Melatonin exacerbates acute experimental autoimmune encephalomyelitis by enhancing the serum levels of lactate: A potential biomarker of multiple sclerosis progression

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Summary
Melatonin has a beneficial role in adult rat models of multiple sclerosis (MS). In this study, melatonin treatment (10 mg/kg/d) was investigated in young age (5-6 weeks old) Lewis rat model of acute experimental autoimmune encephalomyelitis (EAE) followed by assessing serum levels of lactate and melatonin. Results showed that clinical outcomes were exacerbated in melatonin- (neurological score = 6) vs PBS-treated EAE rats (score = 5). Melatonin caused a significant increase in serum IFN-γ, in comparison to PBS-treated EAE rats whereas no considerable change in IL-4 levels were found, although they were significantly lower than those of controls. The ratio of IFN-γ/IL-4, an indicator of Th1/Th2, was significantly higher in PBS- and melatonin- treated EAE rats, in comparison to controls. Moreover, results showed increased lymphocyte infiltration, activated astrocytes (GFAP+ cells) but also higher demyelinated plaques (MBP-deficient areas) in the lumbar spinal cord of melatonin-treated EAE rats. Finally, serum levels of lactate, but not melatonin, significantly increased in the melatonin group, compared to untreated EAE and normal rats. In conclusion, our results indicated a relationship between age and the development of EAE since a negative impact was found for melatonin on EAE recovery of young rats by enhancing IFN-γ, the ratio of Th1/Th2 cells, and astrocyte activation, which seems to delay the remyelination process. While melatonin levels decline in MS patients, lactate might be a potential diagnostic biomarker for prediction of disease progression. Early administration of melatonin in the acute phase of MS might be harmful and needs further investigations.

KEYWORDS
astrocytes, cytokine, experimental autoimmune encephalomyelitis, IFN-γ, lactate, melatonin, multiple sclerosis

1 INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is an experimental animal model of multiple sclerosis (MS), an inflammatory and demyelinating disease. Several studies have suggested a critical role for mitochondria in the pathogenesis of neurological abnormalities. Indeed, increasing evidence suggest that mitochondria is the primary source of neuronal ATPs, which are depleted in MS patients due to the loss of ATPase activity. Different clinical and experimental studies have indicated an imbalance between energy production...
and consumption in MS; characterized by a decline in the ability of mitochondria to supply sufficient ATP for the survival of neurons, which are energy dependent. These energy metabolism impairment and mitochondrial malfunctioning in MS patients are demonstrated by higher levels of compounds derived from ATP catabolism, including hypoxanthine, xanthine, uric acid, uridine and creatinine. Various reports, including proton magnetic resonance spectroscopy studies, have also shown increases in lactate concentration of MS patients in both the cerebrospinal fluid and blood, whereas Fonalledas et al. reported lower levels. Lactate is the final cytoplasmic product of the glycolysis pathway and represents quantitatively the most important mononuclearylate. Indeed, during glycolysis in the Krebs cycle, glucose breaks down to pyruvate, which is subsequently reversibly converted to lactate by the enzyme lactate dehydrogenase. Recently, a number of substances have been shown to be implicated in maintaining mitochondrial function and homeostasis, among which is melatonin. Melatonin, a highly lipophilic hormone, is released from the pineal gland in a circadian pattern and it readily crosses the cell membrane to reach subcellular compartments. Interestingly, some studies have shown that levels of melatonin are inversely correlated with the severity of MS. In addition, melatonin has also been shown to have neuroprotective, regulatory, and immunomodulatory properties. The latter properties include both pro-inflammatory and anti-inflammatory effects. Indeed, melatonin plays a pro-inflammatory role by up-regulating major histocompatibility complex-II (MHC-II) expression and enhancing cytokines in Th-1 cells. In fact, melatonin or constant darkness causes promotion in T-cell-mediated autoimmunity. In contrast, pinealectomy or constant light exposure causes melatonin decline and inhibits disease progression and severity in an animal model of autoimmune arthritis. On the other hand, it was shown that melatonin plays an anti-inflammatory role since twelve week old mice treated with Luzindole, a melatonin receptor antagonist, did not develop EAE, in comparison to controls. Another study, the only one on Lewis rats of 8-12 weeks old, showed amelioration in melatonin-treated EAE rats, through suppression of intercellular adhesion molecule-1 (ICAM-1). Similarly, other researchers have used animals between 8 and 12 weeks old, without any comparison to animals at an earlier age. Importantly, an old study in the 1970s by Janković et al. revealed neurologic deficits and exacerbation of EAE clinical outcomes in 80% of neonatally pinealectomized Wistar rats, in comparison to the same rats at 6 weeks old. These studies showing mitochondrial dysfunction in MS patients, coupled with a decreased level of melatonin, suggested the possibility of using serum lactate levels as a diagnostic biomarker.

