.05$.

3. RESULTS

3.1. The kinetics of hepatocytic membranes oxidation with time.

Incubation of hepatocytic membranes in the absence of iron and ascorbic acid (control) did not modify any of the parameters including membrane fluidity, MDA + 4-HDA, and protein carbonylation. However, the addition of $FeCl_3$ and ascorbic acid significantly decreased membrane fluidity and increased the other two indicators of oxidative damage in a time-dependent manner (Fig. 2). The results showed that membrane fluidity decreased, and lipid peroxidation and protein carbonylation increased in a progressive manner during the 120 min of the incubation period. Statistically significant differences between control and treated groups wrer detected at the 20, 30, 60 and 120 minutes of time points, respectively. According to these results, an incubation time of 120 min was selected for the following experiments.

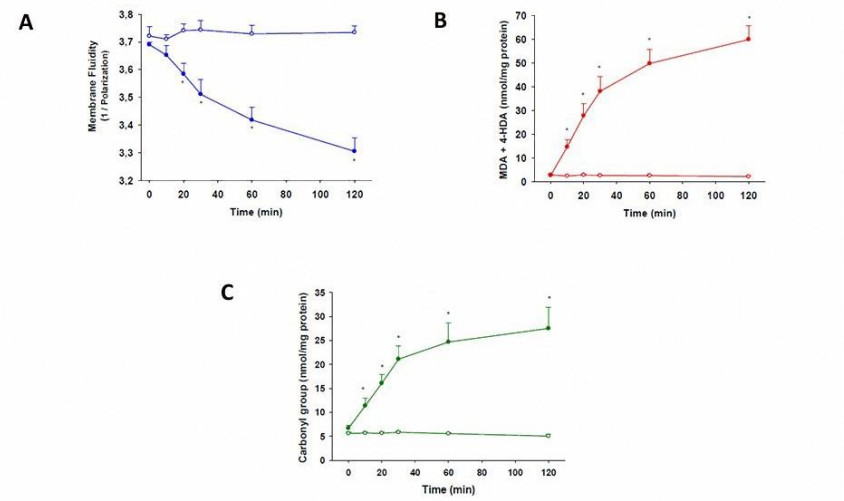

Fig. 2: The kinetics of oxidative alterations with time on hepatocytic membrane.

*A: Kinetics of membrane fluidity; B: lipid peroxidation C: formation of carbonyl moieties. Open cycle: control; solid cycle: with $FeCl_3$ 0.1 mM and ascorbic acid 0.1 mM. Results are given as means ± standard errors (N = 5). * $p \leq 0.05$ vs. time 0. MDA, malondialdehyde; 4-HDA, 4-hydroxyalkenals.*

3.2.Effects of 5-MTOH on oxidative membrane injury.

5-MTOH reduced the membrane rigidity induced by oxidative stress in a dose-dependent manner at all concentrations evaluated. The maximum efficiency was observed at its concentration of 1 mM. The lipid and protein oxidative damages induced by $FeCl_3$ and ascorbic were significantly inhibited by 5-MTOH at all concentrations studied. The minimal effective concentration of 5-MTOH that significantly reduced oxidative damage was 0.1 mM ($p \leq 0.05$) (Fig. 3). The required concentrations preventing oxidative damage to lipids and proteins by 50%, (IC_{50}) were 0.62 mM and 0.63 mM, respectively. For the membrane fluidity stabilization, it was difficult to calculate IC_{50} of 5-MTOH since its protective capacity on this index against oxidative damage was decrease when its concentration was >1 mM. Data are illustrated in Fig 3.

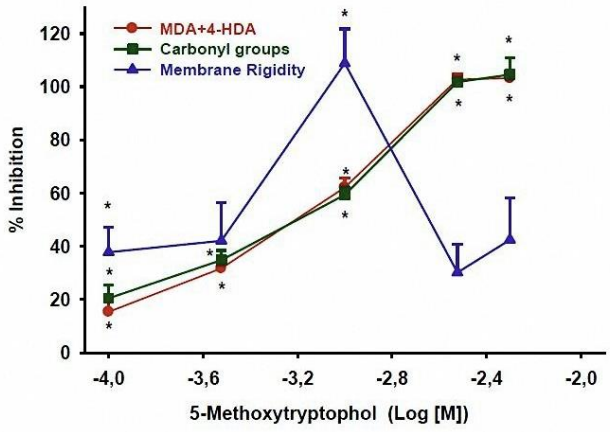

Figure 3. Effects of 5-MTOH on hepatocytic membrane oxidative damage.

*▲: cell membrane rigidity; ●: lipid peroxidation and ■: formation of protein carbonyl. The concentrations are expressed as the decimal logarithm of its molar concentration. Results are given as means ± standard errors (N = 5). * $p \leq 0.05$ compared to peroxidized membranes. MDA, malondialdehyde; 4-HDA, 4-hydroxyalkenals.*

3.3.Effects of 5-MIAA on oxidative membrane injury.

