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MELATONIN AGAINST NEUROINFLAMMATION VIA NF-κB PATHWAY IN SH-SY5Y CELL LINE: A SYSTEMATIC REVIEW

MRINALINI R, SUMITHRA M* AND CHITRA V

Department of Pharmacology, SRM College of Pharmacy, SRM Institute of Science and Technology, Kattankulathur, Chengalpettu – 603203, India

***Corresponding Author: Dr. Sumithra Mohan: E Mail: sumi26379@gmail.com**

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ABSTRACT

Melatonin has a great potential towards the treatment and prevention of neuroinflammation-associated neuro-disorders. This systematic review aims to determine the various effects and activities exhibited by melatonin in a human neuroblastoma cell line like SH-SY5Y. As per the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) parameters, this systematic review was performed by employing electronic databases like PubMed, Science Direct, and Google Scholar, to choose articles, which evaluated the anti-inflammatory activities of melatonin on SH-SY5Y. 11 studies were chosen from 11,836, which met the parameters of the inclusion criteria and were included for this systematic review. All studies considered proved melatonin's potential against neuroinflammation by showing anti-inflammatory effect in the treated SH-SY5Y cell line.

Keywords: Melatonin, neuroinflammation, NF-κB, SH-SY5Y, systematic review

INTRODUCTION

Neuroinflammation is a wide range of immune reactions of the CNS (central nervous system), primarily involving neuroglia cells like microglia and astrocytes. The BBB (blood-brain-barrier), a super-specialized endothelium in the brain, is permeable to pro-inflammatory mediators derived from peripheral inflammation, and is also capable of both releasing and transmitting such mediators while allowing leucocyte migration into the brain **[1, 2]**. Such neuro-inflammatory response leads to an impairment in synapse, neuronal cell death and exacerbation of neurodegenerative diseases **[3-5]**. Neuroinflammation is a trickier condition to resolve due to its dual functions in human body. Inflammation clears and controls the original insult, for example, via phagocytosis and/or inflammasome activation, apoptosis **[6]**. However, although it is beneficial, an exceeding amount of neuroinflammation can cause tissue damage in itself and propagate disease pathology. This is because, on activation, neuro-inflammatory mediators not only target the initial inflammation site, but also nearby and surrounding sites **[7]**. Hence, neuroinflammation can neither be completely eliminated nor retained for its both a friend and foe.

Neuroinflammation is characterized by an abnormal activation of microglia. This activation is the cause for the pathology of various neurodegenerative disorders, namely Alzheimer's disease (AD), Multiple Sclerosis (MS), Parkinson's disease (PD), meningitis, and brain trauma. Neuroinflammation is also seen commonly in lifestyle-oriented disorders like stress, schizophrenia, depression, hypertension, Type II Diabetes Mellitus, and obesity **[8]**. Microglia aging accelerates the aging of brain as well, often seen to lead to cognitive decline during old age as well as in dementia-associated AD **[9]**. Chronic neuroinflammation stimulates this microglial aging, sometimes even during the earlier human years, proving the occurrence of neurodegenerative diseases at a much earlier age than usually seen. This is exactly why we need nutrients or drugs to delay brain aging by reversing/preventing brain aging. However, the drawback of therapeutically dealing with neuro-disorders is that neuroinflammatory responses begin way earlier, before the symptoms begin to arise, by when the disease is already progressed into a clinically significant form **[10]**. Almost all neurodegenerative diseases have neuronal cells degenerating, eventually where the whole brain size shrinks. As a

result of which, the brain will possess hardly any nerve cells than earlier with much lesser mitochondrial function and lose of synaptic connections in-between every neurones. Oxidative stress develops in the due course followed by mitochondrial dysfunction rendering the neurons of patients vulnerable **[11]**. This is why evaluating ROS (Reactive Oxygen Species) and mitochondrial parameter are important for studying neuroinflammation studies. In microglial cells, mitochondrial dysfunction causes excessive ROS generation, thereby promoting a redox imbalance, hence inducing the pro-inflammatory gene transcription while releasing cytokines, like IL-1β, TNF-α, and IL-6, which is why evaluating the levels of these cytokines are important for studying neuroinflammation. Most antioxidant agents inhibit the generation of TNF-α, IL-6 and Nitric Oxide in a concentration-dependent manner **[12]**.