In the present study, and for the first time, the relationship between EAE, pineal gland and melatonin was investigated taking into account the age parameter, which was not considered in previous studies. We then investigated the changes in the concentrations of melatonin and lactate following the induction of acute EAE. Finally, the effects of melatonin administration on EAE severity was then evaluated in order to validate the lactate changes as a potential biomarker.

### Results

#### 2.1 Melatonin treatment exacerbates neurological scores of EAE

The effects of melatonin on neurological scores of EAE rats were determined by daily administration of melatonin, beginning on day 10 post immunization. Symptoms in EAE rats progressed as follows: weakness of tail tonus followed by paralysis of the tail, affliction of hind limbs and ending by complete paralysis of one hind limb. The maximal neurological score was as high as 5 in these rats (n=5). Therapeutic intervention with melatonin (10 mg/kg) resulted in a considerable increase in EAE severity, at the onset of EAE symptoms, when compared to EAE-PBS-treated rats (Figure 1). The peak clinical score of melatonin-treated rats was higher (6 at days 13 and 14), in comparison to EAE-PBS-treated rats (5.5 at day 13 and 5 at day 14). At the end of the study (day 18), these values declined to 4 in melatonin-treated rats and to 2 in PBS-treated rats. Paralysis of the melatonin-treated rats progressed to both hind limbs in addition to forelimb weakness, with the maximal score of 6. Results clearly indicated that melatonin stimulates the development and progression of EAE.

#### 2.2 The effect of melatonin on pro- and anti-inflammatory cytokines

Interferon (IFN)-γ and interleukin (IL)-4 are the major cytokines that direct Th-1 and Th-2 development in EAE. Results showed that serum levels of IFN-γ in PBS-treated EAE rats were significantly higher (P<0.05) than those of controls (250±15.2 vs 189±16.8 pg/mL, Figure 2A). In addition, melatonin treatment caused a significant increase in IFN-γ levels (2.2±0.15 pg/mL, Figure 2B). IL-4, on the other hand, showed a significant decrease (P<0.05) in melatonin-treated rats (5.5±1.2 pg/mL, Figure 2C). The effects of melatonin on IFN-γ and IL-4 levels were also measured in PBS-treated rats, and no significant changes were observed.

In conclusion, melatonin exacerbates neurological symptoms of EAE by stimulating the development and progression of Th-1 cytokines and inhibiting the production of Th-2 cytokines. These findings suggest that melatonin may be a potential therapeutic target for the treatment of autoimmune diseases.
increase (P<.05) in serum IFN-γ, in comparison to PBS-treated EAE rats (333.02±6.2 vs 250±15.2 pg/mL, Figure 2A). In contrast, IL-4 levels in PBS- and melatonin- treated EAE rats were significantly lower (P<.001) than those of controls (205.10±7.3 vs 366.3±8.12 pg/mL, P<.001). However, melatonin treatment caused a significant increase of IL-4 levels, in comparison to PBS-treated EAE rats (236.66±9.2, P<.0001). Data are shown as mean±SEM.

