MELATONIN'S EFFECTS ON FREE RADICAL LEVELS IN RAT HIPPOCAMPUS, MEASURED BY ELECTRONIC PARAMAGNETIC RESONANCE.

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Summary

Associated with aging is the idea of neurodegeneratives diseases that are linked to an increase of free radicals. Melatonin has been postulated as the principal free radical scavenger in the organism. The present work analyzes the chronically applied melatonin effects on the free radical levels in the hippocampus of Wistar rats. It was determined by means of Electronic Paramagnetic Resonance that in melatonin-rats as well as control rats there was an increase in the hippocampal free radical levels in a directly proportional fashion with the age of the animals. However, the melatonin-rats exhibited lower free radical levels than the control rats, the most significant difference at 14-month-old. The analyzed frozen-dried tissue exhibited a stable free radical. On the control samples was spotted a radical with characteristics (g value and magnetic field) that look like similar to an ascorbyl radical which was not observed in the melatonin-treated animals. It suggests that melatonin could produce a"neuronal protection" on the hippocampal cells by reducing the free radicals on this central nervous structure.

Keywords: Scavenger, hippocampal area, indolic hormone, antioxidant, aging.

Introduction

During aging, the brain suffers both morphological and functional modifications. The basis of these changes are unidentified but many studies implicate reactive oxygen species (ROS) and mitochondrial damage (1). Antioxidants, although possibly involved in protection against various age related diseases, do not seem to control the rate of aging (2).Melatonin has protective actions in various models of oxidative stress (3, 4). This hormone is a potent free radical scavenger and an indirect antioxidant (5, 6, 7). Melatonin is an effective hydroxyl radical (°OH) scavenger (8) and detoxifies other reactive oxygen and nitrogen species including single oxygen [1O2] (9, 10, 11, 12). Moreover, melatonin stimulates the activities of enzymes that metabolize reactive species (13, 14). Melatonin is easily absorbed when it is administered by any route and crosses all the morphological barriers including the blood-brain barrier. It seems to enter every part of the cells where it can prevent oxidative damage and preserve mitochondrial function (5). Melatonin also has been postulated as a molecule able of retarding brain aging (12, 15). This pineal indol significantly declines with aging, for this reason, it has been suggested that age related neurodegenerative diseases, as Alzheimer's disease, may be related to melatonin deficiency (16).

Considering these data, the aim of this work was to investigate the "protective" role of melatonin by reducing the free radical levels measured at hippocampus in several ages in Wistar rats.

Method

Animals

Male Wistar rats, weighing 35 ± 5 g, were used in this study. All animals were kept under the same laboratory conditions of temperature (25 ± 2 °C) and light (12:12 h light/dark cycle) and allowed free access to laboratory chow and tap water. Animals were randomly assigned to control or melatonin treatment group. They were injected everyday at the same hour. The used animals were manipulate in a such a way to avoid pain, following the NIH norms (Publication No.80-23).

Chemicals

Melatonin was purchase form Sigma-Aldrich (M-5250, mol. Wt. 232.3) and polyethylene glycol 200 was purchase from Fluka, 88446.

Experimental protocol.

Rats were divided into two groups. The control group (12 rats) received polyethylene glycol (1%, melatonin's vehicle) injections. The experimental group (12 rats) was given melatonin injections (1mg/kg body weight). The i.p. injections were applied for several months (6, 10 and 14). The animals were sacrificed 24 hours after the last injection and the brains were quickly removed isolating a small piece of hippocampus (0.7g), frozen at –70 °C and lyophilized for seven hours for posterior EPR analysis.

Free radicals determination by Electronic Paramagnetic Resonance (EPR).

The measurements of EPR were made in quartz tube at room temperature with a Jeol JES-TE300 spectrometer operating at X-Band fashions at 100 KHz modulation frequency and a cylindrical cavity in the mode TE011. The external calibration of the magnetic field was made with a precision gaussmeter, Jeol ES-FC5 and microwave frequency with a frequency counter 5350B HP. The spectrometer settings for all spectra were as follows: center field, 335.5 mT; microwave power 1 mW, microwave frequency 9.43 GHz; sweep width, ± 2.5 mT, modulation width, 0.125 mT; time constant, 0.3 s; amplitude, 160; sweep time 120 s; accumulation, 3 scans. The measurement of the concentration was made by double integration of the signal using 4-hidroxy tempo as standard. Spectral acquisition and manipulations were performed using the program ESPRIT-382, v1.916. The EPR spectra were recorded as a first derivation and the main parameter such as g- factor values were calculated according to Weil (17).