There are many pathways associated with neuroinflammation, however it's the nuclear factor kappa enhancer binding protein (NF-κB) pathway that happens to be the most basic and most common pathway involved with neuroinflammation from all etiologies. TLRs are vital signal transduction membrane proteins, which are the first line of defense, a wide range of which is expressed in microglia. TLR activation results in phagocytosis of any invading pathogen. These receptors are made of some conserved structural domains, containing both ligand as well as co-receptor's binding sites. TLRs recognize specific ligands and then start the inflammatory response, by activation of signaling molecules like the NF- κB. This activation causes microglial activation, release of cytokines and expression of pro-inflammatory molecules. TLRs possess an extracellular LRR (leucine rich repeat) domain for recognizing specific pathogens, and a TIR (Toll/IL-1 receptor) domain for the signaling process. MyD88 (Myeloid differentiating factor 88), an adaptor protein, binds to TLR through TIR, thus activating various signal transduction pathways, leading to NF-κB activation-induced inflammation **[13]**.

Except for TLR3, all other TLRs are activated by MyD88; in fact, MyD88 can even be restricting the TLR3-mediated signaling. MyD88 pathway is a major contributor towards astrocyte activations in CNS infections, optic nerve injury, AD, and PD. MyD88 might be also involved in optic nerve injury, PD and AD **[13, 14]**. This whole signaling cascade is called TLR4/MyD88/NF- κB **[15]**. NF-κB regulation is based on its nuclear

translocation while associated with IκBα inhibitory molecule. NF-κB dimers are in passive form in the cytoplasm, when associated with the IκB inhibitory family, however, the same NFκB dimer is by removal of IκB protein and the nuclear translocation of the liberated NF-κB dimer occurs, after proper post-translational modifications of the NF-κB occurs. This pathway is activated, by ligands like Tumour Necrosis Factor-alpha (TNF-α) and/or Interleukin 1β (IL-1β), which ironically are also the end products of this pathway. In the due course of the kinase signaling cascades, a ternary IKK complex stimulates the phosphorylation of IκBα inhibitory protein, causing its ubiquitination, and eventually proteasome-induced degradation. The interaction among IκBα and NF-κB is deranged, hence the latter is liberated, followed by its nuclear translocation. Once inside the nucleus from the cytoplasm, NF-κB binds to certain promoter DNA sequences, which activates the expression of all pro-inflammatory genes **[15, 16]**.

Because of its importance in regulating pro-inflammatory gene expression, various techniques have been used or developed for monitoring NF-κB activity at different steps and stages of the activation cascade. Such techniques or parameters that will help us determine the activation of NF- κB activation is discussed here, and further tabulated in this systematic review. For instance, NF-κB and IκB undergo various site specific post-translational modifications such as acetylation, phosphorylation and/or ubiquitination; these can be employed in measuring NF-κB activation. These modifications serve a vital mechanism for regulating NF-κB activation and cessation, while recruiting other transcriptional regulators **[17-19]**. All processes of modifications like phosphorylation, ubiquitination and acetylation, can be measured using Western Blot analysis, with specific-antibodies for the specific proteins. A key function of NF-κB, which is measured is the DNA binding at the κB enhancer motifs present in the NF-κB target genes **[20]**. Such DNA-protein binding is measurable by electro-mobility shift assay **[21]**, or by improved quantitative ELISA No-Shift assay **[22]**. There are many chromatin immunoprecipitation methods, which use sequence-specific NF-κB antibodies for isolating the NF-κB-DNA complexes, which can then be characterized using Polymerase Chain Reaction or using chromatin immunoprecipitation-sequence to give a genomic level of measuring the binding of NF-κB at all regulatory locus **[23, 24]**. Yet

another method of measuring NF-κB activation at a transcriptional level is the gene reporter assay, where an exogenous NF-κB promoter sequence linked to a quantifiable reporter gene like the luciferase is introduced **[25]**. Since NF-κB nuclear translocation needs degradation of IκB, monitoring the movement of NF-κB from cytoplasm to nucleus is helpful in measuring NF-κB activity. Two widely popular techniques to test NF-κB translocation are the Cell fractionation and the Image-based tracking of NF-κB proteins. The former technique is where, the cytoplasmic and nuclear fractions are isolated and subjected to Western Blotting for quantifying NF-κB protein levels **[26-29]**. The latter technique is where in which the dynamics of NF-κB is monitored by antibody-staining or by NF-κB-fused fluorescent protein **[30-32]**.