FIGURE 3 Histogram of TH-1/TH-2 ratio. A higher Th-1/Th-2 ratio is seen in the melatonin group, compared to other groups (normal: 0.51%, EAE + PBS: 1.22%, EAE + melatonin: 1.40%)

2.3 | Melatonin increases inflammatory cells infiltration and demyelination in EAE spinal cord

The effect of melatonin concentration on inflammation and demyelination of the central nervous system (CNS) was then evaluated on lumbar spinal cords (SCs) in acute EAE rats at day 19 post immunization. Hematoxylin and eosin staining and qualitative analysis showed inflammatory cell infiltration into CNS white matter in the lumbar SCs of PBS-treated rats, which was even more profound and significantly higher in the melatonin-treated group (Figure 4B,C). Indeed, blind quantitative analysis revealed that the mean number of infiltrated cells/field in melatonin-treated EAE rats (435±22.7) was significantly higher in comparison to PBS-treated EAE group (269±31.3, P<.001) or controls (84±19.8, P<.001) (Figure 5A). Similarly, the percentage of demyelination plaques increased in the melatonin-treated rats. Surface areas of demyelination plaques at day 19 were markedly developed in melatonin-treated EAE rats (1.04±0.11), in comparison to PBS-treated EAE rats (0.48±0.08) (Figures 4B and 5B).

2.4 | Immunofluorescent assessment of oligodendrocytes and astrocytes

Immunofluorescent analysis of lumbar SCs clearly showed increased demyelinated plaques, as demonstrated by Myelin Basic Protein (MBP) deficient areas in EAE groups, which were widespread in the melatonin group, in comparison to controls (Figure 6). Moreover, induction of EAE caused the activation of astrocytes, as demonstrated by GFPA positive staining, in comparison to control rats. Finally, melatonin-treated EAE rats did not reveal an increase in MBP in demyelination areas whereas they showed intense GFAP positive staining (astrocytes), in comparison to PBS-treated EAE group, with moderate staining, or to controls (Figure 6).

2.5 | Assessment of serum lactate and melatonin

The circulating serum lactate and melatonin levels were then measured for all experimental groups (Figure 7). The serum means lactate values increased from 1.13±0.18 in normal rats to 1.68±0.16 in the PBS-treated group (not significant) and further to 2.46±0.14 for the melatonin group (P<.01) (Figure 7A). On the other hand, the serum melatonin levels decreased from 5.23±0.39 mg/L in normal rats to 2.86±0.52 in the EAE-PBS-treated rats (P<.01) (Figure 7B). However,
FIGURE 4  Melatonin treatment enhanced histological outcomes in spinal cords of experimental autoimmune encephalomyelitis (EAE) rats. In order to detect inflammatory infiltration and demyelination, serial sections were analyzed by hematoxylin and eosin (A and B) and Luxol Fast Blue staining (C); respectively. A, Inflammatory infiltration with extensive perivascular cuffing was widespread in melatonin-treated EAE rats, in comparison to EAE-PBS treated rats. C, Represent infiltrated cells only, which is a modified figure of (B) without background tissue. C, Red arrow indicates demyelination area which is more prominent and amplified in melatonin group compared to EAE-PBS treated rats. Scale bar = 200 μm
serum melatonin increased significantly ($P<.01$) to $5.63\pm0.34$ in the melatonin group, in comparison to PBS-treated mice, but was not affected when compared to controls (Figure 7B). These observations demonstrated that the induction of EAE had different effects on the levels of serum lactate and melatonin, where a decrease in melatonin was compensated by an increase in lactate concentration.

3 | DISCUSSION

In the current study, we investigated inflammation and demyelination processes in a young rat model of acute EAE and evaluated the serum levels of lactate as a biomarker of MS progression, following low doses of melatonin treatment. Previously, it has been confirmed that inflammatory cytokines and the ratio of Th-1/Th-2 play an important role in neuroimmune diseases. Typically, the ratio of IFN-$\gamma$/IL-4 acts as an indicator of Th-1/Th-2. Here, we have found that melatonin had a dramatic impact on the increase in pro-inflammatory cytokine, IFN-$\gamma$. Moreover, while the anti-inflammatory cytokine IL-4 decreased in EAE animals, melatonin-treated animals demonstrated a statistically higher level of IL-4, in comparison to EAE-PBS-treated animals; however, this increase was not as prominent as that of IFN-$\gamma$. These results are in line with an in vitro study showing that melatonin could stimulate Th-1 cells to increase the production of cytokines such as IL-2 and IFN-$\gamma$, but which had a negligible effect on IL-4 production. In accordance with this study, it seems that Th-2 cells act in a hormone-independent manner whereas Th-1 cells are strongly influenced by hormones. In disagreement with our results, Raghavendra et al. showed a reduced IFN-$\gamma$ secretion through Th-1 cells stimulated with CD3 antibody in presence of antigen-presenting cell following administration of melatonin at 10, 20 and 25 mg. Also, it has been reported that IL-4 had the ability to inhibit the production of IFN-$\gamma$ via activated T cells. Moreover, Th-2 cells are sensitive to IFN-$\gamma$, which can strongly inhibit its production. In this study, it appears that a sharp increase in IFN-$\gamma$ inhibited Th-2 cells and prevented an increase in IL-4 secretion.

