

Relative efficacies of α -tocopherol, *N*-acetyl-serotonin, and melatonin in reducing non-enzymatic lipid peroxidation of rat testicular microsomes and mitochondria

Mariana Gavazza · Angel Catalá

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Abstract In this study, we examined the relative efficacies of α -tocopherol, *N*-acetyl-serotonin, and melatonin in reducing ascorbate- Fe^{2+} lipid peroxidation (LPO) of rat testicular microsomes and mitochondria. Special attention was paid to the changes produced on the highly polyunsaturated fatty acids (PUFAs) C20:4 n6 and C22:5 n6. The LPO of testicular microsomes or mitochondria produced a significant decrease of C20:4 n6 and C22:5 n6. Both long-chain PUFAs were protected when the antioxidants were incorporated either in microsomes or mitochondria. By comparison of the IC50 values obtained between α -tocopherol and both indolamines, it was observed that α -tocopherol was the most efficient antioxidant against the LPO induced by ascorbate- Fe^{2+} under experimental conditions in vitro, IC50 values from the inhibition of α -tocopherol on the chemiluminescence were higher in microsomes (0.14 mM) than in mitochondria (0.08 mM). The protective effect observed by α -tocopherol in rat testis mitochondria was higher compared with microsomes, associated with the higher amount of [C20:4 n6] + [C22:5

n6] in microsomes than that in mitochondria. Melatonin and *N*-acetyl-serotonin were more effective in inhibiting the LPO in mitochondria than that in microsomes. Thus, a concentration of 1 mM of both indolamines was sufficient to inhibit in approximately 70% of the light emission in mitochondria, whereas a greater dosage of 10 times (10 mM) was necessary to produce the same effect in microsomes. It is proposed that the vulnerability to LPO of rat testicular microsomes and mitochondria in the presence of both indolamines is different because of the different proportion of PUFAs in these organelles.

Keywords α -tocopherol · *N*-acetyl-serotonin · Melatonin · Lipid peroxidation · Rat testicular microsomes and mitochondria

Introduction

The content of fatty acids in testis is very high with a prevalence of polyunsaturated fatty acids (PUFAs), thus approximately 70% of the fatty acids located in rat testicular microsomes and mitochondria are polyunsaturated with a prevalence of arachidonic C20:4 n6 and docosapentaenoic C22:5 n6 acids [1] which renders these organelles very susceptible to lipid peroxidation (LPO). The most biologically active form of the vitamin E homologues is α -tocopherol, which is present in the membranes of cells and cellular organelles, where it plays an important role in the suppression of LPO. α -tocopherol accumulates at those sites within the cell where oxygen radical production is greatest and thus where is most required, for example, in the membranes of heavy mitochondria, light mitochondria, and endoplasmic reticulum. Therefore, protection against the peroxidation of membrane lipids by α -tocopherol depends

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Angel Catalá is Member of Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina.

M. Gavazza
Cátedra de Bioquímica, Facultad de Ciencias Veterinarias,
Universidad Nacional de La Plata, La Plata, Argentina

A. Catalá (✉)
Instituto de Investigaciones Físicoquímicas Teóricas y
Aplicadas, (INIFTA-CCT La Plata-CONICET), Facultad de
Ciencias Exactas, Universidad Nacional de La Plata,
La Plata, Argentina
e-mail: catala@inifta.unlp.edu.ar

