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Protective Mechanism of Melatonin on Kainic Acid Induced Immune Modulatory Effect on Lymphocytes Derived from Mouse Spleen

Gayathri V and Mohanan PV*

Division of Toxicology, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India

Abstract

Toxicity occurs when cells develop inflammation due to exposure to several toxic substances. The study was aimed to assess the damage (toxicity) induced by kainic acid on splenic lymphocyte and enriched T lymphocyte under *in vitro* conditions and the protective role of exogenous melatonin against this damage (toxicity). The study included assessment of inflammatory mechanism by the expression of immune modulatory cytokine mediators using real time PCR. Oxidative stress (reactive oxygen species) and nitrosative stress (reactive nitrogen species) were also studied to determine the free radical production. Interestingly, kainic acid caused severe splenic lymphocytes and enriched T lymphocyte damage which was evident from deleterious alterations in various parameters. Kainic acid treatment (1 mM) resulted in increased mRNA expression of cytokines like tumour necrosis factor-beta, interleukin 6, interleukin 1, interferon gamma, mitogen-activated protein kinase gene-14, inducible nitric oxide synthase and decreased interleukin 10 mRNA expression. Tritiated (^{3}H) Thymidine Incorporation study signifies that kainic acid treatment (1 mM) increased the proliferation of splenic and enriched T lymphocytes. These changes were normalized by exogenous administration of melatonin (0.25-1.0 mM) in combination with kainic acid. Flow Cytometry Analysis using Annexin V apoptosis assay kit revealed an increase in apoptotic and necrosis (double positive cells) in splenic lymphocytes treated with kainic acid alone. However, melatonin treated in combination with kainic acid, showed attenuation to apoptosis and necrosis on splenic lymphocytes. This study depicts that kainic acid induced inflammatory toxicity could be attenuated by exogenous melatonin treatment as evident by the decreased levels of the inflammatory cytokines, immune reactions and free radical production.

Keywords: Kainic acid; Melatonin; Splenic lymphocytes; T lymphocytes; Cytokines; Reactive oxygen species (ROS); Reactive nitrogen species (RNS); Apoptosis; Necrosis

Abbreviations: KA: Kainic Acid; MLT: Melatonin; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; IL10: Interluekin 10; IL6: Interleukin 6; TNF-β: Tumour Necrosis Factor-beta; INF γ: Interferon Gamma; MAPK 14: Mitogen-activated Protein Kinase gene-14; iNOS: Inducible Nitric Oxide Synthase

Introduction

Kainic acid (KA) is an agonist for a subtype of ionotropic glutamate receptor, and administration of KA has been shown to increase production of reactive oxygen species, mitochondrial dysfunction, and apoptosis in neurons in many regions of the brain, particularly in the hippocampal sub regions of CA1 and CA3, and in the hilus of dentate gyrus (DG) [1]. This is associated with intense stimulation of microglia in the brain, monocyte infiltration, oxidative stress, induction of expression of genes such as IL-1β, TNF-α, iNOS and COX-2 and intense inflammatory responses [2-4]. High levels of free radicals are known to damage cells [5]. KA influences immune system, generates excess free radicals and reduces the levels of anti-oxidant enzymes and induces oxidative stress not only in the brain but also in the liver and kidneys of mice [6].

Melatonin is a free radical scavenger, anti-oxidant and immune modulator [7-9]. It is known to protect cells from mitochondrial dysfunction and related disorders caused by oxidative stress [10]. It is also known to ameliorate KA-induced oxidative stress, neuro-toxicity, DNA damage etc. KA causes an increase in the levels of 8-hydroxy-deoxyguanosine in brain and liver; melatonin reduces this increase [11].

Lesions induced by KA on cerebellar fastigial nucleus participates in the modulation of lymphocyte functions and that the hypothalamus and sympathetic nerves innervating lymphoid organs are involved in this neuro immunomodulation [12]. KA-induced hippocampal neurodegeneration influences adaptive immune response and B cell and T cell subsets may contribute differently to the pathogenesis [13].

IL-10 family cytokines are essential for maintaining the integrity and homeostasis of tissue epithelial layers. Members of this family can promote innate immune responses from tissue epithelia to limit the damage caused by viral and bacterial infections. These cytokines can also facilitate the tissue-healing process in injuries caused by infection or inflammation [14].

Fundamental inflammatory responses such as the induction of cyclooxygenase type 2, increases expression of adhesion molecules, or synthesis of nitric oxide are indistinguishable responses of both IL-1 and TLR ligands. Both families nonspecifically affect antigen recognition and lymphocyte function. IL-1 beta is the most studied member of the IL-1 family because of its role in mediating autoinflammatory diseases [15]. IL-6 exerts stimulatory effects on T- and B-cells, thus favoring chronic inflammatory responses [16]. MCP-1-induced ICAM-1 expression was predominantly dependent on NF-kappa b activation [17].

Nitric oxide (NO) is an inflammatory mediator, which acts as a

cytotoxic agent and modulates immune responses and inflammation p38 mitogen-activated protein kinase (MAPK) signal transduction pathway is activated by chemical and physical stress and regulates immune responses [18]. Studies have shown that p38 MAPK pathway regulates NO production induced by inflammatory stimuli [19].

The rational of this study was to elucidate the effect and mechanism of action of KA induced inflammatory effect on splenic lymphocytes and enriched T lymphocytes, ensuing attenuation by the treatment of melatonin.

The effect of KA on mRNA expressions of cytokines IL-10, IL-6, TNF-β and INF γ on splenic lymphocytes and enriched T lymphocytes isolated from spleen is not well established. Therefore, influence of KA on these cytokines, signaling pathways involved during inflammation and the role of melatonin on the same was also studied.

