**Neuroprotection of Melatonin in Lipopolysaccharide-induced Alzheimer's Disease in rats**

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**Abstract:**

**Background:** Increased levels of the amyloid β protein (Aβ), oxidative damage, mitochondrial dysfunction and possibly down-regulation of cholinergic neurons are early neuropathological markers of Alzheimer's disease (AD). Melatonin; the neurohormone, may act as a protective agent in disease conditions such as Parkinson's, Alzheimer’s, aging, sepsis and other disorders including ischemia/reperfusion. However, the protective mechanisms of melatonin against AD and its relation to autophagy is still essentially unknown.

**Aim:** The present study was designed to evaluate the effects of early melatonin supplementation on cognitive deficits and brain biochemical changes in lipopolysaccharide (LPS)- induced Alzheimer's disease in adult male rats.

**Materials and Methods:** The study was conducted on 24 male adult albino rats. The experimental animals were randomly divided into 3 groups, 8 rats each. Group I: control treated with vehicle, Group II (AD); lipopolysaccharide(LPS) treated (0.8 mg/kg i.p. single dose), Group III: melatonin +LPS co-treatment , melatonin (10 mg/kg i.p. at 17:00 hr. for 6 weeks - 5days / week) and LPS (0.8 mg/kg i.p. once). Melatonin administration was started just after the single i.p. injection of LPS. Cognitive function tests were done by T-maze during the last week of treatment. At the end of the experimental period, all rats were sacrificed and brains were excised for the determination of the level of peroxynitrite, cardiolipin(CL) and choline acetyl transferase-activity (ChAT) and the gene expression of Amyloid β precursor protein (APP) and Beclin-1.

**Results:** It was observed that melatonin treated rats showed a significant improvement of cognitive function in the form of increased percentage of alternations in T-maze, indicating improved working memory. Furthermore melatonin treatment was shown to reduce the expression of APP gene and enhance Beclin-1 gene expression significantly compared with the AD rats. In addition, there was significant increase in CL and ChAT activity together with reduced peroxynitrite in melatonin treated rats.

**Conclusion:**

Our results indicate that neuroprotection by early melatonin supplementation is partly related to reducing amyloid formation and protection of the cholinergic system. We suggest that the neuroprotective effects of melatonin are mediated by its antioxidant capacity with the improvement of mitochondrial function, increase activity of ChAT and induction of autophagy . Early rational melatonin interventions may be one of the most promising strategies in the development of approaches to prevent or retard Aβ-mediated disease progression

**Keywords:** β-amyloid Precursor Protein, Alzheimer's disease; autophagy; choline acetyltransferase; melatonin.
Introduction

Alzheimer’s disease (AD) is an age-associated neurodegenerative disease and characterized by progressive loss of cognition and other neurobehavioral manifestations. Pathological hallmarks of AD include extracellular senile plaques (SP), mainly consisting of β-amyloid (Aβ), and intracellular neurofibrillary tangles (NFTs), mainly composed of abnormally hyperphosphorylated tau, a microtubule-associated protein [1] and a profound loss of basal forebrain cholinergic neurons that innervate the hippocampus and the neocortex [2].

In spite of a large number of studies undertaken, the etiology of AD is largely unknown. Many mechanisms have been proposed, including genetic predispositions (e.g. expression levels and subforms of presenilins (PS) and Apolipoprotein (Apo) E, inflammatory processes associated with cytokine releasing, oxidative stress and neurotoxicity by metal ions [3–7]. Amyloid precursor protein (APP) is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. Its primary function is not known, though it has been implicated as a regulator of synapse formation and neural plasticity and mediating adult neurogenesis. APP is best known and most commonly studied as the precursor molecule whose proteolysis generates amyloid beta (Aβ), a 39- to 42-amino acid peptide whose amyloid fibrillar form is the primary component of amyloid plaques found in the brains of Alzheimer's disease patients [8-10].