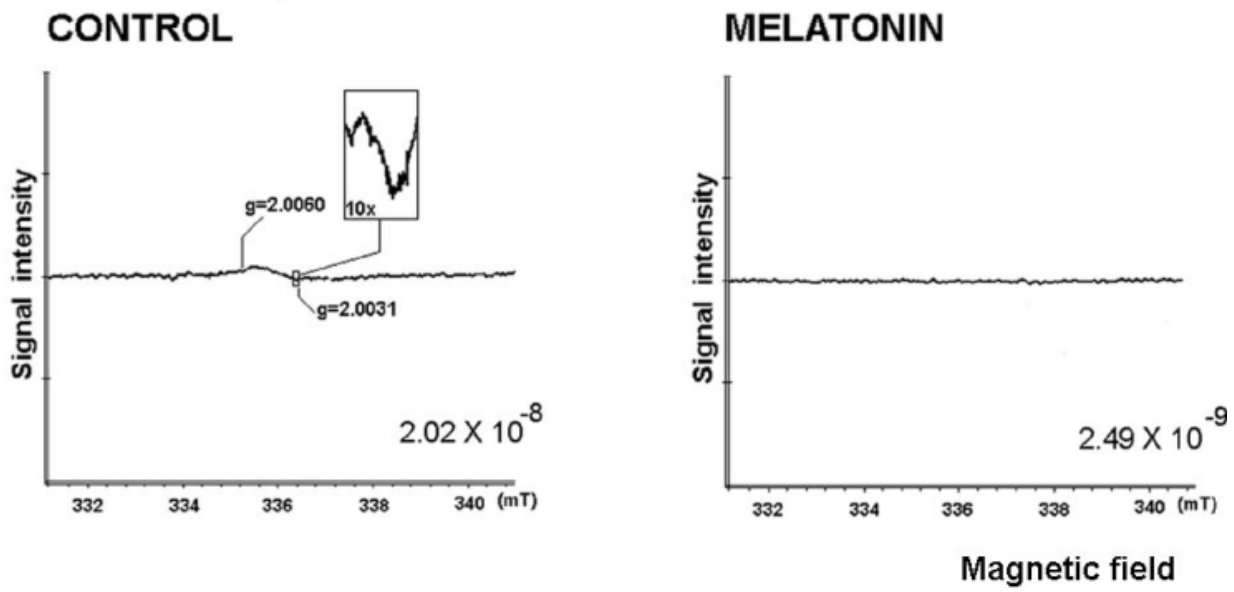
Statistical analysis.

All data was evaluated with two factors ANOVA method, the free radical levels were analyzed according with the age and with the treatment of each rat.

Results

The data we presented is the result of four control and four melatonin rats at 6, 10 and 14 months-old. On the lyophilized hippocampal tissue a stable free radical form with an axial signal with $g^{\perp} = 2.0061 \pm 0.0004$ and $g_{\parallel} = 2.0031 \pm 0.0004$ and $\Delta H = 0.87 \pm 0.01$ mT was detected.

The figures 1-3 are some examples of the EPR spectra with an anisotropic signal with $g_{\parallel} < g_{\perp}$. In some control spectra it was possible to identify a doublet form signal with ah=0.18 mT y g= 2.0058. This was not observed in any melatonin spectra. When a simulated spectrum was compared with the doublet form signal founded, the results showed an equivalent to an ascorbyl radical (18). Figure 4.The results showed a significant difference $p<0.001$ in the hippocampal free radical levels when both the age and treatment are interrelate but only at



fourteen months of age. Figure 5

Figure1. Presents the spectrum of free radicals in the hippocampus from six months-old rats. The control spectrum exhibits a clear wave assigned to free radicals with a $2.02x10^{-8}$ concentration while in the melatonin spectrum is not visible this wave, however there are free radicals to a concentration of $2.49x\ 10^{-9}$, this data can result from noise and the real radicals accord to the calculation made by the program.