Methods

Chemicals and reagents

Agarose, Kainic acid and Melatonin were procured from Sigma Chemical Co, St Louis, Missouri, USA; RNase, Ethanol, Bromophenol blue, Ethidium bromide, 100 bp DNA ladder and Taq Polymerase was purchased from Fermantas, USA; Mouse oligonucleotide primers for interleukin-1(IL 1), mitogen-activated protein kinase gene-14 (MAPK 14), interleukin-6 (IL 6), interleukin-10 (IL 10), inducible nitric oxide synthase (iNOS), interferon-gamma (INF γ), tumour necrosis factor beta (TNF β) and gylceraldehyde phosphate dehydrogenase (GAPDH) were procured from Eurogentec, Belgium; RPMI-1640 medium from Himedia, Mumbai, India; RT^2 SYBR green ROX q PCR master mix, RNeasy lipid tissue mini kit, Qiazol lysis reagent and RT^2 first strands kit from Qiagen, Hilden, Germany; GenElute mammalian genomic DNA Miniprep kit from Sigma/Aldrich, USA; Griess reagent from Sigma/Aldrich, USA; Mouse T cell enrichment kit catalog# 19751 A (Easy Sep) from Stem Cell Technologies Inc, Canada; ^3H thymidine from BRIT, India. All the other chemicals used were of analytical grade and were purchased from qualified local vendors.

Animals

Swiss albino mice were selected for this study. The animals were procured from the Division of Laboratory Animal Sciences of Biomedical Technology Wing (BMT), Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Thiruvananthapuram, Kerala, India with prior approval from Institutional Animal Ethics Committee and in accordance with approved Institutional protocol. Animals were maintained in a 12 h light and dark cycle at controlled environmental conditions of temperature $(22 \pm 3°C)$ and humidity (50-70%). They were fed with standard pellet diet and provided water *ad libitum*.

All animals were handled with care, without causing pain or distress. The care and management of the animals comply with the regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

Collection of splenic lymphocytes and enriched T lymphocytes

Six healthy swiss albino mice were sacrificed by cervical dislocation. Spleen was dissected out immediately and washed with sterile Phosphate Buffered Saline (PBS). The splenic lymphocytes were collected by disrupting the isolated spleen in PBS as described elsewhere [20]. The enriched T lymphocytes were isolated from the single cell suspension of the splenic lymphocytes, isolated as above in

the automated cell separator (ROBOSEP) using the kit according to the manufacture's protocol.

Experimental design

The study was an *in vitro* approach. Briefly, single cell suspension of splenic lymphocytes was divided in to several groups; each group contained 1 ml of the splenic lymphocytes single cell suspension in triplicate. The concentration of kainic acid and melatonin were fixed according to the authors earlier studies [20-33]. First group I was kept as normal control 9 without any treatment); group II was treated with 1 mM of kainic acid alone (KA); group III was treated with 1 mM melatonin alone (MLT); group IV was treated with KA (1 mM)+MLT (0.25 mM) in combination; groups V was treated with KA (1 mM)+MLT (0.5 mM) in combination, groups VI was treated with KA (1 mM)+MLT (1 mM) in combination. In all the combination groups of KA and MLT, MLT was added simultaneously together with the KA [20-33].

Similar experiment was carried out independently with enriched T lymphocytes isolated from the single cell suspension of splenic lymphocytes as mentioned above. All the above groups were used to determine various biochemical and molecular parameters.

Tritiated (3H) thymidine incorporation assay using splenic lymphocytes and enriched T lymphocytes

Isolated spleen from mice (as mentioned above) was used for the isolation of splenic lymphocytes and enriched T lymphocytes to study the cell proliferation by tritiated thymidine incorporation assay in cell culture. Viability of splenic lymphocytes and enriched T lymphocytes were assessed using trypan blue dye exclusion method [21]. Splenic lymphocytes and enriched T lymphocytes were cultured at a density of $2×10^5$ and $1×10^4$ cells/ml respectively in a 96 well plate with RPMI-1640 medium supplemented with 10% FBS, streptomycin (100 μg/ml) and penicillin (100 Units/ml) for 24 h at 37°C in a CO_2 incubator. After 24 h of incubation the cultured wells were treated with1 mg/ml physiological saline (normal control), kainic acid alone (KA-1 mM), melatonin alone (MLT-1 mM), KA and MLT in combinations in various concentrations (as mentioned in experimental design) were added in the respective 96 well plate. After 48 h of incubation at 37°C, the culture treated wells were labeled with 3H-thymidine at a concentration of 1 μci/ml and incubated further for 24 h at 37°C. Cells were harvested after 72 h and radioactivity in terms of counts per minute (cpm) were measured by Liquid Scintillation Counter (Triathler, Hidex).

Real time PCR analysis for determining m-RNA expression of cytokines, iNOS and MAPK 14 gene on splenic lymphocytes and enriched T lymphocytes

Single cell splenic lymphocytes suspension and isolated enriched T lymphocytes were cultured separately at a density of $2×10^5$ and $1×10^4$ cells/ml respectively in a 35 cm^2 culture dish with RPMI-1640 medium supplemented with 10% FBS, streptomycin (100 μg/ml) and penicillin (100 Units/ml) for 24 h at 37°C in a CO_2 incubator for the isolation of total RNA. After 24 h of incubation, various concentrations of KA and melatonin in combinations (as mentioned in Experimental Design) were added in the respective culture dish. Cells treated with 1 mg/ml physiological saline served as normal control. After 48 h of incubation, total RNA was isolated from cultured splenic lymphocytes and enriched T lymphocytes using RNeasy lipid tissue mini kit (Qiagen, Germany). Following the manufacture's protocol quantity and purity was analyzed spectrophotometrically. 100 ng of mRNA were used for cDNA synthesis of IL-6, IL-10, IL- 1, TNF-β, MAPK 14, iNOS, and GAPDH in a

reaction volume of 20 μl using RT2 first strand kit (Qiagen, Germany). The synthesis was carried out in Eppendrof master cycler, Germany.