On the other hand, one of the main sources of pro-inflammatory cytokines involved in histological CNS injury are activated astrocytes. A study by Wensky and colleagues showed that IFN-$\gamma$ determines distinct clinical outcomes in autoimmune encephalomyelitis models. Several studies have clearly demonstrated that astrocytes stimulated by IFN-$\gamma$ affect MBP and present myelin-peptides to T cells in an MHC class II-restricted manner, leading to their activation. Activated astrocytes have been shown to present the oligodendrocytes to T cells, hence amplifying the demyelination process in injured regions. In accordance, our results showed increased GFAP positive cells (astrocytes) in the melatonin group, concomitant with high levels of IFN-$\gamma$, which could explain the greater demyelinated areas in the melatonin group, compared to controls. Although demyelination is not quite prominent in EAE rat models, melatonin treatment caused demyelination areas to be more visible. In agreement, a recent study showed that astrocytes activated with lysophosphatidyl choline inhibit myelin repair by increasing the remyelination inhibitor Endothelin-1 (ET-1). Finally, in line with our study which demonstrated more activated glial cells in the melatonin-treated EAE group, it has been reported that inhibition of glial cell activation ameliorates the severity of EAE.

In addition, this experimental study is the first to evaluate serum levels of lactate as a biomarker of MS progression following melatonin treatment in a young rat model of acute EAE. Melatonin administration was shown to have a significant effect on mitochondrial function, as demonstrated by an increase in serum lactate concentration, in an acute EAE model in Lewis rats, compared to controls. This increase in serum lactate levels coincided with the progression in neurological scores and histopathological outcomes of EAE. In contrast, the serum levels of melatonin were lower in EAE rats, but were enhanced following melatonin therapy, in comparison to controls.

At the cellular and molecular levels, oligodendrocytes are coupled to astrocytes which in turn provide metabolites to myelin-forming oligodendrocytes, early in development, and supply energy to neurons in the form of lactate. Metabolic support for axonal function is provided by oligodendrocytes, however axonal functions are offered.
by glial cells of astrocytes through glycolysis products in the form of lactate or pyruvate, rather than glucose itself. Astrocytes and oligodendrocytes can import glucose, lactate, or pyruvate into their cells, but glucose needs to be converted to pyruvate or lactate.\textsuperscript{62,63} The flux of lactate from the blood or astrocytes into oligodendrocytes provides the main substrate that can be used by the latter to make ATP and fatty acids,\textsuperscript{64} required to synthesize myelin during myelination.

The role of astrocytes and oligodendrocytes in providing lactate as a main substrate for myelination suggests that astrocyte activation could be a plausible mechanism to explain increases in lactate levels, observed in this study. Importantly, our results showed a low rate of remyelination and provided evidence of astrocyte activation following melatonin treatment, in comparison to control rats, which leads to a reduction in lactate consumption. In conclusion, a delay or decrease in remyelination, concomitant with an increase in astrocyte activation, is the most plausible mechanism explaining lactate increases observed following melatonin therapy in EAE rats.

Our results showed a negative impact of melatonin on recovery of acute EAE rats at 5-6 weeks of age by enhancing the concentration of IFN-\(\gamma\) and the ratio of Th-1/Th-2 cells. In fact, an increase in IFN-\(\gamma\) serum levels and activation of astrocytes caused a delay in the remyelination process. Therefore, we suggest that astrocytes and Th-1 cells are more affected by exogenous melatonin whereas Th-2 cells are less or not affected. Since our rats are young in age, in contrast to other studies, we suggest a relationship between age and the development of EAE. In addition, serum levels of lactate and melatonin were found to be affected by EAE induction. These findings suggest that measurement of serum lactate in MS patients might be useful as a potential diagnostic biomarker for prediction of disease progression, as well as for confirmation of the decline in melatonin serum levels in response to treatment. Interestsingly, administration of melatonin caused marked increases in serum levels of both melatonin and lactate, while no correlation was found between melatonin and lactate levels. We believe that early administration of melatonin at pharmacological doses in acute phases of MS might be harmful and needs to be investigated further.