Traditional methods used in determining NF-κB nuclear translocation lack statistical robustness as is the case with microscopy method or they lack the capability to discern heterogeneity within the sampled populations as is the case with Western blotting and Gel Shift assays. New techniques like the ImageStream platform combines both the high image content obtained from microscopy along with the high throughput and multi-parameter analysis obtained from flow cytometry, thus overcoming the drawbacks of conventional assays. Hence, moving onto such new techniques is most essential for a more consistent and accurate results **[32]**.

Melatonin (MLT) is a neuroimmunoendocrine hormone synthesized from the pineal gland during the dark or no light period. MLT's secretion is associated with circannual and circadian rhythm regulation. Several studies have demonstrated the activity of MLT in reducing chronic as well as acute inflammation. The immunomodulatory effects of MLT are well-established; through regulation of cytokine production in immune-competent cells. MLT also decreases adhesion molecules as well as the pro-inflammatory cytokines and also alters the serum inflammatory parameters. As a reason of which, MLT ameliorates the clinical illnesses with an inflammatory etiology. Also MLT possesses a supportive action to being anti-inflammatory, by acting as both direct and indirect anti-oxidant, by either scavenging the free radicals or by stimulating antioxidant enzymes, also by potentiating the effects of other anti-oxidants or by protecting other anti-oxidant enzymes from the toxic effects of oxidative damage. MLT has even been implicated as a neuro-protectant in

neurodegenerative disorders. The effect of MLT on various neurological diseases with inflammatory pathologies, like dementia, dementia-induced Alzheimer disease, Parkinson disease, multiple sclerosis, stroke, CNS injuries, and brain ischemia has been reported. This implicates the role of MLT CNS-related health and diseases. The mechanism of MLT indicating such benefits is through sleep or by maintaining the circadian or biological rhythm intact. Yet, with all the information and data available, MLT is still not seen more used in a clinical setup much. Hence, this molecule was chosen to gather all the information available using it *in vitro* using the aforementioned NF-κB pathway **[33]**.

In vitro studies are the new in-trend systems for pharmacological and/or toxicological analysis. Most scientists and ethical organizations support all practices that inculcate, uplift, and follow the 4R approach (Reduce, Replace, Refine, Rehabilitate). *In vitro* studies allow a more species-specific, a simpler, a more convenient, and a whole lot more detailed analyses, which cannot be done with a whole intact organism. Just as *in vivo* studies replace human trials, *in vitro* studies replace *in vivo* studies. An *in vitro* model widely used in AD, PD, ischaemic cascade and neuroiflammation research is the neuroblastoma cell line called SH-SY5Y. This is a sub-line of SK-N-SH cell line, established in 1970 from the bone marrow of a four-year-old metastatic neuroblastoma patient **[34]**. The initial characterization of the SH-SY5Y demonstrated the cell line to possess a moderate dopamine-β-hydroxylase level with negligible choline acetyl-transferase level. The characterization also showed the cell line to have acetylcholinesterase and butyryl-cholinesterase **[34]**, with basal noradrenaline release **[35]** and tyrosine hydroxylase activity **[36]**. Hence, SH-SY5Y is the most commonly used and is one of the most leading neuro cell line used in cell culture studies **[37]**.

MATERIALS AND METHODS

Search terms

This systematic review involves original articles from the PubMed, Science Direct, and Google Scholar databases from January 2000 to January 2022. The search terms used were “Melatonin”, “SH-SY5Y”, and “Inflammation”. The articles were selected in the order of reading the title first, followed by the abstract and then the full text.