The mouse oligo nucleotide forward and reverse primer sequence used to determine specific mRNA gene expressions were procured from Eurogenetc, Belgium are depicted in Table 1. The real time PCR reaction was carried out with RT2 SYBR Green ROX qPCR master mix (Qiagen, Germany) of total reaction volume of 25 ml; real time PCR amplifications were done using a Chromo 4 System, Bio- Rad (MJ Research, CA) for 40 cycles as per manufacture's protocol. Glyceraldehyde Phosphate Dehydrogenase (GAPDH) was used as the house keeping gene. The level of gene expression is reported as the ratio between the mRNA level of the target gene and the GAPDH, a reference gene using the comparative 2δδct method [22].

Estimation of oxidative stress by reactive oxygen species (ROS) generation by DCFH-DA (2'7'-dicholoroflorescien acetate) on splenic lymphocytes and enriched T lymphocytes

ROS generation in splenic lymphocytes and enriched T lymphocytes treated with KA, MLT and various combinations of KA and MLT combinations as mentioned in the experimental design were assayed using DCFH-DA (2'7'-dicholoroflorescien acetate), a non fluorescent, cell permeant dye. DCFH-DA is converted to fluorescent DCF by the free radicals generated by the cell during oxidative stress. This can be monitored by using fluorescent spectrophotometer. The isolated cells were treated with DCFH-DA according to the protocol of Benedetti et al. [23]. Splenic lymphocytes and enriched T lymphocytes were incubated with DCFH-DA for 30 minutes independently. The supernatant was discarded and different concentrations of KA and MLT (as mentioned in experimental design) were added and incubated for 4 h. After the period, fluorescence was read with excitation wavelength at 450 nm and emission wavelength at 535 nm.

KA alone and MLT alone treated controls were also used for the study. Untreated cells served as normal control.

Nitrite determination to assess nitrosative stress by reactive nitrogen species (RNS) generation using Griess reagent on splenic lymphocytes and enriched T lymphocytes

Production of nitric oxide free radical (NO) by inducible nitric oxide synthase (iNOS) was analyzed. Auto-oxidation of NO·results in production of nitrite which is measured by using Griess reagent [24]. Splenic lymphocytes and enriched T lymphocytes were treated with KA, MLT and various concentrations of KA and MLT in combinations as mentioned in the experimental design. Treated culture was incubated for 24 h independently. Added equal volume of Griess reagent (Sigma, USA) to the cultured cells and further incubated at room temperature for 10 minutes. Standard curve was plotted using sodium nitrite at different concentrations. Absorbance was measured at 540 nm. The concentration of nitrite in the sample was determined by the standard curve.

Live/dead/apoptotic assay using isolated splenic lymphocytes by flow cytometry ananlysis on splenic lymphocytes

Apoptosis assay was carried out to evaluate the cytotoxicity and apoptosis induced by kainic acid and the attenuation of apoptosis induced by the treatment of melatonin in various concentrations on splenic lymphocytes using Vybrant Apoptosis Assay Kit (Molecular Probes). After 72 h, culture of splenic lymphocytes and enriched T lymphocytes, cells were harvested (as described earlier) and washed with PBS later stained with Annexin V–Alexa flour 488 and Propidium iodide (PI) according to manufacturer's protocol. Analysis was carried out by Flow Cytometry and percentages of live, apoptotic and dead cells (necrotic) were estimated using BD FACS Diva software.

Statistical analysis

All the samples were run in triplicates; statistical comparison was done with three or more groups using one-way Analysis of Variance (ANOVA) followed by Dunnetts' test. P values<0.005 and <0.05 were considered significant. Kainic acid control group was compared with normal control group. Kainic acid and melatonin treated combination groups were compared with the Kainic acid control group to assess the statistical significance using ANOVA.

Results

Effect of melatonin on kainic acid induced changes in mRNA expressions of iNOS and MAPK 14 gene on splenic lymphocytes and enriched T lymphocytes from mouse spleen using real time PCR

As shown in Figure 1A, KA (1 mM) treatment resulted in a drastic increase in the levels of mRNA expression of iNOS by 5 and 4 fold in both splenic and enriched T lymphocytes respectively when compared to normal control and MLT alone treated groups. However, exogenous MLT treatment showed concentration dependent attenuation in the mRNA expression of iNOS in combination groups. KA and MLT combination group i.e, KA (1 mM)+MLT (0.25 mM)