\textbf{FIGURE 6} Immunohistochemical staining of glial fibrillary acid protein (GFAP) and myelin basic protein (MBP). Melatonin did not cause an increase in MBP (green) in demyelination area whereas GFAP positive cells (red) were increased. The arrows show demyelinated region without MBP positive cells. Scale bars = 100 \(\mu\)m
4 | MATERIALS AND METHODS

4.1 | Animal handling and housing

Young female Lewis rats (5-6 weeks old, 150-175 g) were purchased from DarouPakhsh Institute (Karaj, Iran). The Institutional Animal Care and Use Committee (IACUC) of Yasuj University of Medical Science approved all experimental procedures and animal use in this study. Rats were maintained and housed under pathogen-free conditions with constant temperature and humidity control at the Animal Breeding Center. Surgical procedures were performed under deep anaesthesia. Housing of the animals and all animal experimental procedures were carried out in accordance with the guidelines of the Iranian Agriculture Ministry and of the European Communities Council Directive (86/609/EEC). All efforts were made to reduce the number of animals used and suffering.

4.2 | EAE induction

Rats were anaesthetized with isoflurane (Abbott Labs, Lake Bluff, IL, USA) and then injected subcutaneously over the flank with 200 μL of a 1:1 (vol/vol) mixture of 1 g guinea pig SC in 1 mL PBS and Complete Freund’s Adjuvant (CFA, Sigma Aldrich, St Louis, MO, USA) and 1 mg/mL Mycobacterium tuberculosis bacteria enriched.

4.3 | Clinical evaluation

Animals were evaluated and scored for clinical signs of the disease by at least two investigators from day 9 to day 19 post immunization using modified 0-8 point scale from that described previously.43 A 0-8 point scale offers greater sensitivity than 0-5 point scales to detect statistical differences between compared groups. Briefly, the scoring system was as follows: 0, normal; 1, flaccid/limp tail; 2, hind limb weakness causing righting difficulty from a supine position; 3, hind limb weakness causing righting inability ≥8 seconds from a supine position; 4, hind limb weakness causing limping and abnormal gait; 5, partial (one limb) hind limb paralysis or extensive hind limb weakness such that the hind limbs cannot contribute to mobility; 6, total (both) hind limb paralysis plus forelimb weakness; 7, hind limb paralysis and forelimb weakness or paralysis resulting in a side resting position; 8, moribund requiring euthanasia or inadvertent death.

4.4 | Treatment of animals

All EAE animals were randomly divided into three groups of: normal control rats (n=5), PBS-treated EAE rats (n=5), and melatonin (10 mg/kg) treated rats of EAE (n=5). All administrations were done orally. In order to have a clinically relevant treatment protocol, treatment was given for 6 consecutive days starting on the day of clinical symptom onset (score ≥3).

4.5 | Quantifying serum cytokines by enzyme-linked immunosorbent assay

After sacrifice, blood samples were collected from all rats via cardiac puncture. Serum was obtained following centrifugation (2500 rpm, 10 minutes), and frozen in −80°C until enzyme-linked immunosorbent assay (ELISA) test was performed. Serum levels of IL-4 and IFN-γ were measured using ELISA Kit (Sigma Aldrich) according to manufacturer’s protocol.