on its incorporation into membranes and the extent to which the quantity of α -tocopherol is present in the membranes [2]. Damage to testicular long-chain fatty acids adversely affects their normal physiology and may lead to disturbances during the process of spermatogenesis [3]. In a very recent report, we have showed that melatonin (*N*-acetyl-5-methoxytryptamine), the main secretory product of the pineal gland, may act in “vivo” and in “vitro” as antioxidant protecting long-chain polyunsaturated fatty acids present in rat liver microsomes from the deleterious effect by a selective mechanism that reduces the loss of docosahexaenoic and arachidonic acids [4]. The predominant lipids of whole testis are phospholipids, with smaller amounts of acylglycerols (chiefly triacylglycerols) free and esterified cholesterol and even smaller amounts of gangliosides and sulfolipids [5]. The phospholipids of testis are characterized by extremely high proportions of long chain highly PUFAs with a prevalence of 4,7,10,13,16-docosapentaenoic acid, C22:5 n6 [6]. In addition to the saturated and unsaturated fatty acids commonly found in mammalian tissues, testicular lipids have been shown to be enriched with 20- and 22-carbon polyenes and to contain 24-carbon polyenes [7]. The efficient synthesis of C22:5 n6 may also partly explain why this is the major 22-carbon fatty acid in rat testis [8]. Many studies related with lipid chemistry and metabolism of testicular tissue has led to the suggestion that polyenoic acids, particularly C22:5 n6 have an important role in the process of spermatogenesis in the rat [7]. Karbownik et al. [9] have demonstrated that melatonin and related indoles at pharmacological concentrations protect against both the autoxidation of lipids and induced peroxidation of lipids in testis. In doing so, these agents would be expected to reduce testicular cancer that is initiated by products of LPO. *N*-acetyl-serotonin is the immediate precursor of melatonin in the metabolism of tryptophan in the pineal gland. Chemically, *N*-acetyl-serotonin only differs from melatonin in the substitution of a hydroxy group for the methoxy group in position 5 of the indole ring. Both indolamines are free radical scavengers and indirect antioxidants because of their stimulatory effect on antioxidative enzymes. Besides the pineal gland, melatonin is reported to be produced in a number of extrapineal sites, where it could act as an intracellular mediator or paracrine signal in addition to its endocrine effects. Using the reverse transcription-polymerase chain reaction (RT-PCR) method, Stefulj et al. [10] have determined the tissue-specific expression of mRNAs encoding two key enzymes of the melatonin biosynthesis: serotonin-*N*-acetyltransferase (NAT) and hydroxyindole-*O*-methyltransferase (HIOMT). At 35 cycles, only gut, testis, spinal cord, raphe nuclei, stomach fundus, and striatum yielded positive signals for both enzymes. The aim of the current study was to evaluate the relative efficacies of α -tocopherol, *N*-acetyl-serotonin,

and melatonin in reducing non-enzymatic LPO of rat testicular microsomes and mitochondria. Chemiluminescence and fatty acid composition of both organelles were used as an index of the oxidative destruction of lipids.

Materials and methods

Chemicals

α -Tocopherol, melatonin, *N*-acetyl-serotonin, butylated hydroxytoluene (BHT), and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, USA). Standards of fatty acids methyl esters were from Nu Chek Prep, Inc., Elysian, MN, USA. All other reagents and chemicals were of analytical grade from Sigma.

Animals and preparation of mitochondria and microsomes

Male wistar rats, 2-months old, weighing 200–250 g were used. Rats were maintained on a commercial standard pellet diet and tap water ad libitum. The use of the experimental animals has been approved by The University of La Plata-related authority. The diet contained 4% of total lipids with a fatty acid composition of 19.14% palmitic acid C16:0, 0.184% palmitoleic acid C16:1n-7, 4.10% stearic acid C18:0, 19.34% oleic acid C18:1 n-9, 51.53% linoleic acid C18:2 n-6, and 4.83% linolenic acid C18:3 n-3.