Inclusion and Exclusion criteria

The articles were chosen and excluded on the basis of the selected parameters of the inclusion and exclusion criteria **(Table 1)**.

Table 1: Inclusion criteria and exclusion criteria used to make this review

Parameter	Inclusion criteria	Exclusion criteria
Language	English	Languages other than English
Publication Type	Research articles	Review articles, editorials, and book chapters
Type of study	*in vitro*	Any other type of study (*in vivo, ex vivo)*
Cell line used	SH-SY5Y	any other cell line
Year of publication	January 2000 - January 2022	any other year/month from the range
Drug	Melatonin	any other drug
Mechanism	NF-κB	Any other transcription factors or mechanisms
Data collection resources	Databases including Science direct, PubMed, and Google Scholar.	Other than the mentioned databases and articles

Quality evaluation

The chosen articles were evaluated for their quality based on assigning quality scores to them. Quality scores were provided on the basis of Number of Gene/Protein Parameters evaluated (maximum 2 points), Cell Line tests *in vitro* (maximum 1 point), number of assays performed (maximum 2 points), type of assays performed (maximum 3 points), and use of standard controls (maximum 1 point).

Quality evaluation of the parameters

Number of Gene/Protein Parameters evaluated

- If only 5 parameters are evaluated - 1 point
- If more than 5 parameters are evaluated - 2 points

Cell Line tests in vitro

- If *in vitro* test is not done or done on other cell lines apart from SH-SY5Y - 0 point
- If *in vitro* test is done in SH-SY5Y cell line - 1 point

Number of assays performed

- If only one assay of any nature performed - 1 point
- If two or more assays of any nature performed - 2 points

Determination of pathway used

- If pathway used by the drug to obtain the end-result not determined - 0 point
- If pathway used by the drug to obtain the end-result determined - 1 point

Type of assays performed

- If the performed assay(s) is/are cellular level - 1 point
- If the performed assay(s) is/are DNA or gene expression analysis and/or cell cycle analysis - 2 points
- If the performed assay(s) are cellular, DNA, gene and protein levels - 3 points

Use of inflammation markers such as TNF, ILs in determination

- If inflammation markers were not determined - 0 point
- If inflammation markers were determined - 1 point

Quality ranges

Articles falling within the quality score ranges of 8 - 10, 5 - 7, and 0 - 4 are considered high, intermediate and low in quality respectively.

Data extraction

From each study, information such as author names, published year, country, objective and results of the study were collected and utilized to draft this systematic review.

RESULTS

Selection of studies

The preliminary search in the chosen databases resulted in 11,836 articles. No more articles were added through manual addition. After deducting the duplicates from the collecting articles, there were 11,164 articles remaining. On screening those 11,164 articles on the basis of the inclusion criteria, 11,153 articles were excluded, since they did not satisfy the inclusion criteria or met the exclusion criteria. The rest of the 11 articles were included for this systematic review and was assessed for the eligibility using the full-text. **Figure 1** depicts the work flow of the search results employed in this systematic review.