S.No	Primer	Primer Sequence	Accession no.
1	Interleukin 10 (IL-10)	F-TAAGGCTGGCCACACTTGAG R-GTTTTCAGGGATGAAGCGGC	NM 010548.2
2	Interleukin 1 (IL-1)	F-CTCTCCCCAGCTTTTCCAGG R-TCTCTGGGCTTGACTGCTTG	NM 001177975.1
3	Tumour Necrosis Factor beta (TNF β)	F-TGCCAGCTCCAGGATTTCAG R-CTCAGCCCTCACTTGACCTG	NM 011610.3
4	Mitogen-Activated Protein Kinase 14 (MAPK14)	F-AGGCTCACGTCACCAAGTCCC R-TGGTCTCGAAAGCTACGTGGGAGG	NM 001168514.1
5	Interleukin 6 (IL-6)	F- GAGAGCATTGGAAATTGGGGTA R- GAGAGCATTGGAAATTGGGGTA	NM 031168.1
6	Inducible Nitric Oxide Synthase (iNOS)	F- CAGCTGGGCTGTACAAACCTTC R- CAGCTGGGCTGTACAAACCTTC	NM 010927.3
7	Interferon gamma (ING-γ)	F- AGGCTCACGTCACCAAGTCCC R- TGGTCTCGAAAGCTACGTGGGAGG	M 34815.1
8	Glyceraldehyde Phosphate Dehydrogenase (GAPDH)	F-GCGTGGGGACAGCCGCATCTT R-ATCGGCAGAAGGGGCGGAGA	BC 023196.1

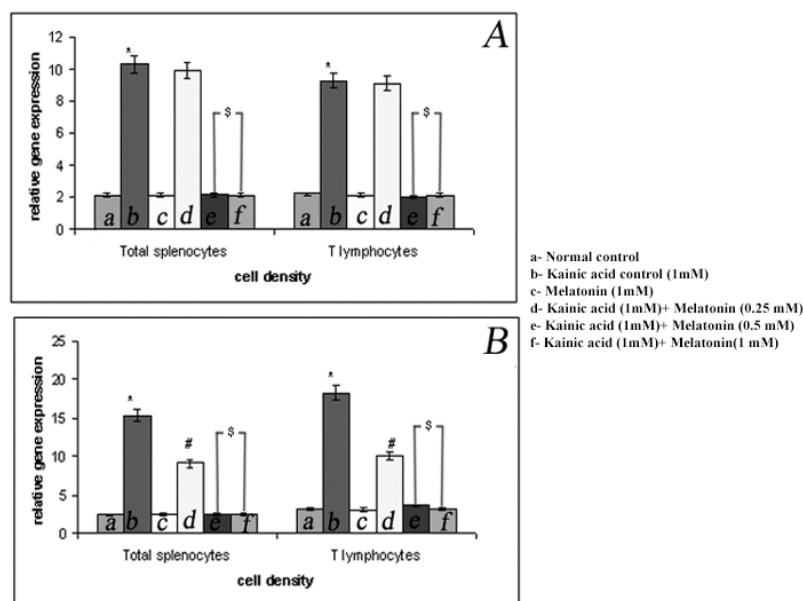**Table 1:** Semi-quantitative real time PCR primer sequences.

FIG 1

Figure 1: (A) Effect of melatonin on kainic acid induced iNOS mRNA expression on splenic lymphocytes and enriched T lymphocytes from mouse spleen using real time PCR. (a) Normal control, (b) Kainic acid control (1 mM), (c) Melatonin (1 mM), (d) Kainic acid (1 mM)+Melatonin (0.25 mM), (e) Kainic acid (1 mM)+Melatonin (0.5 mM), (f) Kainic acid (1 mM)+Melatonin (1 mM). Values are mean ± S.D; *$P<0.005$ (compared to respective normal control); $P<0.005$ (compared to Kainic acid control). n=3 in each group. (B) Effect of melatonin on kainic acid induced alterations on MAPK 14 gene mRNA expression of splenic lymphocytes and enriched T lymphocytes from mouse spleen using real time PCR. (a) Normal control, (b) Kainic acid control (1 mM), (c) Melatonin (1 mM), (d) Kainic acid (1 mM)+Melatonin (0.25 mM), (e) Kainic acid (1 mM)+Melatonin (0.5 mM), (f) Kainic acid (1 mM)+Melatonin (1 mM). Values are mean ± S.D; *$P<0.005$ (compared to respective normal control); #$P<0.05$(compared to Kainic acid control) and $P<0.005$ (compared to Kainic acid control). n=3 in each group.

showed no significant changes in the iNOS mRNA expression when compared with KA (1 mM) alone treated group in both splenic and enriched T lymphocytes. However higher concentrations of KA and MLT combination groups, KA (1 mM)+MLT (0.5 mM) and KA (1 mM)+MLT (1 mM) showed significant attenuation in iNOS mRNA expression when compared with KA (1 mM) alone treated group by 4 fold in both splenic and enriched T lymphocytes (Figure 1A).

As shown in Figure 1B, KA alone (1 mM) treated group significantly increased MAPK 14 gene mRNA expression of by 4 fold when compared to normal control group. Whereas KA treated lymphocytes when combined with MLT at various concentrations showed a concentration dependent reduction in MAPK 14 gene mRNA expression levels. However KA (1 mM)+MLT (0.25 mM) group did not show significant changes in the MAPK 14 gene mRNA expression levels when compared with KA (1 mM) alone treated group. Whereas higher concentrations of KA and MLT combination groups, KA (1 mM)+MLT (0.5 mM) and KA (1 mM)+MLT (1 mM) showed significant attenuation by 5 and 6 fold in MAPK 14 gene mRNA expression levels when compared with KA (1 mM) alone treated group in both splenic and enriched T lymphocytes respectively (Figure 1B).