The rats were killed by cervical dislocation and testis rapidly removed. To prepare homogenates, testes were decapsulated, weighed, cut into small pieces, and washed extensively with 0.15 m NaCl. A homogenate of the tissue was prepared in solution A (0.25 m sucrose, 10 mm Tris-HCl pH 7.4, and PMSF 0.001 m), 3-ml of solution per gram of tissue, using the Potter-Elvehjem homogenizer. The homogenate was spun at 3000g, pellets were discarded, and then the supernatant was spun at 20,000g for 10 min to obtain testis mitochondria [11]. After centrifugation, 5 ml of the resultant supernatant was applied to a Sepharose column (1.6 × 12 cm) equilibrated and eluted with 10 mM Tris-HCl (pH 7.4), 0.01% NaN₃. The microsomal fraction appearing in the void volume (12–20 ml) was used and cytosol (30–40 ml) discarded. All operations were performed at 4°C. Microsomes that are of similar composition with regard to concentrations and activities of certain Microsomal enzymes to that obtained by ultracentrifugation can be prepared by Sepharose gel filtration chromatography of post mitochondrial supernatant. In experiments designed to study the interaction of microsomes with soluble proteins, these microsomes are preferable to those prepared by centrifugation as they are

not contaminated with cytosolic proteins [12]. Mitosomes and mitochondria were stored at -84°C and used within a week, after one cycle of freezing and thawing.

Non-enzymatic lipid peroxidation of mitochondria and microsomes

Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to mitochondrial or microsomal preparations [13]. Mitochondria and/or microsomes (1 mg of protein) were incubated at 37°C with 0.01 M phosphate buffer (pH 7.4), 0.4 mM ascorbate, final volume 1 ml. Phosphate buffer contained sufficient iron to provide the necessary ferrous or ferric iron for LPO (final concentration in the incubation mixture $2.15\ \mu\text{M}$) [14]. Stock solutions of α -tocopherol ($100\ \mu\text{mol}/\mu\text{l}$), *N*-acetyl-serotonin ($100\ \text{nmol}/\mu\text{l}$), and melatonin ($100\ \text{nmol}/\mu\text{l}$) were prepared in ethanol. Simultaneous assays with microsomes treated with ascorbate 0.4 mM and increased concentrations of α -tocopherol (0.031, 0.062, 0.125, 0.5, and 1 mM), melatonin (1, 2.5, 5, and 10 mM), or *N*-acetyl-serotonin (0.25, 0.5, 1, 2.5, 5, and 10 mM) or mitochondria treated with ascorbate 0.4 mM and increased concentrations of α -tocopherol (0.031, 0.062, 0.125, 0.5, and 1 mM), melatonin (0.031, 0.062, 0.125, 0.25, 0.5, and 1 mM), or *N*-acetyl-serotonin (0.031, 0.062, 0.125, 0.25, 0.5, and 1 mM) and microsomal and mitochondrial preparations which lacked ascorbate were carried out simultaneously. Membrane light emission was determined over a 180-min period; chemiluminescence was recorded as cpm every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. A maximal response was obtained between 90 and 120 min after the addition of ascorbate. Chemiluminescence was measured as counts per min in a liquid scintillation analyzer Packard 1900 TR.

Measurement of fatty acid composition

Rat testicular mitochondrial or microsomal lipids were extracted with chloroform/methanol (2:1 v/v containing 0.01% BHT as antioxidant) [15]. Fatty acids were transmethylated with 20% F3B in methanol at 60°C for 3 h. Fatty acid methyl esters were analyzed with a GC-14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a packed column (1.80 m \times 4 mm id) GP 10% DEGS-PS on 80/100 Supelcoport. Nitrogen was used as a carrier gas. The injector and detector temperatures were maintained at 250°C , the column temperature was held to 200°C during 60 min. The fatty acid methyl esters were identified by comparison of retention times with standard compounds. All compositions were expressed as% by area of total fatty acids.

Protein determination

Proteins were determined by the method of Lowry et al. [16] using BSA as standard.

Statistical analysis

Results were expressed as means \pm SD of three independent determinations. Data were evaluated statistically by one-way analysis of variance (ANOVA), Tukey test, and Student's *t*-test. Statistical significance at different *P*-values is indicated in each case.