Oxidative stress may contribute to the etiology of AD by dysregulation of APP metabolism. Overexpression of the APP gene could result in an increased secretion of neurotoxic Aβ peptides, while preventing the overexpression might be protective. Studer et al. [11] reported that the antioxidant N-Acetyl-L-Cystein (NAC) downregulates APP gene transcription in human neuroblastoma cells. The effect is reversible when cells are returned to NAC free medium. These results open up new possibilities for the development of therapeutic agents that intervene at the transcriptional level.

It had been reported that reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by the mitochondria are involved in the pathophysiology of AD [12].

As mitochondrion-derived ROS result in enhanced amyloidogenic amyloid precursor protein processing, and that Aβ itself leads to mitochondrial dysfunction and increased ROS levels and a vicious cycle is triggered that contributes to the pathogenesis of AD [13]. Increasing evidence suggests that oxidative damage to proteins and other macromolecules is a salient feature of the pathology of Alzheimer’s disease.

Peroxynitrite, a powerful oxidant produced from the reaction of superoxide with nitric oxide, is involved in Alzheimer’s disease. Peroxynitrite is a source of hydroxyl radical-like reactivity, and it directly oxidizes proteins and other macromolecules [14].

Convincing evidence indicates that Aβ can mediate neurotoxicity through a complex series of interactions that involves increasing free radicals, raising intracellular calcium concentrations, and even triggering apoptosis and impairments in cellular energy metabolism [15]. It become
clear that beta-amyloid represents a potent molecular target for pharmacological manipulation to perhaps prevent the onset and progression of Alzheimer's disease[16].

Melatonin (N-acetyl-5-methoxytryptamine), a tryptophan metabolite, is synthesized mainly by the pineal gland. Melatonin has a number of physiological functions, including regulating circadian rhythms, clearing free radicals, improving immunity [17]. Generally it inhibits the oxidation of biomolecules by different mechanisms [18]. It is generally accepted that melatonin deficit is closely related to aging and age-related diseases. Decreased melatonin in serum and cerebrospinal fluid (CSF) and the loss of melatonin diurnal rhythm are observed in AD patients [19].

In mammals, melatonin exerts some of its functions through two specific high-affinity membrane receptors, melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2). Decreased MT2 immunoreactivity and increased MT1 immunoreactivity have been reported in the hippocampus of AD patients [20]. Increased pineal monoamine oxidase A (MAOA) activity might contribute to the reduced pineal melatonin production in AD [21].

It has been considered that melatonin enters freely into most of cells by passive diffusion through the cell membrane[22]. It is selectively taken up by mitochondrial membranes, a function not shared by other antioxidants. Melatonin is known to retard aging and to inhibit the lethal effects of septic shock or I/R lesions by maintaining respiratory complex activities, electron transport chain, and ATP production in mitochondria [23].

Cardiolipin, a phospholipid located at the level of inner mitochondrial membrane, is required for several mitochondrial bioenergetic processes as well as in mitochondrial-dependent steps of apoptosis. Alterations in cardiolipin structure, content, and acyl chain composition have been associated with mitochondrial dysfunction in various tissues under a variety of pathophysiological conditions [24]. Melatonin was reported to protect the mitochondria from oxidative damage by preventing cardiolipin oxidation and this may explain, at least in part, the beneficial effect of this molecule in mitochondrial physiology [24].

Autophagy is regarded as a major protein degradation pathway that is critical for stress-induced and constitutive protein turnover as well as the maintenance of normal cellular homeostasis [25]. Conditional deletion of ATGs in the central nervous system of mice results in loss of autophagic activity and the concomitant accumulation of ubiquitinated proteins, leading to significant neurodegeneration[26]. Rapamycin, an mTOR inhibitor which also functions as a tumor suppressor and an autophagy inducer, is able to reduce Aβ and Tau pathology in animal models of AD[27]. In addition, a small-molecule enhancer of rapamycin (SMER) has recently been proven to promote the clearance of Aβ and APP-CTFs from cultured cells. Further suggesting that autophagy could be a legitimate pharmacological target of AD[28].