Kainic acid- induced alterations in the mRNA expression of various cytokines (IL-6, IL-1, IL-10, TNF-β, INF-γ) on splenic lymphocytes and enriched T lymphocytes isolated from mouse spleen and cytokine attenuation by melatonin treatment

From Figures 2A and 2B, it is evident that treatment of splenic and enriched T lymphocytes with KA (1 mM) resulted in a remarkable decrease in the levels of IL-10 mRNA expression an anti inflammatory

marker; whereas the mRNA expression of TNF-β was increased by 4 and 3 fold respectively in both the cell systems when compared to normal control. The observations also showed a 4 and 3 fold significant increase in the IL-1 mRNA expression in KA alone treated groups when compared with normal control group in both splenic and enriched T lymphocytes respectively. IL-6 and INF γ mRNA expressions were also significantly increased in both the cell systems in KA (1 mM) alone treated groups by 3 and 2 fold respectively than normal control (Figures 2A and 2B).

Interestingly, MLT treatment in the combination groups attenuated these changes in mRNA expression in a concentration dependent manner. KA+MLT (1 mM) completely prevented KA-induced changes in the mRNAs expression of the IL-1, IL-6, TNF-β, INF-γ when compared with KA (1 mM) alone treated groups in a significant manner ($P<0.005$) in both splenic and enriched T lymphocytes. Whereas IL-10 expression in these groups was significantly elevated by 5 and 4 fold in both splenic and enriched T lymphocytes respectively.

However, KA+MLT (0.5 mM) and KA+MLT (0.25 mM) partially prevented KA-induced changes in the m-RNA levels. IL-6, IL1, TNF-β, INF-γ mRNA expression were decreased by 2 fold in splenic lymphocytes and by 3 fold enriched T lymphocytes respectively. Similarly, IL-10 mRNA expression was elevated by 2 fold in both splenic and enriched T lymphocytes.

KA (1 mM)+MLT (0.25 mM) did not show any significant changes in the proinflammatory cytokine i.e, IL-6, IL1, TNF-β, INF-γ mRNA expression when compared with KA (1 mM) alone treated group.
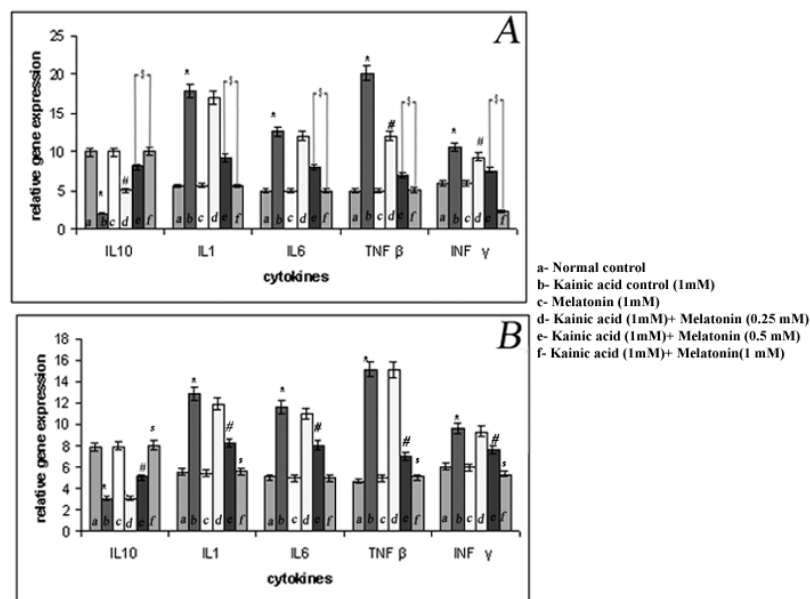
Figure 2: (A) Kainic acid-induced alterations in the expression of various cytokine (IL-10, IL-1, IL-6, TNF-β, INF-γ) mRNA from splenic lymphocytes isolated from mouse spleen and their attenuation by melatonin treatment using real time PCR. (a) Normal control, (b) Kainic acid control (1 mM), (c) Melatonin (1 mM), (d) Kainic acid (1 mM)+Melatonin (0.25 mM), (e) Kainic acid (1 mM)+Melatonin (0.5 mM), (f) Kainic acid (1 mM)+Melatonin (1 mM); IL10-interleukin 10; IL6-interleukin 6; TNF-β: tumour necrosis factor beta; INFγ: interferon gamma. Values are mean ± S.D; *P<0.005(compared to respective normal control); #P<0.05 (compared to Kainic acid control) and $P<0.005 (compared to Kainic acid control). n=3 in each group. (B) Kainic acid-induced alterations in the expression of various cytokine (IL-10, IL-1, IL-6, TNF-β, INF-γ) mRNA from enriched T lymphocytes isolated from mouse spleen and their attenuation by melatonin treatment using real time PCR. (a) Normal control, (b) Kainic acid control (1 mM), (c) Melatonin (1 mM), (d) Kainic acid (1 mM)+Melatonin (0.25 mM), (e) Kainic acid (1 mM)+Melatonin (0.5 mM), (f) Kainic acid (1 mM)+Melatonin (1 mM); IL10-interleukin 10; IL6-interleukin 6; TNF-β: tumour necrosis factor beta; INFγ: interferon gamma. Values are mean ± S.D; *P<0.005 (compared to respective normal control); #P<0.05 (compared to Kainic acid control) and $P<0.005 (compared to Kainic acid control)). n=3 in each group.
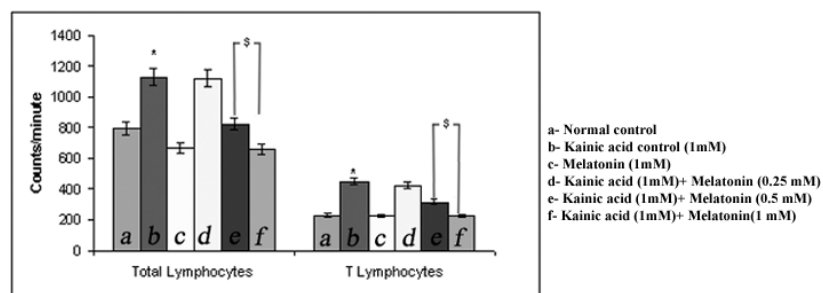

