Research Article

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Simultaneous comparison of L-NAME and melatonin effects on RAW 264.7 cell line’s iNOS production and activity

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Abstract

**Background:** NO (nitric oxide) inhibition could be used for evaluating the drug’s efficacy for NO-mediated inflammatory disorders. The aim of this study was to investigate the influence of L-NAME and melatonin on different NO production levels in RAW 264.7 cell line as an in vitro model for inflammatory diseases.

**Materials and methods:** RAW 264.7 macrophage cell line was used to compare the effects of L-NAME and melatonin on basal and Lipopolysaccharide (LPS)-induced iNOS levels. The cells were treated using L-NAME and melatonin for 1 h, afterward incubated with/without LPS for 8 and 24 h. Finally, iNOS mRNA, protein, activity, and nitrite concentrations were evaluated.

**Results:** Inhibition rate of nitrite by 1 mM L-NAME compared with LPS control were 78% and 80% during 8 and 24 h, respectively. Real-time PCR showed that in the LPS-treated group, 1 mM L-NAME could result in 14% increase of iNOS-mRNA compared with the control group during 8 h. Dose-dependent activity of iNOS in LPS-induced cells from non-treated to 4 mM L-NAME showed 79% reduction while at the same concentrations of melatonin this decrease was 32% (p-value <0.05).

**Conclusion:** L-NAME showed lower iNOS expression modulating efficacy than melatonin. The result concluded lower potential of the NOS synthetic inhibitors rather than melatonin in the treatment of NO-related disorders.

**Keywords:** Melatonin; Nitric oxide; L-NAME; Inflammation.

Introduction

Nitric oxide (NO) is a compound derived from L-arginine which could be produced by three distinct nitric oxide synthase (NOS) isoforms including endothelial (eNOS), neuronal (nNOS), and inducible NOS (iNOS) [1]. Among these isoforms, iNOS is exceedingly expressed in macrophages with the highest NO production upon induction. The induced NO in macrophages acts as the anti-tumor and anti-microbial molecule. Moreover, NO overproduction could be regarded as a marker of inflammation and its blocking by inhibition of iNOS, providing a possible approach for evaluation of the drugs on NO-mediated pathological disorders such as migraine headaches and other inflammatory diseases [1, 2]. Previous studies have reported that monocytes of migraine patients release more NO than healthy individuals [3], which could be due to increased expression and activity of iNOS [3, 4].

A natural inhibitor of NOS is melatonin, an indolamine mostly secreted by the pineal gland in mammals [5]. Due to the nocturnal decrease in plasma and urinary melatonin levels in patients with migraine [6], it could be regarded as a biological marker of this disease [7]. It seems that NOS inhibition is one of the possible mechanisms of melatonin in the prevention of migraine headache [8].
previous studies, it has been indicated that melatonin acts as an efficient anti-inflammatory and antioxidant which inhibits the production of excessive amounts of NO in lipopolysaccharide (LPS)-induced macrophages [9].

Previously it has been suggested that neurovascular headaches, such as migraine are probably the result of trigeminovascular pain activation structures projecting to the trigeminocervical complex of neurons in the caudal brain stem and upper cervical spinal cord. It has been proposed that N-nitro-L-arginine methyl ester (L-NAME) which is a non-selective competitive inhibitor of NOS enzymes, could improve the condition via inhibiting the Fos protein expression. This is suggesting a possible therapeutic approach for these patients [10].

For these reasons, NOS inhibitors are under consideration for future application against inflammatory disorders including migraine [11]. Although these evidence are somewhat convincing, some studies have reported the inefficiency of the NOS inhibitors in migraine treatment [12]. In agreement with the finding, an in vitro study showed that L-NAME despite the decrease in NO induced by LPS in cell supernatants, induces iNOS protein as synergistic to LPS [13]. In order to resolve this controversy, evaluating the effect of L-NAME on all iNOS expression levels could help to resolve this problem.

Due to restricted literature on comparing the NO inhibition efficacy of L-NAME and melatonin, the present study aimed to address this issue. The originality of this study was to simultaneously investigate the influence of L-NAME and melatonin on different NO production level in RAW 264.7 cell line as an in vitro model for inflammatory diseases. Since the murine iNOS as an in vitro model of migraine could serve as a suitable alternate for the iNOS assays in human [14, 15], we decided to use this cell line for this study.