5-MIAA appeared no significant effects on the formation of MDA, 4-HDA and protein carbonylation induced by oxidative stress at the concentrations tested. The only observed effect of 5-MIAA was that it slightly increased the fluidity of the membrane at its highest concentration (5 mM); however, this increase exhibited statistical significance compared to the control group.

5-MIAA, at its high concentrations (3-5 mM), slightly reduced the protein oxidation but without significant difference compared to the control. It had no protective effect on lipid peroxidation induced by iron in its highest concentrations tested. Therefore, its IC_{50} could not be established in none of these parameters studied (Figure 4).

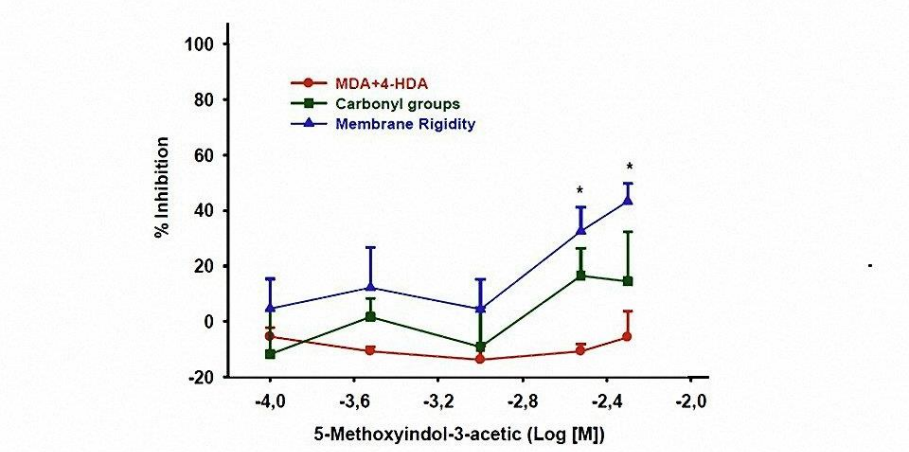

Figure 4. Effects of 5-MIAA on hepatocytic membrane oxidative damage.

*▲: Cell membrane rigidity; ●: lipid peroxidation and ■: formation of protein carbonyl. The concentrations are expressed as the decimal logarithm of its molar concentration. Results are given as means ± standard errors (N = 5). * $p \leq 0.05$ compared to peroxidized membranes. MDA, malondialdehyde; 4-HDA, 4-hydroxyalkenals.*

4. DISCUSSION

In the current study, the iron and ascorbic acid (0.1 mM, respectively) were used to induce the *in vitro* oxidative membrane damage which was also reported by others (40, 53). This model is based on that high level of ascorbic acid reduces ferric iron to the ferrus iron which can further reduce the H_2O_2 to form hydroxyl radical by the Fenton reaction (36). This model is widely accepted to incude oxidative stress in different experimental conditions (16, 17, 42, 43, 54). Lipid peroxidation is well known to decrease membrane fluidity in cell membranes (13, 19, 20, 54-56). The oxidative injury of the molecular components of cell membranes includes the loss of polyunsaturated fatty acids (57), the formation of cross-linking of lipid–lipid and lipid–protein moieties (57-59), production of MDA, 4-HDA and carbonyls group, protein fragmentation and aggregation, protein peroxide formation, and enzyme inactivation (60), and thus causes structural changes that modify the fluidity of the membrane. All of these are the basic mechanisms for membrane rigidity (13).

Optimal biological membrane fluidity is crucial for cell functions; even slight change of the membrane fluidy will cause aberrant function and pathology (44). Currently, there is a substantial interest in the identification of molecules that reduce membran rigidity and facilitate the membrane stability to resist oxidative stress. In previous studies, it has

been demonstrated that melatonin, a main pineal indoleamine, exhibits cellular protection and antioxidant activity (61). In addition, its structurally related molecules, including 5-MTOH, also stabilize cell membranes due to their powerful free radical scavenging activity and thus, prevents membrane lipid peroxidation (13, 19).

5-MIAA and 5-MTOH, both generated on the MAO pathway, are synthesized maily in pineal gland in the light phase because serotonin, its precursor, has highest concentrations during the day in the pineal gland. This rhythm is opposite to melatonin's rhythm in pineal gland (25, 28). The role of 5-MIAA in oxidative stress is not fully investigated yet. In the present study, 5-MIAA has failed to prevent the formation of MDA + 4-HDA, or oxidation of proteins induced by the combination of iron and ascorbic acid. In its highest concentrations (3 and 5 mM), it showed only a weak protective effect on the rigidity of hepatocytic membranes oxidative stress. These results suggest that 5-MIAA possesses little or no antioxidant activity to protect cell membranes. Our results were consistant with Wang *et al*. who reported that this molecule was unable to prevent the oxidation of low density lipoprotein serum using MDA formation as a biomarker (62). Its high concentration on membrane fluidity may be independent from an antioxidant activity. Ng *et al.*, also observed that several pineal indoles except 5-MIAA inhibit erythrocytic hemolysis and lipid peroxidation in brain homogenates and liver membranes and these molecules all potently suppressed superoxide radical formation (63). It is suggested that free radicals promote different pineal indoles to responses in different extents, probably depending on the structure of pineal indoles. It is known that the antioxidative effects of phenolic molecules are related to the number of hydroxyl groups and the conjugated system of these molecules (64). It has been also described the variable rates of reaction of this indoleamines with •OH and ROO• when the pH of the incubation medium was markedly acid (65). Wang *et al*. and our work were conducted at physiological pH. However, a recent publication showed that this indoleamine acted as an antioxidant against Fe^{3+} induced oxidative stress on synaptosomal membranes isolated from the rat brain (18). The inconsistence may be related to the different methods used and the different membrane models. It requires futher investigation.