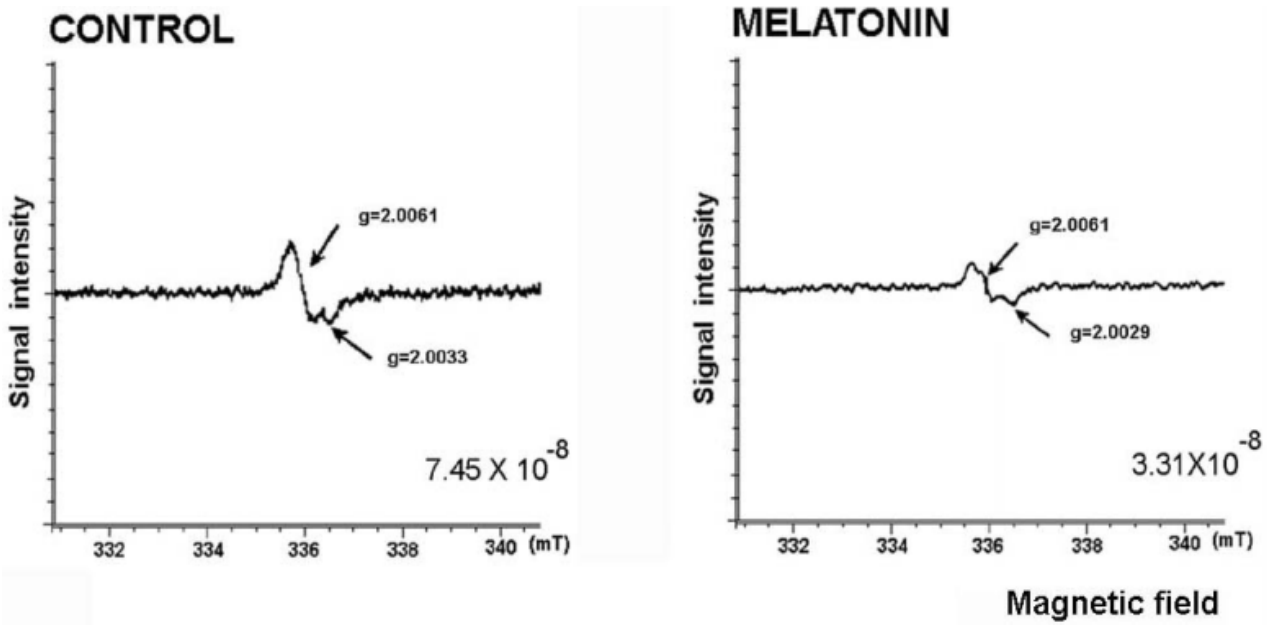


Figure.2. Shows the spectra of free radicals in the hippocampus from ten months-old rats. The control spectrum exhibits a clear wave in the free radicals field with a 7.45×10^{-8} concentration while in the melatonin spectrum the intensity of this wave is lower, $3.31x\ 10^{-8}$.