Results

Inhibition of LPO (light emission) of rat testicular mitochondria and microsomes by α -tocopherol, *N*-acetyl-serotonin, and melatonin

The data for the time-course of light emission during LPO of rat testis microsomes or mitochondria exposed to ascorbate- Fe^{2+} is given in Fig. 1. In the absence of ascorbic acid, light emission was very low (not shown). Ascorbic acid added to this system caused an increase of chemiluminescence. A decrease of chemiluminescence (inhibition of LPO) was observed when the antioxidants were added to the incubation medium containing organelles. The inhibition of LPO (light emission) of rat testicular mitochondria or microsomes by α -tocopherol, *N*-acetyl-serotonin, and melatonin at 1 mM concentration exposed to ascorbate- Fe^{2+} is depicted in Fig. 1a–c, respectively.

The inhibitory effect of α -tocopherol, *N*-acetyl-serotonin, and melatonin at different concentrations on ascorbate- Fe^{2+} induced LPO of testis organelles are shown in Fig. 2. The 0% inhibition was calculated by subtracting the total cpm originated in the control without ascorbate from peroxidized organelles (with ascorbate). The IC₅₀ was determined by calculating the concentration of each antioxidant required to inhibit 50% of the light emission (LPO) in rat testis microsomes and mitochondria. By comparison of the values of IC₅₀ obtained between α -tocopherol and both indolamines (Table 1), it was observed that α -tocopherol was the most efficient antioxidant against the LPO induced by ascorbate- Fe^{2+} under experimental conditions in vitro in both organelles. Therefore, it was observed that this antioxidant was more efficient in mitochondria than in microsomes. In both organelles the maximal inhibition percent reached was 70%, with a concentration of 1 mM of α -tocopherol. It is important to note that both inhibition curves are coincident. The data show that IC₅₀ was higher in microsomes than in mitochondria

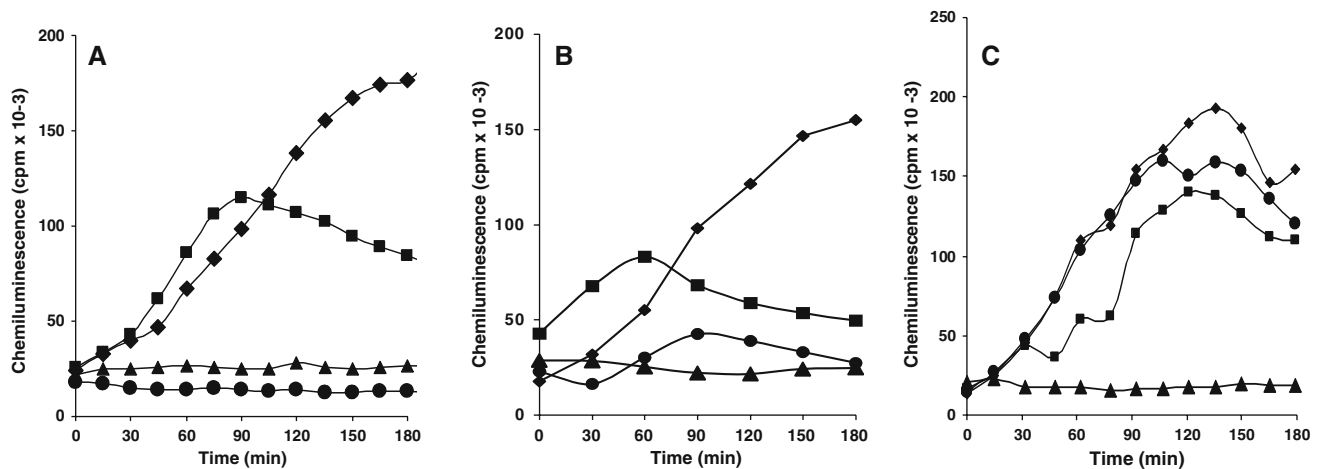


Fig. 1 Effect of antioxidants: (a) α -tocopherol, (b) *N*-acetyl-serotonin, and (c) melatonin, on lipid peroxidation of rat testicular microsomes and mitochondria. Microsomes without antioxidant

(■—■), plus 1.0 mM antioxidant (●—●), mitochondria without antioxidant (◆—◆), plus 1.0 mM antioxidant (▲—▲)

when both indolamines were present. In our experimental conditions *N*-acetyl-serotonin and melatonin was determined to be at about 18.0 and 7.4 times more potent in mitochondria than in microsomes.