The present study was designed to investigate the possible relation between the level of expression of amyloid precursor protein (APP) and mitochondrial oxidative damage in the development and progression of AD and their relation to autophagy. In addition to evaluate the short-term influence of melatonin treatment on cognitive deficits and brain biochemical changes in lipopolysaccharide (LPS)-induced Alzheimer's disease in adult male rats.
Materials and Methods

Experimental animals:

24 adult male albino rats with initial body weight range between 150-200g were used in this study. The rats were supplied by the Animal House Unit of Kasr Al-Aini- Faculty of Medicine, Cairo University. They were housed in cages in the animal house of Physiology Department belonging to the same Faculty. They were kept at room temperature and normal dark-light cycles. They had free access to laboratory rat chow and tap water throughout the experimental protocol for 6 weeks. The experiments were carried out during November-December 2013.

Animal Groups:

Group I: control treated with vehicle, Group II: (LPS-AD), LPS(from Sigma) treated (0.8 mg/kg i.p.once)[29]. Group III: (MT); melatonin +LPS co-treatment group, melatonin :from Sigma, (10 mg/kg i.p. [30] at 17:00 hr. [31] for 6 weeks - 5days / week) and LPS (0.8 mg/kg i.p. once). Melatonin administration was started just after the single i.p. injection of LPS. Cognitive function tests were performed for all rats by T-maze. These experiments were carried out between 09:00 and 12:00 h. during the last week of experimental period. At the end of the experimental period, all rats were sacrificed and brains were excised for the determination of the level of Peroxynitrite, Cardiolipin( CL) and Choline Acetyl Transferase-activity (ChAT) and the gene expression of Amyloid β precursor protein (APP) and Beclin-1.

Cognitive function studies in T-maze:

The T-maze was used to test spontaneous alternation behavior. These tests are based on the innate interest of rodents to explore a new environment [32].

The spontaneous alternation task was conducted using a manual T-maze apparatus. It was constructed of white wooden runways (Fig.1A).

The maze was settled so that the central partition was in place and all guillotine doors are raised. The animal was placed in the start area, and was allowed to choose a goal arm ;side arm (Fig.1B). It was confined in the chosen arm by quietly sliding the door down. After 30 s, the central partition was removed, and then the animal was guided back into the start box for the next trial. The guillotine door of the sample arm was raised (the other arm door should already be up) and a choice phase (the animal’s choice between the sampled and unsampled arms) was allowed. The trials were run closely together, like a continuous trials procedure[32]. Each group were subjected to a spontaneous alternation protocol for 1day (one session consisting of 5 pairs of trials), after 3 days of habituation according to the criteria of Deacon & Rawlins [32] during the last week of the experimental period.

In these trials, the number of spontaneous alternations for each rat in each session was measured which is a good reflection of working memory [33,34]. The correct response was counted for each rat and the% of alternations then was calculated from the following formula:

\[ \% \text{ of alternations in T-maze} = \frac{\text{No. of spontaneous alternations/total arm entries} - 1}{1} \times 100 \]

This formula was deduced from Maurice et al. [35].
Also, the time from the animal being placed in the start area until the selected criterion (whole body plus tail tip on goal arm) is reached (s) for each rat in each trial was measured by stopwatch. The mean was calculated for each rat; it was expressed as: the time consumed in T-maze, and it is an index for the cognitive performance as well, according to Shoji et al. [33].

**Figure 1(A)**: T-maze plan. Dimensions are in cm, Walls= 30 cm (according to Deacon & Rawlins [32])

**Figure 1(B)**: T-maze spontaneous alternation task.

*Biochemical measurements:*

**Detection of peroxynitrite**

Peroxynitrite was assessed by electron paramagnetic resonance according to [36], briefly, for electron paramagnetic resonance (EPR) measurements, brain tissue homogenate were incubated in 200 ul phosphate buffer saline containing 120 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). After 15 min, buffer were removed and analyzed by EPR. EPR spectra were recorded on a Bruker ECS106 spectrometer. The DMPO-OH signal generated from brain tissue homogenate was quantified by comparison with 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy standard.