Figure 3: Effect of melatonin on kainic acid induced variations in 3H thymidine incorporation on splenic lymphocytes and enriched T lymphocytes isolated from mouse spleen. (a) Normal control, (b) Kainic acid control (1 mM), (c) Melatonin (1 mM), (d) Kainic acid (1 mM)+Melatonin (0.25 mM), (e) Kainic acid (1 mM)+Melatonin (0.5 mM), (f) Kainic acid (1 mM)+Melatonin (1 mM). Values are mean ± S.D; *P<0.005 (compared to respective normal control); $P<0.005 (compared to Kainic acid control). n=3 in each group.

Effect of melatonin on kainic acid induced variations in 3H thymidine incorporation on splenic lymphocytes and enriched T lymphocytes isolated from mouse spleen

KA (1 mM) treatment increased ^{3}H-Thymidine incorporation in both splenic lymphocytes and enriched T lymphocytes. MLT, in a concentration dependent manner attenuated this increase in ^{3}H-Thymidine incorporation. KA-induced modulation of ^{3}H-Thymidine incorporation was significantly suppressed by MLT treatment in the combination groups i.e, KA (1 mM)+MLT (0.5 mM) and KA (1 mM)+MLT (1 mM) by 2 fold when compared with KA (1 mM) alone treated group in both splenic and enriched T lymphocytes.

While lower concentration of MLT, KA (1 mM)+MLT (0.25 mM) showed no significant effect compared to KA (1 mM) alone treated group in both splenic and enriched T lymphocytes on ^{3}H-Thymidine incorporation (Figure 3).

Counter effect of melatonin on ROS and RNS production induced by kainic acid on splenic lymphocytes and enriched T lymphocytes isolated from mouse spleen

KA (1 mM) treatment increased oxygen free radicals significantly in both splenic lymphocytes and enriched T lymphocytes. MLT in a concentration dependent manner attenuated this increase in reactive
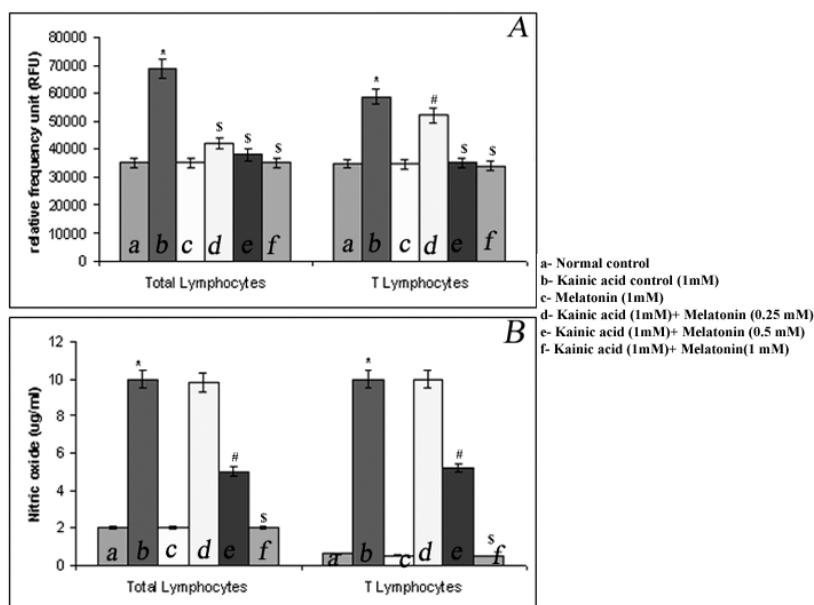
Figure 4: (A) Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay to assess the counter effect of melatonin on ROS production induced by kainic acid on splenic lymphocytes and enriched T lymphocytes isolated from mouse spleen. (a) Normal control, (b) Kainic acid control (1 mM), (c) Melatonin (1 mM), (d) Kainic acid (1 mM)+Melatonin (0.25 mM), (e) Kainic acid (1 mM)+Melatonin (0.5 mM), (f) Kainic acid (1 mM)+Melatonin (1 mM). Values are mean ± S.D; *P<0.005 (compared to respective normal control); #P<0.05 (compared to Kainic acid control) and $P<0.005 (compared to Kainic acid control). n=3 in each group. (B) Determination of Nitrite production (RNS) by Griess reagent induced by kainic acid on splenic lymphocytes and enriched T lymphocytes isolated from mouse spleen and its counter effect by melatonin treatment. (a) Normal control, (b) Kainic acid control (1 mM), (e) Melatonin (1 mM), (d) Kainic acid (1 mM)+Melatonin (0.25 mM), (e) Kainic acid (1 mM)+Melatonin (0.5 mM), (f) Kainic acid (1 mM)+Melatonin (1 mM). Values are mean ± S.D; *P<0.005 (compared to respective normal control); #P<0.05 (compared to Kainic acid control) and $P<0.005 (compared to Kainic acid control). n=3 in each group.
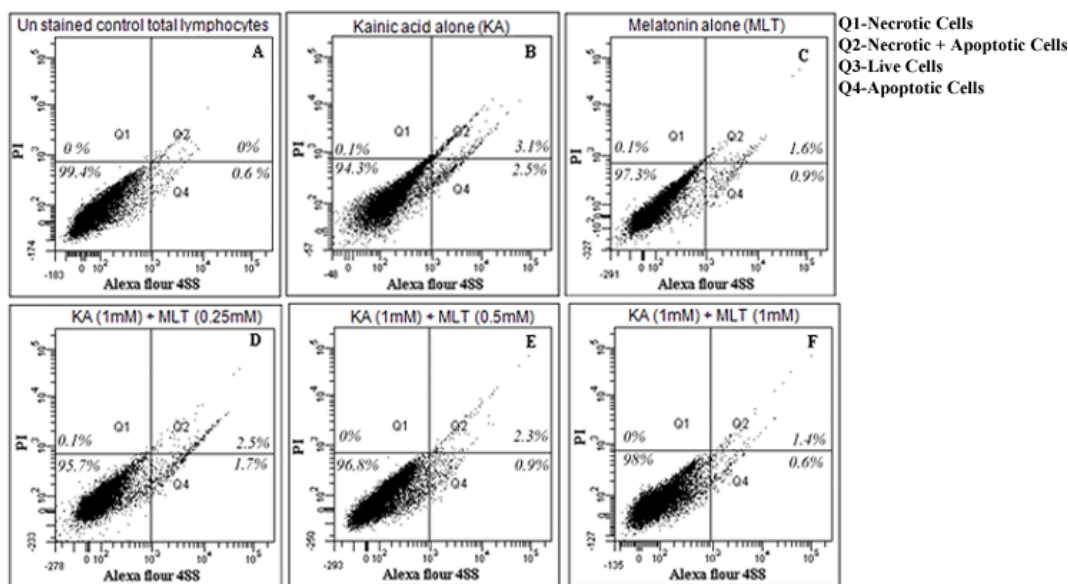