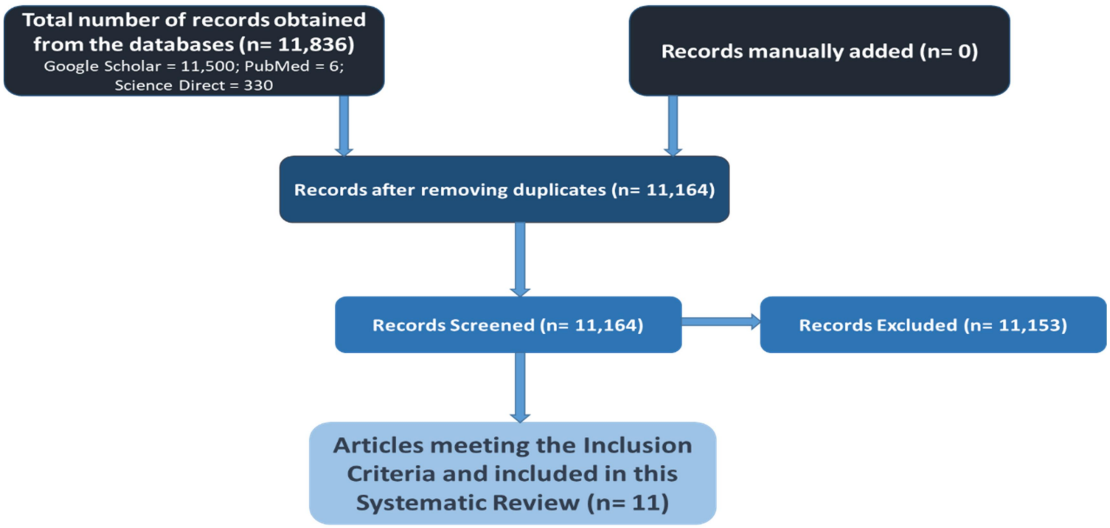

Figure 1: Systematic review study workflow

Study Characteristics

Among the 11 articles included for this review, 7 studies demonstrated the cell metabolic activity of MLT through MTT assay (Chetsawang *et al*., 2006 **[38]**; Permpoonputtana *et al*., 2012 **[39]**; Martínez

et al., 2019 **[40]**; Nopparat *et al*., 2017 **[41]**; Zhi *et al*., 2020 **[42]**; Chaudhary *et al*., 2016 **[43]**; Martínez *et al*., 2020 **[44]**). Western immunoblotting was employed by 9 studies to estimate the quantitative measurements of proteins or genes of interest (Chetsawang *et al*., 2006 **[38]**; Panmanee *et al*., 2015 **[45]**; Wongprayoon *et al*., 2015 **[46]**; Permpoonputtana *et al*., 2012 **[39]**; Nopparat *et al*., 2017 **[41]**; Zhi *et al*., 2020 **[42]**; Chaudhary *et al*., 2016 **[43]**; Nopparat *et al*., 2017 **[47]**; Sinjanakhom *et al*., 2015 **[48]**). About 7 studies employed Real Time-Polymerase Chain Reaction for evaluating the inflammation markers (Panmanee *et al*., 2015 **[45]**; Wongprayoon *et al*., 2015 **[46]**; Permpoonputtana *et al*., 2012 **[39]**; Martínez *et al*., 2019 **[40]**; Nopparat *et al*., 2017 **[41]**; Zhi *et al*., 2020 **[42]**; Martínez *et al*., 2020 **[44]**). Inducing methods such as Hydrogen Peroxide (H_2O_2) was used by 5 studies (Chetsawang *et al*., 2006 **[38]**; Panmanee *et al*., 2015 **[45]**; Nopparat *et al*., 2017 **[41]**; Nopparat *et al*., 2017 **[47]**; Sinjanakhom *et al*., 2015 **[48]**).

Results of each study

All the 11 studies involved in this review, evaluates the anti-inflammatory properties of MLT in SH-SY5Y neuroblastoma cell line. Some reports among these even included the supportive data proving the mechanism by which MLT exhibited the neuroprotective effects. **Table 2** tabulates certain significant features such as the important and main findings of each study in detail.

Quality Evaluation of the Studies

Table 3 can clearly depict the quality assessment conducted for all the 11 studies individually based on the inclusion and exclusion criteria and the quality evaluation parameters as mentioned above. The total average score was found to be 9.09±1.04. This proved that the overall data used for this review was of high quality, which we found as appropriate for drafting this systematic review.

Table 2: Main characteristics of the included studies

Reference	Country	Overall Study Objective	Neuroinflammation Tests		Markers Determined	Main findings using Melatonin against SH-SY5Y cell line
			Methods	Controls		
Chetsawang *et al*., 2006[38]	Thailand	To decipher the cellular mechanisms underlying the neuronal cell degeneration caused due to oxidative stress as well as to understand the neuro-protective roles of melatonin on cell death	Induction: H_2O_2-induced neuronal cell degeneration Tests: MTT assay, Western immunoblotting, Immunocytochemistry analysis.	Negative Control: culture medium Positive Control: H_2O_2	phosphorylated and total NF-κB, Bax, and Bcl-2	Inducing agent H_2O_2 significantly reduced cell viability and MLT reversed this. Ac-DEVD-CHO, a caspase-3 inhibitor, significantly improved cell viability in the disease control by inhibiting the enzyme caspase activity. The transcription factor, nuclear factor kappa