**Detection of cardiolipin**

Cardiolipin was measured by elisa kit supplied by (DIAGNOSTIC AUTOMATION, INC.USA) according to manufacturer’s instruction [37].

**Measurement of activities of choline acetyltransferase (ChAT)**

Measurement of ChAT activities were performed by spectrophotometric method [38], briefly, 10ul of 0.5M buffer (sodium phosphate), 6 mM acetyl coenzyme A, 1 M choline chloride, 0.78 methyl neostigmine sulphate 3M sodium chloride, 1.1 mM EDTA, 20 ul of 1 M creatine HCL and 120ul distilled water to make total volume of 200ul, the mixed solution were incubated at 37°C for 5 min. the supernatant (0.1ml) containing enzyme solution was added mixed and kept at 37°C for 20 min, mixture were boiled for 5 min to stop the reaction then added with 0.4ml of distilled water. After cooling the denatured protein was removed by centrifuge at 15000rpm for 10 min then 10 ul of 3M dithiopyridine was added to 0.5 ml of supernatant. The absorbance of enzyme activity was read at 320 nm and the level of enzyme activity was calculated from standard curve.
Detection of APP and beclin gene expression using quantitative real time PCR (qRT–PCR):

Total RNA was extracted from brain tissue using the SV Total RNA extraction kit (Promega, Madison USA). Concentration of RNA was quantified by measuring absorbance at 260 nm, and RNA integrity was verified on a agarose gel electrophoresis stained with ethidium bromide. Reverse transcription (RT) was performed for cDNA synthesis by incubating 1 μg total RNA, 500 ng random hexamers, 0.5 mmol of dNTPS, 1x reaction buffer, 20 U Rnasin, and 100 U MMuLV RT in a final volume of 20 μl. This mixture was incubated at 42°C for 1 h.

For real-time quantitative PCR, 5 μLof first-strand cDNA was used in a total volume of 25 μL, containing 12.5 μL 2x SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 ng of each primer, which shown in table 1. PCR reactions consisting of 95°C for 10 min (1 cycle), 94°C for 15 s, and 60°C for 1 min (40 cycles), were performed on step one plus Real Time PCR system (Applied Biosystems). Data were analyzed and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the GAPDH genes [39].

Table 1. Primer sequences used for RT-PCR

<table>
<thead>
<tr>
<th>primer</th>
<th>Sequence</th>
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</table>
| APP    | Forward 5′ GATGGCGGTGAAGACAAAGT 3′  
         | Reverse 5′ CATCAGCTTCTCTCTTTCTCAA3′ |
| beclin | Forward 5GGCCAATAAGATGGGTTCTG-3  
          | Reverse 5- GCTGCACACAGTCCAGAAAA -3 |
| GAPDH  | Forward 5′ TGCTGGTGCTGATGTGTTG 3′  
         | Reverse 5′ TTGAGAGCAATGCCAGCC 3′ |

Data Treatment and Statistics

The data was coded and entered using the statistical package SPSS version 15. The data was summarized using descriptive statistics: mean ± standard deviation, minimal and maximum values for all variables. Statistical differences between groups were tested using ANOVA (analysis of variance) for quantitative normally distributed variables. When a significant F was obtained, multiple comparison post tests were used to determine which groups were significantly different. Correlations were done to test for linear relations between variables. P-values less than or equal to 0.05 were considered statistically significant.
**Results:**

The results of cognitive function tests in T-maze were summarized in Table 2 and Figures (2&3).

Working memory in our study was evaluated by measuring the rate of spontaneous alternations and total duration of each trial in T-maze. % of alternations was significantly reduced in AD group as compared with the control (P<0.001). However, melatonin treatment markedly improve this task to be comparable with that of the control. However, time consumed in T-maze was shown to be significantly increased in LPS induced AD rats compared with the control group (P<0.001). This indicating prolonged latency in performance, which might be caused by generally altered cognition or a lack of interest. Although it was significantly decreased in the melatonin treated rats (P<0.001), but it was still significantly more than that of the control animals (P<0.001). This indicated that melatonin can relatively improve working memory.