Figure 5: Effect of melatonin on kainic acid induced apoptosis and necrosis on splenic lymphocytes isolated from mouse spleen by Annexin V assay using flow cytometry. (A) Un-stained splenic lymphocytes control; (B) Kainic acid control (1 mM); (C) Melatonin (1 mM); (D) Kainic acid (1 mM)+Melatonin (0.25 mM); (E) Kainic acid (1 mM)+Melatonin (0.5 mM); (F) Kainic acid (1 mM)+Melatonin (1 mM). Repeated thrice for statistical evaluation.

oxygen species (ROS) (Figure 4A). Whereas KA (1 mM)+MLT (0.25 mM) showed moderate significant changes (P<0.05) in the ROS production when compared with KA (1 mM) alone treated group in both splenic and enriched T lymphocytes. However higher concentrations of

KA and MLT combination groups, KA (1 mM)+MLT (0.5 mM) and KA (1 mM)+MLT (1 mM) showed significant 2 fold decrease ROS production in when compared with KA (1 mM) alone treated group by 4 fold in splenic lymphocytes. ROS production by KA (1 mM)+MLT

(0.5 mM) and KA (1 mM)+MLT (1 mM) in enriched T lymphocytes showed a more than 2 fold (P<0.005) decrease ROS production in when compared with KA (1 mM) alone treated group.

Similarly nitrite production was also increased in both splenic lymphocytes and enriched T lymphocytes by KA (1 mM) treatment. MLT suppressed the reactive nitrogen species (RNS) generation by KA in a concentration dependent manner (Figure 4B). Whereas, KA (1 mM)+MLT (0.25 mM) showed no significant changes in the RNS generation when compared with KA (1 mM) alone treated group in both splenic and enriched T lymphocytes. However higher concentrations of KA and MLT combination groups, KA (1 mM)+MLT (0.5 mM) and KA (1 mM)+MLT (1 mM) showed significant decrease RNS production in when compared with KA (1 mM) alone treated group in both splenic lymphocytes and enriched T lymphocytes (P<0.005) respectively (Figure 4B).

Effect of melatonin on Kainic acid induced apoptosis and necrosis on splenic lymphocytes isolated from mouse spleen by Annexin V assay using flow cytometry

The Flow Cytometry analysis (Figure 5) for apoptosis using Annexin V assay revealed that KA (1 mM) induced apoptosis, precisely necrosis in splenic lymphocytes. MLT alone and in combination groups abolished apoptosis and necrosis (double positive cells Q2) in a concentration dependent manner (Figures 5A-5E). Similarly double positive cells (Q2) were also increased in splenic lymphocytes by KA (1 mM) treatment. MLT suppressed the necrosis and apoptosis of cells induced by KA in a concentration dependent manner (Figure 5). KA (1 mM)+MLT (0.25 mM) showed moderate changes in necrosis and apoptosis (Q2) and apoptosis alone (Q4) of cells (P<0.05) when compared with KA (1 mM) alone treated group in splenic lymphocytes. However higher concentrations of KA and MLT combination groups, KA (1 mM)+MLT (0.5 mM) and KA (1 mM)+MLT (1 mM) showed significant decrease in necrosis and apoptosis (Q2) and apoptosis alone (Q4) of cells (P<0.005) when compared with KA (1 mM) alone treated group.

Discussion

Kainic acid is a neurotoxin isolated from sea weed. When injected into animals it mimics the action of the excitatory amino acid neurotransmitter, glutamate [25]. Kainic acid produces acute and subacute epileptiform activity that can last for days. Ultimately, it causes widespread irreversible neuropathological changes that involve both neurons and glia [26]. Kainic acid binds to and activates a subtype of ionotropic glutamate receptor. In addition to inducing brain lesions directly, probably *via* processes involving free radicals, kainic acid also provokes the discharge of potentially neurotoxic quantities of glutamate from nerve endings [27].