						B (NF-κB)'s phosphorylation was elevated in the disease control, which was effectively abolished in MLT-treated group. The process of translocation of the phosphorylated NF-κB into the nucleus, studied through immunofluorescence, was seen to happen at an escalated scale in the disease control than in the control cells, which was again abolished in the MLT-treated cells. Additionally, induction of Bcl-2 and Bax proteins was seen in disease control, whereas Bax was induced but not Bcl-2 in MLT-treated cells.
Panmanee *et al*., 2015[45]	**Thailand**	**To check the potential of MLT in regulating inhibition BACE1 and PS1/PS2 while enhancing ADAM-10 expression in SH-SY5Y cell line.**	**Induction: H_2O_2-induced neuronal cell degeneration (for western blot analysis of phosphorylated NF-κB, BACE1 and PS1); Go 6983 (for western blot analysis of ADAM-10)** **Tests: Western Blotting, Semiquantitative RT-PCR, β-secretase fluorimetric assay, luciferase and β-galactosidase enzyme assay kits.**	**Normal control: Ethanol**	**BACE1, PS1, ADAM 10, APP, GAPDH, and NF-κB p65**	**MLT inhibits BACE1 and PS1 (by attenuating NF-κB phosphorylation) while stimulates ADAM10, concentration dependently via MT1 and MT2 receptors. MLT regulates β and γ secretases while activating α secretase by activating protein kinase C, producing non-amyloidogenic pathway.**
Wongprayoon *et al*., 2015[46]	**Thailand**	**The effect of MLT was investigated on methamphetamine stimulated overexpression of TNFα and activation of NFκB.**	**Induction: Methamphetamine** **Tests: Cellular fractionation, siRNA transfection, Western Blot, Semi-quantitative RT-PCR.**	**Normal control: Serum-free media**	**IκBα, NF-κB p65, TNFα, and GADPH**	**MLT pre-treatment prevents the methamphetamine-induced TNFα overexpression, IκB degradation and NFκB nuclear translocation. This preventive effect was abolished by pretreatment with luzindole (MT1 and MT2 antagonist). MT2 knockdown abrogated the anti-inflammatory effect of MLT.**
Permpoonputtana *et al*., 2012[39]	**Thailand**	**To examine the anti-inflammatory action of MLT against Methamphetamine-induced neuroinflammation**	**Induction: Methamphetamine** **Tests: MTT Assay, Western Blot, Semi-quantitative RT-PCR, Immunocytochemistry.**	**Normal control: Serum-free Media** **Positive control: Methamphetamine**	**iNOS, Nrf2, pNF-κB, TNFα, and GADPH**	**MLT reduces the methamphetamine-induced expression of iNOS protein and TNF-α mRNA levels. The levels of pNF-κB is reduced**