**TABLE 2. Cognitive function studies in T-maze: % of alternations and Time consumed in (s) of studied groups in Mean ±SD (n=8 in each group)**

<table>
<thead>
<tr>
<th>Tests</th>
<th>% of alternations in T-maze</th>
<th>Time consumed in T-maze (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group(I)</td>
<td>92.5±8.864</td>
<td>10.625±1.408</td>
</tr>
<tr>
<td>LPS-AD Group(II)</td>
<td>50.0±9.258&lt;sup&gt;A&lt;/sup&gt;</td>
<td>132.875±9.598&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>MT Group(III)</td>
<td>86.25±9.161&lt;sup&gt;B&lt;/sup&gt;</td>
<td>24.125±4.486&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>A</sup> = Statistically significant as compared to control [ Group I ].

<sup>B</sup> = Statistically significant as compared to LPS-AD [ GroupII ].
FIGURE 2. The effect of early melatonin treatment on the % of alternations in T-maze in rat model of Alzheimer’s disease (LPS-AD). (n=8 in each group).

A = Statistically significant as compared to control [ Group I ].
B = Statistically significant as compared to AD [ GroupII ].

FIGURE 3. The effect of early melatonin treatment on the time consumed in T maze (s) in rat model of Alzheimer’s disease (LPS-AD). (n=8 in each group).

A = Statistically significant as compared to control [ Group I ].
B = Statistically significant as compared to AD [ GroupII ].

The level of Brain Peroxynitrile, Cardiolipin and Choline Acetylene Transferase; ChAT, activity were summarized in Table 3 and Figures (4,5&6).

There was statistical significant elevation (P<0.001) of brain peroxynitrile together with significant decrease of cardiolipin(P<0.001) and ChAT activity(P<0.001) in AD rats compared with control rats.

As regards the effect of melatonin treatment on these changes, there were significant reduction (P<0.001) in peroxynitrile and increase in cardiolipin(P<0.001) and ChAT activity(P<0.001) in group III compared with AD rats. This indicates the presence of antioxidant effect of melatonin which could be related to improvement of the mitochondrial function as well as the activity of acetylene choline secreting cells.

TABLE 3. The level of Brain Peroxynitrile; (μmol/mg.ptn.), Cardiolipin; CL (ng/ml) and Choline Acetylene Transferase; ChAT, activity (nmol/mg/min) in the studied groups in Mean ±SD (n=8 in each group)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Peroxynitrile</th>
<th>Cardiolipin</th>
<th>ChAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group(I)</td>
<td>0.366 ± 0.058</td>
<td>129.513 ± 5.550</td>
<td>4.325 ± 0.567</td>
</tr>
<tr>
<td>LPS-AD Group(II)</td>
<td>2.275 ± .333 A</td>
<td>37.825 ± 8.685 A</td>
<td>0.915 ± 0.146 A</td>
</tr>
<tr>
<td>MT Group(III)</td>
<td>0.678 ± 0.117 AB</td>
<td>83.712 ± 10.723 AB</td>
<td>2.060 ± .320 AB</td>
</tr>
</tbody>
</table>

A = Statistically significant as compared to control [ Group I ].
B = Statistically significant as compared to LPS-AD [ GroupII ].
The changes in the level of gene expression of brain amyloid β precursor protein (APP) & Beclin-1 in studied groups were summarized in Table 4 and Figures (7&8).

The brain of AD rats exhibited notably statistical significant increase (P<0.001) of APP gene expression. Although it was significantly lowered in the melatonin treated rats (Group III) (P<0.001), but it was still significantly higher than the control animals (P<0.001).