Relative efficacies of α -tocopherol, *N*-acetyl-serotonin and melatonin in preserving peroxidizable fatty acids during LPO of rat testicular mitochondria and microsomes

Changes in the fatty acid profiles were used as an index of the oxidative damage to lipids. The long-chain fatty acids mainly affected during the LPO process was the PUFA's arachidonic (C20:4 n6) and docosapentaenoic (C22:5 n6) acids. The percentage of these fatty acids was approximately twice in microsomes (25.17 ± 3.40 – 26.98 ± 3.20) when compared to that in mitochondria (15.02 ± 2.10 – 13.71 ± 1.86), Table 2.

Table 1 Comparative inhibition of lipid peroxidation in rat testis by different antioxidants (Chemiluminescence)

Antioxidant	Mitochondria IC50	Microsomes IC50
α -Tocopherol	0.078	0.144
Melatonin	0.67	4.98
<i>N</i> -acetyl-serotonin	0.25	4.50

The IC50 (mM) was determined by calculating the concentration of each antioxidant required to inhibit 50% of the light emission (lipid peroxidation) in rat testis microsomes and mitochondria

The fatty acid composition of microsomes and mitochondria isolated from rat testis was substantially modified when subjected to non-enzymatic LPO with a considerable decrease of arachidonic acid C20:4 n6 and docosapentaenoic acid C22:5 n6. The degradation of both polyunsaturated fatty acids was preserved when α -tocopherol, *N*-acetyl-serotonin,

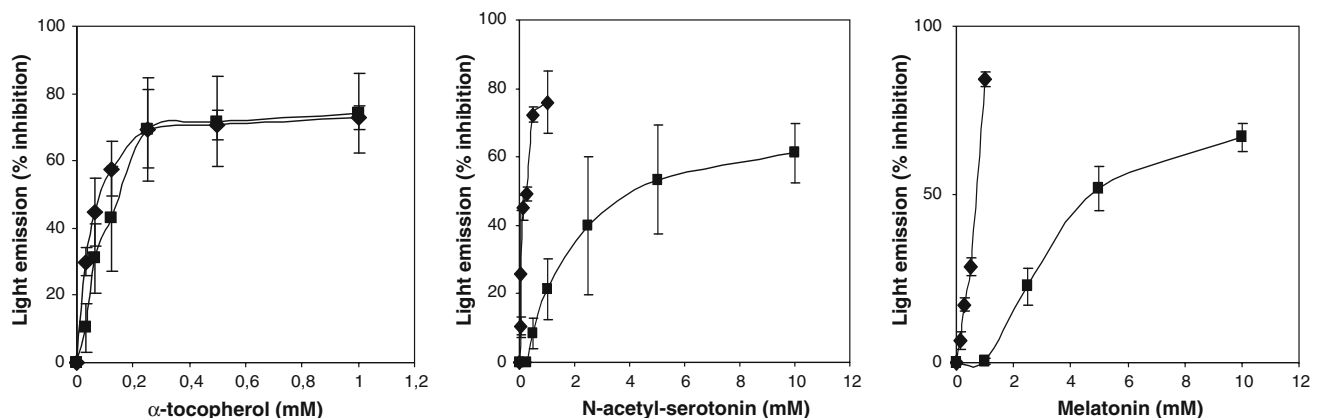


Fig. 2 Inhibition of lipid peroxidation (light emission) of rat testis mitochondria (◆) and microsomes (■) by *N*-acetyl-serotonin, melatonin, and α -tocopherol. Each point is the mean \pm s.e.m. of three independent experiments

Table 2 Protections during lipid peroxidation of arachidonic and docosapentaenoic acids by α -tocopherol, *N*-acetyl-serotonin, or melatonin in rat testicular mitochondria and microsomes