						while Nrf2 expression is elevated with MLT pre-treatment.
Martínez *et al*., 2019[40]	Spain	To examine the antioxidant effect of MLT against cyfluthrin-induced oxidative stress, apoptosis, and neuroinflammation	Induction: Cyfluthrin Tests: MTT Assay, DCFH Assay, Tsvetkov's NQO1 activity, MDA Assay, RNA isolation and cDNA synthesis, Griess Assay, Quantitative RT-PCR, Real-Time PCR array analysis, Ingenuity pathway analysis	Normal control: DMSO Standard control: MDA (MDA assay), Sodium Nitrate (Griess assay)	Bax, Bcl-2, Casp-3, BNIP3, AKT1, p53, APAF1, NFκB1, TNFα, and Nrf2	MLT partially reduces the cyfluthrin-induced increase in the mRNA levels of Bcl-2, Bax, Casp-3, AKT1, BNIP3, p53, APAF1, Nrf2, TNF-α, and NF-κB1. MLT also reversed the greater fold change induced by cyfluthrin on certain upregulated genes such as AOX1, CYBB, NOS2, and BNIP3.
Nopparat *et al*., 2017[41]	Thailand	To investigate the effect of MLT on hydrogen peroxide-induced inflammation in SH-SY5Y cells.	Induction: H_2O_2 Tests: MTT Assay, SA-βgal staining, Propidium Iodide (PI) staining, Semi-quantitative RT-PCR, Western Immunoblotting, Sandwich ELISA, Immunocytochemistry.	Normal control: Untreated Positive control: H_2O_2	GADPH, IL-1β, IL-6, TNF-α, NF-κB p65, $p21^{Cip1}$ and $p16^{INK4a}$	MLT significantly reduces the H_2O_2-induced increase in $p21^{Cip1}$ and $p16^{INK4a}$ (cell cycle arrest markers), and SA-βgal staining. MLT increases Ki67-positive cells, which decreases on H_2O_2 treatment. MLT attenuated the peroxide-induced increase in IL-1β, IL-6, and TNF-α. MLT also decreases pNF-κB expression and its nuclear translocation, while increases Nrf2 in the H_2O_2-treated cells.
Zhi *et al*., 2020[42]	China	To investigate the effect of MLT in oxygen glucose deprivation / re-oxygenation-stimulated SH SY5Y cells	Induction: OGD/R Tests: MTT Assay, LDH Assay, Hoechst 33258 and Propidium Iodide staining, qPCR assay, Griess Assay, DCFH-DA Assay, MDA assay, FITC/PI assay, Caspase-3 activity assay, Western Blot Analysis	Normal control: Untreated Positive control: OGD/R	NF-κB p65, phosphorylated NF-κB p65, Akt, p-Akt, mTOR, p-mTOR, p70S6K, p-p70S6K Thr389, 4E-BP-1, p-4E-BP-1 Nrf2, HO-1, HMGB1, TNF-α, iNOS, Bcl-2, Bax and GADPH.	MLT increases the cell viability by reducing the death and LDH release of OGD/R insulted cells through blocking NF-κB or reversing Nrf2 or HO-1 knockdown. MLT represses the iNOS, TNF-α, and HMGB1 expression, NO production, and NF-κB activation in OGD/R insulted cells. MLT reduces the ROS, MDA, 4-HNE, 8-OHdG, Nrf2, and HO-1. MLT also activates Akt signaling, to exert anti-apoptotic activity. MLT also repressed OGD/R-challenged SH SY5Y cell autophagy by decreasing the LC-II and beclin-1 levels. MLT also increases

						the phosphorylation of mTOR, p70 ribosomal protein S6 kinase.
Chaudhary *et al*., 2016[43]	India	To investigate the action of MLT against Phytanic acid-induced neurotoxicity in SH-SY5Y cell line	Induction: Phytanic Acid Tests: MTT Assay, Bright Field Imaging, LDH Assay, DCFH-DA Assay, Griess Assay, Western Blot Analysis, Bradford Protein Estimation	Normal control: Untreated Positive control: Phytanic Acid	phosphorylated-NF-κB, Bax, Bcl-2, GAPDH, PARP-1, iNOS, and cleaved caspase-3	MLT exerts anti-apoptotic and anti-inflammatory effects through regulation of Bcl-2, Bax, p-NFκB, and iNOS expressions in cells.
Nopparat *et al*., 2017[47]	Thailand	To study the effect of MLT on autophagy via SIRT1 deacetylation on RelA/p65.	Induction: H_2O_2 Tests: SA-βgal staining, PI-staining-cell cycle distribution analysis, Immunofluorescence, Western Blot Analysis	Normal control: Untreated Positive control: H_2O_2	phospho-NF-κB p65, LC3-II, acetyl-NF-κB p65, Beclin1, and Sirt1	MLT reduces the number of SA-βgal-positive cells. It also increases SIRT1, Beclin1, and LC3-II and reduces acetylated-Lys310 in the p65 subunit of NF-κB in H_2O_2-treated cells.
Sinjanakhom *et al*., 2015[48]	Thailand	To investigate the pro-autophagic activity of MLT through deacetylation of SIRT1 on RelA p65 in H_2O_2-induced senescence in SH-SY5Y cells.	Induction: H_2O_2 Tests: Western Immunoblotting	Normal control: Untreated Positive control: H_2O_2	LC3-II, acetyl-NF-κB p65, Sirt1, and actin	MLT increases SIRT1 and LC3-II and reduces acetylated-Lys310 in p65 in H_2O_2-treated cells. MLT enhances autophagy via SIRT1 pathway.
Martínez *et al*., 2020[44]	Spain	To investigate the neuroprotective role of MLT against glyphosate- and AMPA-induced oxidative stress in SH-SY5Y.	Induction: Glyphosate and AMPA Tests: MTT Assay, LDH Assay, MDA Assay, DAF-2 DA Assay, DCFH Assay, Caspase-Glo 3/7 assay, RNA isolation and cDNA synthesis, RT-PCR, Ingenuity pathway analysis	Normal control: Untreated Positive control: Glyphosate, AMPA	GADPH, IL-6, TNF-α, GAP43, CAMK2A, CAMK2B, TUBB3, Wnt3a, Wnt5a, and Wnt7a	MLT reduces MDA concentration when compared with the positive control group.