In contrast, there was significant decrease in the gene expression of the autophagic marker Beclin-1 (P<0.001) in the brain of AD rats compared with control rats. However, melatonin treatment to these animals caused significant rise in the gene expression of brain beclin-1 as compared with group II (P<0.001), but not to the control level (P<0.001).
TABLE 4. The level of gene expression of brain amyloid β precursor protein (APP) & Beclin-1 by PCR in the studied groups in Mean ±SD (n=8 in each group)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>APP</th>
<th>Beclin-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Group(I)</td>
<td>0.108±0.021</td>
<td>1.700±0.293</td>
</tr>
<tr>
<td>LPS-AD Group(II)</td>
<td>0.981±0.105^A</td>
<td>0.271±0.054^A</td>
</tr>
<tr>
<td>MT Group(III)</td>
<td>0.527±0.104^AM</td>
<td>0.910±0.185^AM</td>
</tr>
</tbody>
</table>

^A = Statistically significant as compared to control [Group I].
^B = Statistically significant as compared to LPS-AD [Group II].

The results of current study showed an inverse correlation between the level of brain peroxynitrite and each of the following biochemical variables: ChAT, cardiolipin and beclin-1 mRNA (r=-0.800, -0.887 & -0.838 respectively and P<0.001 for all). On the other hand there was a positive correlation between peroxynitrite and APP gene expression (r=0.900, and P<0.001).

Also, there was significant inverse correlation between the gene expression of APP and the indicator function of working memory: % of alternations in T-maze (r=-0.827 and P<0.001) in studied groups (Fig.9(A)). In addition, there was also significant inverse correlation between the mRNA of APP and both cardiolipin and Beclin-1 mRNA (r=-0.951, -0.913 respectively and P<0.001 for all) as illustrated in the Fig.9(B,C).
FIGURE 9. The inverse correlation between the gene expression of brain Amyloid B precursor protein (APP) and each of: % of alternations in T-maze (A), cardiolipin (B) and Beclin-1 gene expression (C) in the studied groups.

Discussion:

T-maze alternation has almost universal applicability in detecting cognitive dysfunction[32]. Alternation reflects the motivation of the animal to explore its environment and locate the presence of resources such as food, water, mates or shelter. Animals do not need to be deprived of such resources to show alternation behavior; in this case it is called ‘spontaneous alternation. Moreover, alternation, whether rewarded or spontaneous, is superb at detecting hippocampal dysfunction[32]. Although the septal-hippocampal system is crucially involved in spontaneous alternation, other brain areas are also involved, including the cerebellum, thalamus and substantia innominata [32].

In our study, LPS –induced AD rats displayed behavioral deficits in hippocampus-dependent learning paradigms in the form of decreased rate of spontaneous alternation in the T-maze. This was consistent with previous findings that AD rats showed a severe deficit in this behavioral task [34]. In addition, significant deficit in spontaneous alternation in the Y-maze and T-maze had been reported in other models of AD [29,40].

Olcese et al.[41] revealed that transgenic (Tg) mice given melatonin were protected from cognitive impairment in a variety of tasks of working memory and spatial reference learning/memory function; Tg control mice remained impaired in all of these cognitive tasks/domains. The cortical mRNA expression of three antioxidant enzymes (SOD-1, glutathione peroxidase, and catalase) was significantly increased to non-Tg levels by long-term oral melatonin treatment in Tg mice. This was consistent with our findings showing that melatonin treatment of LPS–induced AD in rats improved cognitive function indicated by significant increase in % of spontaneous alternation and it decreased oxidative stress by decreasing peroxinitrite. Thus, melatonin’s cognitive benefits could involve its antioxidant property.

In addition our results are also supported with a limited controlled clinical trials which indicate that melatonin is useful to treat mild cognitive impairment (MCI) and to prevent progression to AD [42].
In the present study administration of melatonin just after LPS leads to reduction in peroxynitrite and increase ChAT activity, with a significant inverse correlation between the level of brain peroxynitrite and increase ChAT activity. These findings are in agreement with a study of Guermonprez et al [43], who demonstrated that melatonin partially prevented peroxynitrite-induced inhibition of choline transport and ChAT activity.

Feng et al. [44] found significant learning and memory deficit and a profound reduction in ChAT activity in the frontal cortex and hippocampus in eight-month-old APP695 transgenic mice with Aβ deposition, these neuropathological, behavioral and biochemical changes were significantly ameliorated by four-month melatonin treatment. Also our findings showed that melatonin improves the cholinergic system deficit which is involved in the pathogenesis of AD and improves memory deficit [2].