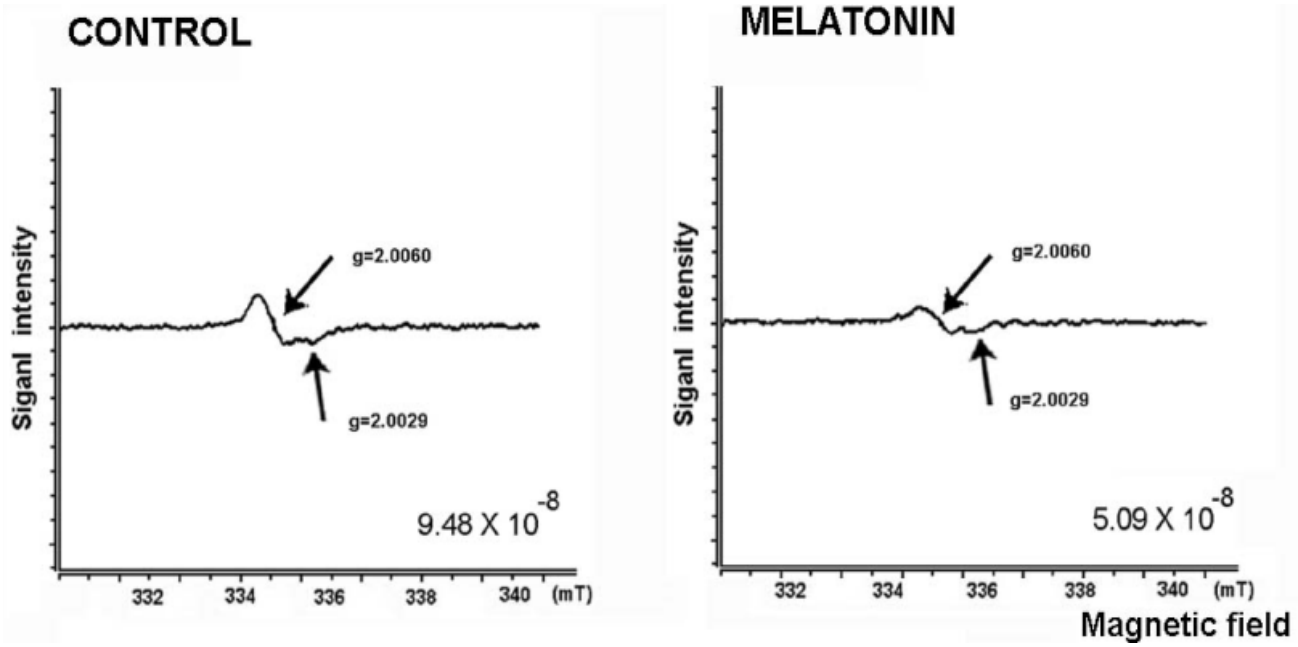


Figure.3. Shows the spectra of free radicals in the hippocampus from fourteen month-old rats. The control spectrum exhibits a clear wave in the free radicals field with a 9.48 x 10^{-8} concentration while in the melatonin spectrum the wave intensity is lower 5.09 x 10^{-8}. Both the g value as the signal wide suggest the presence of an organic free radical.

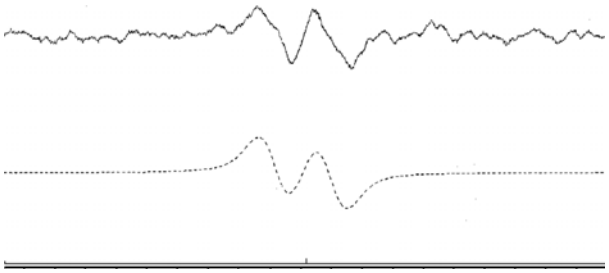


Figure.4.The solid line show the EPR spectra founded in some of lyophilized tissue of control hippocampus, and the dotted line show the simulated spectra with g= 2.0058 , aH = 0.18 mT. This signal was assigned to ascorbyl radical.