4.6 | Histopathological analysis

At the end of the study (day 19 after immunization), rats were deeply anaesthetized with ketamine/xylazine (5/1) and then perfused via the left ventricle with 30 mL PBS (0.1 mol/L) followed by 4% paraformaldehyde (PFA) for 10 minutes. Since the most significant EAE histopathological changes were detected in the lumbar region of the
SC, it was removed and immersed in 4% PFA for 24 hours. Fixed tissues were then paraffin-embedded, and 6 μm sections were prepared from the lumbar SCs. Sections were then de-paraffinized and hydrated through xylol and alcohol with a routine protocol. A total of 10 systematic randomly selected sections of lumbar SC from each animal were stained with luxol fast blue and hematoxylin and eosin (H&E) for measuring the extent of demyelination area and infiltration state, respectively. In each group, at least eight sections per rat obtained from the lumbar SC were examined histologically. Sections were then scanned and captured using a light microscope Olympus BX60 microscope with a digital camera (Spot camera; Diagnostic Instruments Inc., Sterling Heights, MI). ImageJ software, version 1.6.0.24 (National Institutes of Health, USA) was used to determine demyelination percentage and infiltration intensity. Also, in order to better recognize infiltrating cells on tissue section images, a protocol using ImageJ software, version 1.6.0.24 (National Institutes of Health, USA) was designed to eliminate background tissue and to count infiltrated cells in the SC. In summary, the protocol includes these stages: (1: File→open image 2: Image→Adjust→Threshold, change the value in brightness part: upper value = 113, Lower value = 225, Thresholding method = Default, Threshold Color = White, Color space = HSB, check dark background, close the windows. 3: Process→Noise→remove outliers. Change the value Radius = 5.0, Threshold = 50, which outliers = Dark and ok. 4: process→smooth, repeat this stage to get a clearer picture). The infiltrated cells were counted (8 sections/group, 4 fields/section) and results reported as mean number of cells per field per group.

4.7 | Immunohistochemistry

Following de-paraffinization and hydration, sections were permeabilized by 20% tween for 20 minutes for fluorescence immunostaining. Non-specific labelling was blocked with 0.1% BSA in 0.1% Triton X-100/PBS for 60 minutes. Sections were incubated overnight at 4°C with primary antibodies anti-MBP (1:500) and anti-GFAP (1:800). Then, the slides were incubated with appropriate secondary antibodies (Alexa Fluor 488 and 566, 1:500; and Hoechst, 1:1000) for 1 hour. All antibodies were obtained from Abcam, USA. Samples were analyzed using a fluorescent microscope (Olympus IX-71; Olympus, Tokyo, Japan) equipped with a Canon EOS digital camera.

4.8 | Preparation of serum samples

Blood samples were collected from all rats via cardiac puncture on day 19. After 25 minutes at room temperature, blood samples were centrifuged at 700 g for 10 minutes and the resulting sera were frozen at −80°C. To measure lactate and melatonin concentrations, an aliquot of all serum samples was used with no further processing.

4.9 | High performance liquid chromatography

The chromatographic measurements were carried out with a KNAUER smartline HPLC system equipped with micro vacuum degasser, LPG system, UV-VIS Detector (2550 was set at 220 nm) and a MZ ODS-C18 (250 mm×4.6 mm, 5 μm) column. The chromatographic calculations were performed using an EZCHROM elite system. Determination of melatonin was performed by HPLC according to Bechgaard protocol66 at the optimum separation condition with isocratic binary mobile phase consisting of 30:70 (v/v) of methanol: 0.1 mol/L phosphate buffer with 1 mL/min flow rate. A digital pH meter (Inolab-pH730, Weihlim, Germany) was employed for pH measurements. A HERMLE bench centrifuge (Hermle-Labortechnik 2206A, Gosheimer Str., Germany) was used to accelerate the phase separation. In addition, determination of lactate was carried out following the method described by Artiss et al.67 The accuracy and applicability of the proposed method for the extraction and determination of melatonin and lactate levels in the serum were investigated using standard addition method. Pearson’s correlation analysis was used to study the plausible correlation between serum levels of melatonin and lactate using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA).

4.10 | Statistical analysis

Results are presented as an average with error bars indicating the standard error of the mean (mean±SEM). GraphPad Prism (Version 6.01) software was used to perform statistical analyses. Following assessment of normality, ordinary one-way ANOVA followed by Tukey post-hoc test, was used to analyze the data. Significance is indicated by *P<.05, **P<.01, ***P<.001 and ****P<.0001.

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DISCLOSURE

The authors declare no conflict of interests regarding the publication of this paper.

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