Materials and methods

Dulbecco’s modified Eagle’s medium (DMEM) (cat. no. 11885), fetal bovine serum (FBS) (cat. no. 16000-036) were purchased from Gibco company (USA). LPS (cat. no. L2630), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (cat. no. M2128), antibiotics including penicillin/streptomycin and were acquired from Sigma (USA). L-NAME (cat. no. ALX-105-003-G025) was acquired from Alexis Biochemical. Primary anti-β-actin and anti-iNOS antibodies and goat anti-rabbit IgG-HRP for applying as the secondary antibodies were purchased from Abcam (UK).

Cell culture

The RAW 264.7 cells were provided by Iranian Biological Resource Center at fifth passages. All investigations were performed by 1 h pretreatment of L-NAME and melatonin with/without LPS (5 μg/mL) during an 8 and/or 24 h period. 5 μg/mL dose of LPS was preferred to achieve a maximum induction for iNOS in the target cells.

Cell viability test

Mitochondrial respiration was determined via reduction of MTT to formazan [16]. Briefly, RAW 264.7 Cells were processed by L-NAME and melatonin with or without LPS, then incubated by MTT. The supernatants were discharged and the insoluble formazans were re-suspended using dimethylsulphoxide (DMSO). Evaluation of 540 nm optical density was carried out and viabilities of target cells were expressed in comparison with non-treated group. The following equation describes how the cell viability was calculated:

\[
\text{Cell Viability} = \frac{\text{processed samples light absorb}}{\text{light absorb of control group}} 
\]

Nitrite level evaluation

Analyzing the nitrite level was determined through 540 nm spectrophotometry by the Griess reagent [16] using the sodium nitrite (MERK, cat. no CC634149) as the control. In brief, 100 μL from any sample were transferred to into a 96-well microtiter plate. Then, 100 μL Griess reagent (2% sulfanilamide (MERK, cat. no K3967135) in 5% HCl solution (MERK, cat. no K35384014) and 0.1% N-(1-naphtyl) ethylenediamine (MERK, cat. no K31451237) in water was added to the samples. Also, in order to reduce the nitrate compound into nitrite, 100 μL of vanadium chloride III (Sigma, USA, cat. no S5666093) 0.8% was added into the samples. Afterward samples were incubated for an hour at 37°C. At the next step, the nitrite levels of samples were investigated through colorimetry using ELISA-reader device (Anthos 2020 UK, Biochrom Ltd.) at 540 nm.

Measurement of NOS activity

7.2 × 10^4 RAW 264.7 cells were cultivated at wells of 75-cm^2 diameter flask. After pretreatment with L-NAME and melatonin with/without induction with LPS (5 μg/mL), cells...
were detached. At the next step, cells were washed by phosphate buffer solution (PBS), re-suspended in 0.3 mL ice-cold homogenization buffer (50 mM HEPES, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), with supplied protease inhibitor cocktail (Sigma-USA) at pH 7.4 followed by sonication on ice with two 30S bursts. Afterward, cells were 15 times passed through a 26-gauge needle to attach to the syringe. Resultant homogenates were sediment at 20,000 g (4°C, 15 min).

After separation of supernatant, protein concentrations were determined via Bradford method. NOS activity was measured according to the protocol described in Ultrasensitive Colorimetric Assay for NOS kit (Oxford Biomedical Research, UK). Briefly, after incubation of 300 μg of cell lysates with reaction mixture (HEPES/EDTA, NADP+/Glucose 6-Phosphate/L-Arginine and Glucose 6-Phosphate dehydrogenase) in a 250 μL final volume for 5 h at 37°C, the reconstituted nitrate reductase was added to the sample and kept at room temperature for 20 min. Concentration of the nitrite was measured by Griess reagent.

To analyze the direct effect of L-NAME and melatonin on iNOS activity, homogenates of LPS-stimulated cells (LPS, 5 μg/mL, 24 h) were treated by various loads of L-NAME (0.1–4 mM) and melatonin (0.1–4 mM), separately, and iNOS activity was investigated.