Melatonin plays an effective role in regulating mitochondrial homeostasis[45]. Both in vitro and in vivo, melatonin was effective to prevent oxidative stress/nitrosative stress-induced mitochondrial dysfunction seen in experimental models of AD, Parkinson's disease (PD) and Huntington's disease (HD) [46].

Cardiolipin (CL) peroxidation had been shown to play a critical role in aging and neurodegeneration [47]. Studies of Petrosillo et al.[48,49] showed that melatonin prevents CL oxidation both under in vitro or in vivo conditions in mitochondria. Because of the central role played by CL in mitochondrial bioenergetics, the protective effect of melatonin on CL oxidation largely prevented the age-associated alterations of mitochondrial bioenergetics. The previous studies support our results which showed that melatonin administration significantly reduced cardioliipin level in brain tissue of LPS-induced AD in rats. Our results indicate that administration of melatonin at the beginning of the disease protects against AD by improving mitochondrial bioenergetics.

In the present study we found that LPS administration increased APP mRNA in rat brain and this is in agreement with several researches reported increased levels of APP mRNA, APP protein and APP-CTFs in sporadic AD brains[50,51]. Melatonin has been found to have regulatory effects on APP metabolism. Melatonin treatment inhibited normal levels of secretion of soluble APP (sAPP) in different cell lines, by interfering with APP full maturation and reduced Aβ generation [52,53]. Additionally, administration of melatonin efficiently reduced Aβ generation and deposition in vivo [54]. The previous studies are in line with our results showing significant decrease in APP mRNA in rat brain of LPS-induced AD when melatonin was given with the induction of the disease. Our findings indicate that melatonin has the ability to regulate APP metabolism and prevent Aβ pathology when its treatment initiated at the beginning of the disease.

There is mounting evidence that the autophagosome-lysosomal degradation is impaired, which could disturb the processing of APP and provoke AD pathology. Beclin 1 is a molecular platform assembling an interactome with stimulating and suppressive components which regulate the initiation of the autophagosome formation[55]. Recent studies have indicated that the expression Beclin 1 is reduced in AD brain. Moreover, the deficiency of Beclin 1 in cultured neurons and transgenic mice provokes the deposition of amyloid-β peptides whereas its overexpression reduces the accumulation of amyloid-β [56,57]. There are several potential mechanisms, which could inhibit the function of Beclin 1 interactome and thus impair autophagy and promote AD.
pathology. Of these mechanisms: reduction of Beclin 1 expression or its increased proteolytic cleavage by caspases [55].

The previous studies are in line with our results which demonstrated significant increase in APP mRNA and significant decrease in beclin1mRNA. Also there was significant inverse correlation between APP and beclin1. According to our findings we suggest that Beclin1 regulates APP processing and that autophagy play an important role in AD pathology.

Also, our findings regarding melatonin enhanced effect of autophagy in LPS induced AD in rats was supported by the studies of Jeong, etal. [58] and Guo etal [59] in nerve cell injury models. Jeong, etal observed in their study that the protective effect of melatonin against mitochondrial dysfunction was related with autophagy activation and melatonin-treated cells were dose-dependently increased in LC3-II (an autophagic marker) expression [58]. Also Guo etal observed that the protein levels of LC3II and Beclin1 were remarkably increased in ischemia/reperfusion-injured neuronal cells N2a in the presence of melatonin, suggesting that autophagy is possibly one of the mechanisms underlying neuroprotection of melatonin [59].

**Conclusion:** Our results indicate that neuroprotection by early melatonin supplementation is partly related to reducing amyloid formation and protection of the cholinergic system. We suggest that the neuroprotective effects of melatonin are mediated by its antioxidant capacity with the improvement of mitochondrial function, increase activity of ChAT and induction of autophagy. Early rational melatonin interventions may be one of the most promising strategies in the development of approaches to prevent or retard Aβ-mediated disease progression.
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