In the absence of $FeCl_3$ and ascorbic acid, the formation of MDA + 4-HDA or carbonylation of proteins are very limited, and only the highest concentration of 5-MIAA (5 mM) slightly increased the hepatic membrane fluidity. This fact suggests that the increase in membrane fluidity may be due to a direct interaction of 5-MIAA with the phospholipids of the lipid bilayer and changes the dynamics of membrane. It should be noted that several other well-known molecules that act as antioxidants, such as tocopherols (66) and tamoxifen (67), also modify membrane fluidity in the absence of oxidative stress.

In contrast, under current experimental condition, the hydroxylated form of methoxyindoles, 5-MTOH, showed a remarkable antioxidant effect on lipids and proteins in a concentration-dependent manner in all concentrations tested. A significant protective effect of this indoleamine on isolated cell membranes from rat liver was observed at concentrations of 0.1-1 mM including to reduce the membrane rigidity in the presence of iron and ascorbic acid. Nevertheless, there were some publications to report this methoxyindoleamine to reduce free radical-mediated damage and its role as a scavenger against the highly reactive •OH (42, 45). Our data are in agreement with previous reports in which 5-MTOH acts as an antioxidant. Its rates of reaction with •OH and ROO• in the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) model were 0.20×10^9 and 0.45×10^9 $mol \cdot l^{-1} \cdot s^{-1}$, respectively (65). 5-MTOH also inhibited lipid peroxidation caused by iron and ascorbic acid in rat brain, liver and kidney homogenates, and hemolysis of rat erythrocytes (67). It detoxified H_2O_2 in rat brain homogenates (68) and suppressed oxidative damage of lipid and proteins in rat brain synaptosomal membranes treated with

iron (18), and also, protected microsomal liver membranes from damage by free radicals (19).

While ample evidence shows protective effects of melatonin *in vitro* and *in vivo* against free radical damage in a wide range of experimental models (8, 9). Ng *et al*. reported that 5-MTOH had higher antioxidative activity than that of melatonin (63). Results from our study show that 5-MTOH is a more potent antioxidant than 5-MIAA. This reinforces the important role of pineal gland derived methoxyindoles in protecting the biological membranes from oxidative damage. 5-MTOH only acts on the membrane which was under the oxidative stress. For example, in the absence of iron, it did not produce significant modifications on membrane fluidity or lipids and proteins. These results are similar to those reported by us on hepatic microsomes MDA + 4-HDA concentrations, and differ slightly in the assessment of the membrane fluidity in microsomes, where it decreased by exposure to 5-MTOH at the concentrations greater than 0.3 mM (20). Collectively, that 5-MTOH stabilizes hepatocytic membranes and prevents the rigidity induced by model-oxidative is related to its free radical scavenging activity (20). Since alteration of membrane fluidity modifies celular function, a main physiological function of 5-MTOH could be the stabilization of the membrane fluidity which is related to its ability to reduce lipid peroxidation and protein oxidation. This is important because during aging, membrane fluidity decreases as a consequence of lipid peroxidation (20).

5-MTOH differs from 5-MIAA in the substitution of a carboxyl acid by an alcoholic hydroxyl group in carbon attached to position 3 of the indole ring. This could mean that loss of acidity and incorporation of hydroxyl group would improve antioxidant behavior of the molecule, suggesting that protective effect of 5-MTOH is related to its electron donation or reductive capacity. Thus, a free radical scavenging mechanism is responsible for its stabilizing effect on hepatic cell membranes. Nevertheless, the mechanisms by which 5-MTOH modifies lipid dynamics in biological membranes are poorly understood. Finally, our results provide new information related to functional role of 5-MTOH deveried from pineal gland. It appears that 5-MTOH positively contributes to the overall antioxidant action of pineal gland. Studies should be warranted to further understand the molecular mechanism of antioxidant activity of this and other pineal derivatives.

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AUTHORSHIP

MC. Reyes-Gonzales, E. Esteban-Zubero, L. López-Pingarrón, MS. Soria and D. Pereboom contributed to acquisition of data, data analysis and interpretation, drafting of the manuscript and approval of the article. DX. Tan and RJ. Reiter contributed to the data analysis and interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article. J.J. García contributed to the conception and design of the investigation, acquisition of data, data analysis and interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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