### Western blotting

2.4×10^6 RAW 264.7 cells were treated and centrifuged, then the pellet was washed twice using PBS. Then samples were centrifuged at 250 g (five min, 4°C). Resultant pellet was dissolved at 100 μL lysis buffer (50 mM Tris-HCl with pH 7.5), 1% Triton X-100, 1 mM EDTA, 2% sodium deoxycholate, and protease inhibitor cocktail (Sigma-USA). About 60 μg total proteins were separated through 8% SDS-PAGE gel electrophoresis. After transferring the protein bands to a nitrocellulose membrane, the membrane was immersed in the diluted primary antibodies 1:1000 and 1:2000 of iNOS and beta-actin, respectively for 2 h at 37°C. Then, it was processed by diluted horseradish peroxidase-conjugated goat anti-rabbit antibody at 37°C for an hour.

The target protein bands were inspected through application of the Amershams enhanced chemiluminescence kit (GE Healthcare) and exposed to Kodak X-ray autoradiography films inside a film cassette. Equal protein loadings were confirmed through beta-actin immunoblotting. In order to ascertain the changes, each test was repeated three times.

### Real-time PCR

The total RNA content was provided through application of the RNasy mini kit. cDNA was synthesized by cDNA synthesis kit in accordance with the manual provided by manufacturer (Qiagen, Germany). Real-Time PCR analysis was carried out in triplicate using SYBR Premix Ex Taq polymerase II (TaKaRa, Japan). All exon-exon junction designed primers were supplied by Qiagen: beta-actin (Cat. no. QT01136772), iNOS (Cat. no. QT00100275). As an endogenous reference, β-actin protein was used to make the comparison possible.

A standard curve containing five dilution points was evaluated in triplicate. The results of PCR tests displayed efficiencies between 1.8 and 2.0. Gene expression fold changes were evaluated by comparative C_{T} method [17].

### Statistical analysis

Graph pad prism software was used for analysis and presentation of data. Data are presented as the mean±SD. Diversities among groups have been evaluated through analysis of variance (ANOVA) ensuing with post hoc Tukey test for sub-two groups contrast. p-Value <0.05 were considered meaningful.

### Ethical considerations

The conducted research was not related to either human or animal use.

### Results

**Melatonin and L-NAME influences on cell supernatants NO levels**

RAW 264.7 cells displayed an increased nitrite concentration upon processing with LPS during 8 and 24 h in a time-dependent manner. Pretreatment of L-NAME (0.1 mM and 1 mM) and melatonin (0.1 mM and 1 mM) with LPS showed an inhibition at nitrite levels in culture media dose-dependently (p <0.05) (Figure 1). In LPS treated cells, at 1 mM concentration of L-NAME, the mean of nitrite concentration was 2.26 μM at 8 h compared with 7.51 μM at 24 h. In comparison, at the same concentration of melatonin, the nitrite concentration at 8 h was 4.96 μM, when at the 24 h this concentration was 12.6 μM. Inhibitory effects
of nitrite by 1 mM L-NAME compared with untreated LPS-induced were 78% and 80% at 8 and 24 h, respectively. The inhibitory effect for 1 mM melatonin was 53% and 67% in 8 and 24 h, respectively. The cell viability in all samples was up to 90%. Data are presented as mean ± SD of triplicates versus untreated control (CTRL): *p < 0.05; vs. LPS treated control (LPS): **p < 0.05.

MTT assay showed that both L-NAME and melatonin with/without LPS resulted in no considerable influence on the viability of target cells. Figure 1 illustrates how the samples cell viability was up to 90% (p < 0.05). Therefore, the reduced NO production or iNOS suppression in LPS-induced samples were not because of the supplemented components toxic effects.

Analysis of iNOS mRNA expression by L-NAME and melatonin

RAW 264.7 LPS-induced cells showed higher iNOS mRNA level compared with non-induced controls in 8 and 24 h. Without LPS-induction, the fold changes with 1 mM of L-NAME were 1.9, and 0.3 in 8 and 24 h relative to the untreated peer control group, respectively, while for melatonin they were 1.07, and 0.8, respectively. In LPS-induced condition, 1 mM of L-NAME caused 10.7, and 7.9-fold changes at 8, and 24 h, respectively, while 1 mM of melatonin 2.7 and 1.7-fold changes, respectively. Nevertheless, a suppressive effect on iNOS mRNA in 24 h rather than 8 h was observed (Figure 2).