	Mitochondria		Microsomes	
	C20:4 n6	C22:5 n6	C20:4 n6	C22:5 n6
Control	15.02 \pm 2.10	13.71 \pm 1.86	25.17 \pm 3.40	26.98 \pm 3.20
Peroxidized	5.18 \pm 1.90**	2.85 \pm 0.80**	13.37 \pm 1.30*	15.56 \pm 3.60*
Peroxidized + 1.0 mM antioxidant				
α -Tocopherol	12.84 \pm 1.93	9.84 \pm 1.67	23.82 \pm 2.60	24.84 \pm 3.37
<i>N</i> -acetyl-serotonin	11.22 \pm 0.40	9.66 \pm 0.60	4.41 \pm 1.24**	4.79 \pm 1.15**
Melatonin	15.70 \pm 2.10	14.55 \pm 1.70	12.78 \pm 2.30*	14.60 \pm 4.40*

Data shown are given in percentages of total fatty acid content and are mean \pm s.d. of three separated experiments. Control without ascorbic acid. Statistically significant differences between control vs. peroxidized and control versus peroxidized + 1.0 mM (antioxidant) are indicated by * $P < 0.05$ and ** $P < 0.005$ using analysis of variance (ANOVA)

or melatonin was present in the LPO system. Our results clearly demonstrate that preservation of both PUFAs by *N*-acetyl-serotonin and melatonin at a concentration 1 mM, during ascorbate-Fe²⁺ LPO was more effective in rat testicular mitochondria than in microsomes. A higher concentration (10 mM) of both indolamines was necessary in microsomes to protect both polyunsaturated fatty acids from the deleterious process. Therefore it was clearly demonstrated that α -tocopherol at a concentration 1 mM, was the most efficient antioxidant against the LPO induced by ascorbate-Fe²⁺ under experimental conditions in vitro in both organelles.

Discussion

Understanding the consequences of frequent oxidative stress in the male reproductive environment is gaining extensive attention [17–20]. Free radical production and LPO are known to be important mediators in testis physiology [21]. However, elevated levels of reactive oxygen species (ROS) in testis in vivo can result in altered tissue physiology, or induce oxidative damage to DNA, which is of potential danger to reproduction. Recent findings have led to the suggestion that oxidative stress can play a vital role in the etiology of male infertility [22–24].

Certain enzymes play an important role in antioxidant defense, to maintain viable reproductive ability; a protective mechanism against oxidative stress is of importance [25].

Testicular tissue contains a range of antioxidants that can inactivate free radicals; these antioxidant defenses include enzymes SOD, GSH-Px, glutathione reductase (GSH-Rd), and CAT, which convert free radicals or reactive oxygen intermediates to non-radical products. SOD and GSH-Px are major enzymes that scavenge harmful ROS in male reproductive organs [25]. Melatonin is an important component of the antioxidant profile of many

tissues and cells. Reiter et al. [26] documented that melatonin is an efficient scavenger of OH[•], peroxyxynitrite anion (ONOO⁻), O₂, nitric oxide radical (NO[•]), and peroxy radicals. Moreover, it enhances the ability of cells to resist oxidative damage by inhibiting the pro-oxidant nitric oxide synthase [27]. The degree of LPO has been assessed according to MDA formation, which has been routinely used as an index of LPO.

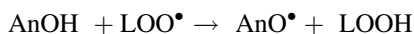
The major lipid soluble antioxidant is vitamin E. Some aqueous antioxidants include ascorbate, glutathione, etc. The pineal product melatonin is a powerful antioxidant in many different tissues. It was reported that melatonin prevented LPO. Although the mechanism of action of melatonin is not fully understood, some investigators believe that antioxidant properties of melatonin are mediated by its ability to directly scavenge reactive oxygen species [28]. Melatonin was proposed to directly trap superoxide anion and hydroxyl radical in both in vitro and in vivo system [29]. The action of melatonin may involve other mechanisms and stabilizing cell membranes [30] and stimulating antioxidative enzymes [31].