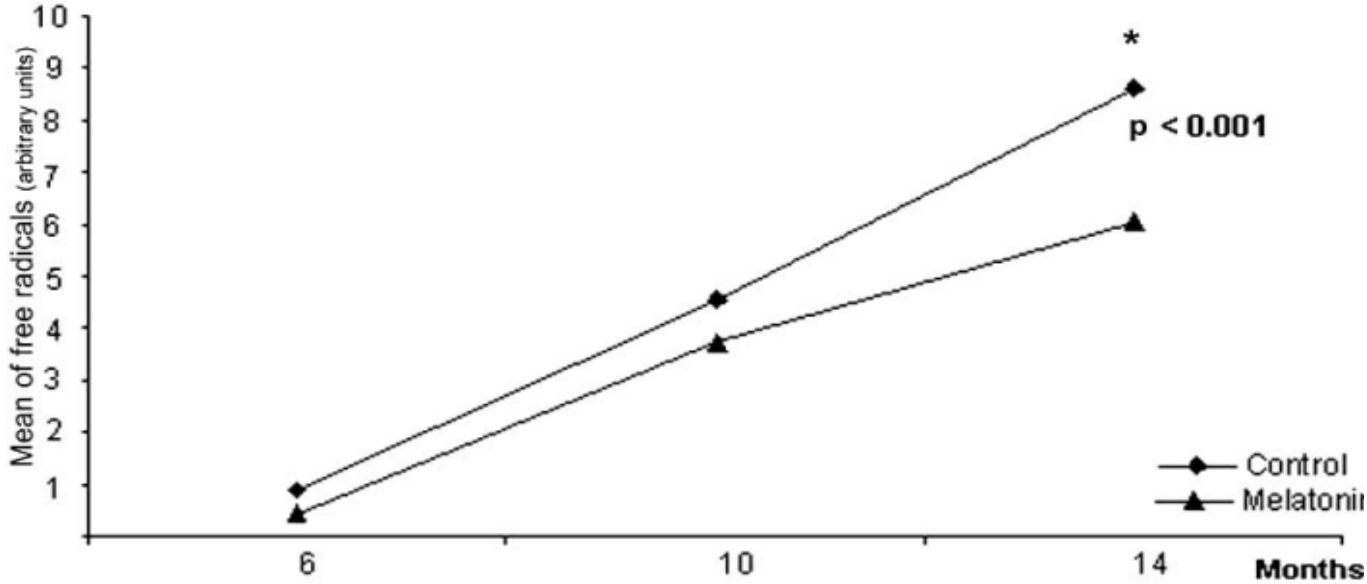


Figure.5.The curves show the treated rats at 6, 10 and 14 months of age. In both groups there is a directly proportional increase in the radical amount with the age. When the control rats are compared with the melatonin ones there is an important reduction in such free radicals concentration, however only at 14 months the difference was statistically significant. P<0.001

Discussion

It's generally known that the body needs oxygen to create energy. Unfortunately, the products resultants of the energy producing chemical reactions sometimes are toxic. These products, naturally produced by aerobic metabolism can damage cell's proteins, carbohydrates, lipids and DNA. There is considerable evidence that point to age-related syndromes can be the result of cellular changes in the CNS, which are, in part, attributable to an imbalance between pro-oxidant and antioxidant factors, particularly, the brain is a highly susceptible organ to the oxidative damage (19).Free radicals produce alterations in cytoskeletal organization, neurite damage and neural loss. This disorder involves loss of axons and dendrites of neurons in the CNS and, consequently, disruption of synaptic connectivity, causing memory and motor deficits, conditions present in many aging neuropathologies (18, 20).

This work shows that free radical levels in both control and melatonin animals are increasing in a parallel way with age, however it shows that the chronically applied melatonin in a doses of 1 mg/Kg elicited a decrement in the quantity of the free radicals in the hippocampus. The data obtained from the six months animals did not exhibit good enough statistical differences neither did the ten months old animals, the significant effects were seen at fourteen months of age with a $p < 0.001$. These data suggests that with the passage of time the free radicals resulting of the cellular metabolism accumulate, it is possible that when the rats are young the antioxidative mechanism are running in an appropriate way and the melatonin is not needful at all, however, when the animals become older there is probably a reduction of the antioxidative mechanisms, that is why the melatonin scavenger effect is evident as registered in the melatonin-rats, while these mechanism in the control rats are evidently decreased. It is interesting to show that the ten months old control animals had higher free radical levels than the melatonin rats and at this point it seems that the concentration difference is beginning to be significant, is the biochemical aging starting?

The EPR data indicate that the g and the magnetic field are related to the free radical signal. In all the spectra there was found a stable free radical form which does not belong to the short life free radicals species (example:hydroxyl radical •OH, Singlet oxygen 1O2, Nitric oxide NO•) because this radical was present even when the tissue was frozen-dried. Mallard (21) showed that on lyophilized tissue it is possible to detect stable free radicals as the semiquinones. Furthermore, it has been suggested that the melatonin is able to reduce the semiquinones formation, reacting with hydrogen peroxide to lower the production of reactive species (22). In the quinone redox cycle the hydrogen peroxide participates (23). If melatonin reduces this hydrogen peroxide, then there will be less quinone radical in animals treated. For this reason, the melatonin-animals exhibit less stable free radical (semiquinone?) levels than the control rats and their spectra have a small signal.

Also, it is possible that melatonin is activating the putative MT3 receptor, which is postulated as the homologue of the human quinone reductase2, (24) and because of this the melatonin could decrease the semiquinone formation reducing the free radical signal observed in the melatonin animals.The doublet form signal found in the control animals is within the magnetic field of an ascorbyl radical, and it was not found in any melatonin tissue sample. This suggests that melatonin could be reducing this radical or, if it is present it is covered by another radical signal coupled. The data presented suggests that melatonin could be protecting the Central nervous system from oxidative effect generated by aging by reducing free radicals.

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