The Real-Time PCR analysis indicated that L-NAME pretreatment in presence of LPS stimulated a considerable augment in iNOS mRNA in comparison with LPS-induced group (p < 0.05). However, without LPS, it led to induction of iNOS mRNA in 8 h and suppression in 24 h compared with untreated control (p < 0.05). iNOS mRNA analysis with melatonin showed that melatonin dose-dependently suppressed gene expression of LPS-induced iNOS, while the presence of melatonin in cultures lacking LPS resulted in no influence on translation of iNOS mRNA.

L-NAME and melatonin effects on iNOS protein with or without LPS

Western blot analysis confirmed the results of the NO assay (Figure 3). Without LPS induction, by adding 1 mM of L-NAME, and melatonin, the mean of protein level relative to the untreated peer groups were 2.8 and 0.9, respectively in 24 h. In LPS-induced samples, 1 mM treated L-NAME caused 9.1 relative fold change compared with

**Figure 1:** L-NAME and melatonin effects on the nitrite concentration and RAW 264.7 cells viability (MTT assay).

**Figure 2:** Real-Time PCR analysis of L-NAME and melatonin effects on iNOS mRNA expression.
Masoumeh Azizi et al.: Simultaneous comparison of L-NAME and melatonin effects on RAW 264.7 non-treated peer group, when these levels for melatonin was 2.5 in 24 h. L-NAME in 24 h incubations with/without LPS increased iNOS levels in comparison with LPS induced and non-induced groups, respectively (p < 0.05). In the same conditions, pretreatment of 1 mM melatonin with LPS in a 24 h time, reduced the iNOS protein levels, while no notable changes were detected without LPS (p < 0.05).

At 8 h period, in LPS-induced cells, 1 mM of L-NAME produced 10.1 nmol nitrite per mg of protein, while in the peer group with melatonin treatment, this production was 24 nmol. These concentrations in 24 h treatment were 15.6, and 22 nmol for L-NAME, and melatonin, respectively. Activity analysis of iNOS protein (Figure 4A, B) showed that pretreatment of L-NAME with LPS significantly increased the iNOS activity in 24 h (Figure 4B) (p < 0.05), but not in 8 h (Figure 4A). In contrast, nitrite measurement in media supernatants of these samples showed an inhibitory effect on nitrite by L-NAME (p < 0.05). When cells were processed with L-NAME, but without LPS, it increased both basal nitrite and iNOS activity in 24 h (p < 0.05).

Moreover, melatonin did significantly inhibit iNOS activity in presence of LPS during 8 and 24 h without important effects on its activity in absence of LPS (p < 0.05). Nitrite measurement in media supernatants related to melatonin was in agreement with the activity assay (Figure 4A, B).

**Figure 3:** Western blotting analysis of iNOS protein expression. (A) Typical Western blot analysis of iNOS gene expression. (B) Semi-quantitative analysis of iNOS protein levels through image J (NIH) for densitometry analysis and then normalized against β-actin as the endogenous control. In LPS-induced group, 1 mM of L-NAME resulted in 1.4 and 2.84 times increase at iNOS protein level in comparison with LPS induced and non-induced groups, respectively. Data are provided as mean ± SD of triplicates in comparison with untreated control (CTRL): *p < 0.05; compared with LPS treated control (LPS): **p < 0.05.

**Figure 4:** Effects of L-NAME and melatonin on the iNOS activity and its related nitrite levels in eight (A) and 24 h (B). Melatonin did not significantly inhibit iNOS activity in presence of LPS during 8 and 24 h without important effects on its activity without LPS. Pretreatment of L-NAME with LPS in 8 h treated group did not pose any considerable changes (A), but during 24 h period, changes were significant (B). When cells were processed with L-NAME but without LPS, it increased both basal nitrite and iNOS activity in 24 h (B). Data are expressed as mean ± SD of duplicates. Compared with untreated control (CTRL): *p < 0.05; vs. LPS treated control (LPS): **p < 0.05.

**Direct effects of L-NAME and melatonin on iNOS extracted from LPS-induced RAW 264.7 cells**

As shown in Figure 5, the mean of iNOS activity at 0.1 mM L-NAME was 35.3 nmol nitrite/min/mg protein, but at 4 mM L-NAME, it 10.9 nmol nitrite/min/mg protein. In comparison, at 0.1 mM melatonin, the activity caused 47 nmol nitrite/min/mg protein while at 4 mM of melatonin it was
only reduced to 34 nmol nitrite/min/mg protein. Dose-dependent inhibition of iNOS in LPS-induced cells from non-treated to 4 mM L-NAME showed 79% reduction while at the same concentrations of melatonin this decrease was 32% (p-value < 0.05).