In the present study we have tried to contribute to the explanation of the LPO process in testis, by exploring the relative efficacies of α -tocopherol, melatonin, and *N*-acetyl-serotonin, the latter molecule being of importance because of its double role in the metabolic pathway of melatonin, both as direct precursor, but also as the probable product of in vivo back transformation: in fact, it has been recently shown that NAS is produced and released by skin cultured cells [32]. Furthermore, a detailed metabolic pathway (via an *O*-demethylase) that can convert melatonin back to *N*-acetyl-serotonin has been demonstrated in various mammalian (including human) tissues.

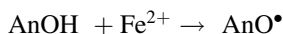
Many in vivo and in vitro studies have claimed a powerful antioxidant ability of MLT, even more effective than vitamin E, especially as a quencher of peroxy radicals [33–35]. However, knowledge of this important property is far from being consolidated, both from the biochemical

point of view and in its physiological and possible therapeutic implications.

The differential antioxidative effects of melatonin and *N*-acetyl-serotonin may depend on the unique chemical structures of these molecules. Antioxidants which possess a reactive hydroxyl group at position 5 of the indole ring are often hydrogen donors, thereby reducing free radicals that promote radical chain reactions and effectively reduce LPO in vitro [30].



At the same time, however, they may autoxidize in the presence of transition metals and increase the formation of primary radicals.



NAS possess a hydroxyl group and appear to be effective against LPO in vitro [30], melatonin do not possess a hydroxyl group.

Siu et al. [36] have compared, in studies in vitro, the efficacy of melatonin, *N*-acetyl-serotonin, and pinoline with vitamin E. They found that vitamin E was the more effective antioxidant followed by *N*-acetyl-serotonin, pinoline, and melatonin. Daniels et al. [37] have reported the effect of melatonin and serotonin as free radical scavengers. These authors have postulated that serotonin exerts its free radical scavenging action in the aqueous phase or at the water membrane interphase, while melatonin protects membrane phospholipids against free radical attack by acting within the lipid bilayer of the membranes, and would thus be ineffective as free radical scavenger on the surface of membranes. *N*-acetyl-serotonin has the OH-group bond to C5 and is more water-soluble and it would scavenge free radicals within the aqueous environment.

To evaluate and compare the role of these three molecules (α -tocopherol, *N*-acetyl-serotonin, and melatonin) in reducing non-enzymatic LPO of rat testicular microsomes and mitochondria, we have chosen as a test system, chemiluminescence, and fatty acid composition as an index to decrease polyunsaturated fatty acids.

The protective effect observed by *N*-acetyl-serotonin and melatonin in rat testis mitochondria was higher than that observed in microsomes which could be explained if we consider that the sum of C20:4 n6 + C22:5 n6 in testis microsomes is twofold than that present in mitochondria.

The data show that IC50 values were higher in microsomes than in mitochondria when both indolamines were present. In our experimental conditions *N*-acetyl-serotonin and melatonin was determined to be at about 18.0 and 7.4 times more potent in mitochondria than in microsomes. The concentrations of each antioxidant required to inhibit 50% of the lipid damage (IC50) in mitochondria were in mitochondria (0.078, 0.25, and 0.67 mM) and in

microsomes (0.144, 4.50, and 4.98 mM) for α -tocopherol, *N*-acetyl-serotonin, and melatonin, respectively. Statistical analysis of the data showed that α -tocopherol treatment always yielded a lower level of chemiluminescence than did the same concentration of melatonin or *N*-acetyl-serotonin.

In summary, our studies have allowed to characterize in detail the changes that arachidonic and docosapentaenoic acids present in microsomes and mitochondria of rat testis experience during non-enzymatic lipid peroxidation and the protective effect exerted by the antioxidants: α -tocopherol, melatonin, and *N*-acetyl-serotonin.

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