In earlier studies the authors have demonstrated KA induced deleterious effects on the antioxidant enzymes as well as on antioxidant parameters both *in vitro* and *in vivo* on brain tissue. Such an effect was abolished with MLT treatment in a concentration dependent manner. The study also showed KA induced alterations in cytokine mRNA expression as well as nucleotide alterations were observed in the cytochrome b sequence of mouse mtDNA. These effects were attenuated by MLT treatment in a concentration dependent manner [20]. The immune modulatory effect of KA and MLT on lymphocytes is not well documented yet. In the present study authors provide valuable information on the effect of KA on lymphocytes.

Given that free radicals are likely involved in the neurodestructive

processes of kainic acid, soon after melatonin was discovered as a free radical scavenger, it was tested for its antiexcitatory efficacy [28].

The authors report here that KA had drastic deleterious effect on lymphocytes which was attenuated by the treatment of MLT. In this study the authors documented that melatonin did not directly inhibit the action of kainic acid on lymphoctes but had an anti-inflammatory and antioxidant mechanism which was evident by the expression mRNA of MAPK 14, iNOS and other inflammatory cytokine levels, However attenuation of free radical species and proinflammatory cytokines was resulted by MLT treatment in a concentration dependent manner. *In vivo* as well, melatonin reduced the ability of kainic acid to kill neurons in both the cerebellum and in the hippocampus [29,30]. Moreover, melatonin reduced the associated neural lipid peroxidation as well as preventing the death of the majority of the rats that received kainic acid [31]. The authors of these reports were convinced that the beneficial effects of melatonin against neurotoxicity of kainic acid were due to melatonin's ability to readily enter the brain and scavenge free radicals and stimulate antioxidant enzyme activities in the affected neurons. The ability of melatonin to shield these neurons from free radical damage is of particular interest since pyramidal neurons are also lost, via mechanisms involving oxidative and nitrosative stress, in a variety of neurodegenerative diseases. In cerebellar granule neurons, Dabbeni-Sala et al. [32] found that kainic acid increased mitochondrial free radical generation (identified with 2',7'-dichlorofluorescein) and impaired the function of complex II of the respiratory chain. Moreover, kainate promoted the activity of nitric oxide synthase thereby elevating the production of NO• and ultimately of ONOO-. In this series of *in vitro* studies, melatonin counter acted oxidative and nitrosative stress associated with the exposure of cerebellar granule neurons to KA.

The authors of the present study have reported that KA induces ROS and RNS production in splenic lymphocytes and enriched T lymphocytes. MLT proved effective in combating both the oxidative and nitrosative stress associated with the exposure of splenic lymphocytes and enriched T lymphocytes to KA.

Further, studies on C57BL/6 mice lacking specific T cell populations (CD4, CD8, and CD4/CD8 cells) and B cells [Igh-6(-/-)] demonstrated that influence of the adaptive immune response on KA-induced hippocampal neurodegeneration and suggest that B cell and T cell subsets may contribute differently to the pathogenesis induced by KA [10].

Confirmation of mitochondrial level of action of melatonin during excitotoxicity was provided by the observations that melatonin also prevented mitochondrial DNA damage [10,32] while also reducing seizures that often accompany kainic acid administration [33]. Neuronal death that occurs in these situations is presumably the result of apoptosis.

Chuang et al. [34] provided direct evidence that in fact apoptosis was the process that leads to cellular implosion after kainate is given. Three days following the intrastriatal injection of kainic acid, these workers noted the dramatic cytotoxic action of the drug along with elevated Bcl-2 immunoreactivity in TUNEL-positive cells. Although detectable, less severe damage was seen in the ipsilateral substantia nigra. Melatonin reduced Bcl-2 expression and preserved neuronal viability (reduced the number of TUNEL-positive neurons). Similar effects of melatonin on Bcl-2 expression in kainic acid-treated rat hippocampal neurons were reported by Yalcin and colleagues [35]. In addition to reducing hippocampal neuronal death due to its direct scavenging activity, melatonin may be protective since it stimulates the antioxidative enzymes, SOD and catalase, in these cells as well [36].

CD4/CD8-deficiency and increased age prevented KA-induced increase of both locomotor and rearing activities. The results suggest that a decline of immunological function is associated with aging, and both of them may contribute to the relative resistance to KA-induced neurotoxicity [37].

Chung and Han [10] surmised that the toxicity of kainic acid in relation to selective neuronal loss in the hippocampus may involve activation of microglia. Thus, besides enhancing the release of excessive amounts of the excitatory amino acid neurotransmitter, glutamate, which leads to damage of the post synaptic neuron, activates microglial cells and the production of ROS which would further exaggerate the neuronal damage kainic acid may also induce oxidative and nitrosative stress on splenic lymphocytes and enriched T lymphocytes. Also, antioxidant properties of exogenous melatonin can be a remedy to this effect of KA on lymphocytes.

Conclusion

The study clearly depicts that KA induces oxidative damage to splenic lymphocytes and enriched T lymphocytes in the form of apoptosis and inflammation which was evident by expression of various cytokines and spleen DNA integrity. This may lead to mutations and altered protein synthesis in due course of time. It is of interest to note that MLT has a protective action on the damage induced by KA by protecting the lymphocytes from such alterations. Hence exogenous MLT can be used as a therapeutic agent against immune toxicity by scavenging the free radical production induced by KA.

Disclosures

The authors have no financial conflict of interest.

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