Analysis of L-NAME and melatonin direct influences on synthetic nitrite and iNOS extracted from LPS-stimulated RAW 264.7 cells showed that L-NAME could have a stronger dose-dependent activity inhibition of iNOS than melatonin.

Discussion

The current research indicated that both L-NAME and melatonin inhibited the cell culture supernatants LPS-stimulated NO with a dose-dependent pattern. Further evaluation demonstrated that L-NAME inhibition is limited to the enzyme activity inhibition while the effect of melatonin mainly suppressed iNOS expression. In addition, L-NAME led to the induction of iNOS with/without LPS during 24 h.

According to the previous studies, it has been demonstrated that L-NAME and N-monomethyl-L-arginine (LNMA), in combination with LPS could effectively increase in vitro rate of iNOS production. Previous studies mostly examined an inductive effect on iNOS in presence of LPS, but this study showed the same effect without LPS [13]. In addition, an in vivo study in rats has earlier reported that long term L-NAME incubation at low doses could increase NO generation and vasorelaxation [18]. Present study by simultaneous evaluation of the effects L-NAME on both LPS stimulated and non-stimulated condition showed that this compound could increase the rate of iNOS expression, and the suppressive results of this compound on NO level are related to the activity of enzyme rather than gene expression.

The results acquired from the L-NAME could be related to autoregulatory properties of NO. NO showed to exerts a biphasic effect on the expression of iNOS. In a low concentration, NO acts as a positive feedback and it can upregulate iNOS activity via reaction with enzyme-bound heme in stimulated rat macrophage cell line [19]. In addition, using a competitive inhibitor of NOS showed the inhibition of iNOS activity itself increases the transcription of this gene [20]. NO interferes in iNOS expression processes by suppressing either nuclear factor kappa B (NF-kB) activation [20–22], its binding to DNA or by induction and stabilization of NF-kB inhibitor [21, 22]. Accordingly, it could be concluded that NO modulates the NOS transcription rate. The signaling pathway of NF-kB transcription factor could be activated using LPS which is apparently a critical step for the induction of iNOS [2]. For these reasons, seemingly NO reduction by L-NAME resulted in NF-kB activation followed by increased iNOS production and vice versa.

Previously, it has been proposed that melatonin could be a promising alternative for prophylaxis usage in migraine patients, but in fact there are not enough proof for its efficacy in preventing the condition [23]. Since this study showed that melatonin modulates the iNOS expression, it could be hypothesized that regardless of presence or absence of LPS, melatonin prevents the NO autoregulatory mechanism. The earlier findings claim that melatonin mostly regulates the release of NO from the cells that express iNOS in the transcriptional and/or posttranscriptional stages [9, 24–27]. Our results are consistent with these reports. According to the fact that induction of iNOS is a process requiring de novo protein synthesis [1, 2], the inhibitory effects results from Real-Time PCR, western blotting and enzyme activity assay indicated that melatonin has played role at iNOS gene transcription expression steps followed by reduced NO production.

The results provided by this study indicated that melatonin could be considered more effective than L-NAME for regulation of iNOS expression. These actions of melatonin occurred in a high micromolar level. The micromolar
concentration showed to be higher than physiological plasma level of melatonin in human (up to 1 nM). These levels could be synthesized locally by the peripheral tissues other than pineal gland, for example GI tract melatonin [5]. The micromolar melatonin has antioxidant effects [26] and blocks the LPS-induced vasodilation [27]. Therefore, the application of pharmacologic concentrations can simulate an extrapineal melatonin level. This is proposing a potential therapeutic strategy for disorders such as migraine headache; where the involvement in inflammatory processes, like iNOS overexpression and vasodilation [12, 28] with melatonin deficiency is reported [8].

Conclusion

This study indicated that L-NAME versus melatonin had lower ability to modulate the iNOS expression, therefore it could exacerbate the processes linked to iNOS overexpression and be the possible reasons for weak efficacy of the NOS inhibitors in treatment of migraine. Nevertheless, this study showed that L-NAME had a useful short time suppressive effect on activity.

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Conflict of interest: The authors state that there is no conflict of interests with any potential to negatively influence the study.

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