Table 3: Quality assessments for in research articles

Author	Resources	Number of Gene/Protein Parameters evaluated	Cell Line tests in vitro			Determination of pathway used	Use of inflammation markers such as TNF, ILs in determination	Total
			SH-SY5Y cell line	No. of assays	Type of assay			
Chetsawang *et al*., 2006 [38]	GS	1	1	2	3	1	0	8
Panmanee *et al*., 2015 [45]	GS	2	1	2	3	1	0	9
Wongprayoon *et al*., 2015 [46]	GS, PM, SD	1	1	2	3	1	1	9
Permpoonputtana *et al*., 2012 [39]	GS, PM	1	1	2	3	1	1	9
Martínez *et al*., 2019 [40]	GS, SD	2	1	2	3	1	1	10

Nopparat *et al*., 2017 [41]	**GS, PM, SD**	**2**	**1**	**2**	**3**	**1**	**1**	**10**
Zhi *et al*., 2020 [42]	**GS, PM**	**2**	**1**	**2**	**3**	**1**	**1**	**10**
Chaudhary *et al*., 2016 [43]	**GS**	**2**	**1**	**2**	**3**	**1**	**1**	**10**
Nopparat *et al*., 2017 [47]	**GS, PM**	**1**	**1**	**2**	**3**	**1**	**0**	**8**
Sinjanakhom *et al*., 2015 [48]	**GS**	**1**	**1**	**1**	**3**	**1**	**0**	**7**
Martínez *et al*., 2020 [44]	**GS, SD**	**2**	**1**	**2**	**3**	**1**	**1**	**10**

PubMed - PM (5), Science Direct - SD (4), Google Scholar - GS (11)

DISCUSSION

Cell Source

Chetsawang *et al*., 2006, Panmanee *et al*., 2015, Wongprayoon *et al*., 2015, Permpoonputtana *et al*., 2012, Nopparat *et al*., 2017, Zhi *et al*., 2020, Sinjanakhom *et al*., 2015 had their cell lines from the American Type Culture Collection, Manassas, USA.

Martínez *et al*., 2019, Martínez *et al*., 2020 had their cell line from the European Collection of Authenticated Cell Cultures (94030304), Sigma-Aldrich.

Chaudhary *et al*., 2016 had their cell line from the National Centre for Cell Science, Pune, India.

Culture Conditions

Chetsawang *et al*., 2006, Panmanee *et al*., 2015, Wongprayoon *et al*., 2015, Permpoonputtana *et al*., 2012, Nopparat *et al*., 2017, Sinjanakhom *et al*., 2015 had grown their cell lines in a complete media, made with 45% Minimum Essential Media, 45% Ham's F-12, 10% inactivated fetal bovine serum, and 100 U/mL penicillin/streptomycin, at 37^{o}C under 5% carbon dioxide and 95% humidified air.

Martínez *et al*., 2019, Zhi *et al*., 2020, Chaudhary *et al*., 2016, Martínez *et al*., 2020 maintained their cell line in a DMEM-F12 medium supplemented with a 10% heat-inactivated Fetal Bovine Serum, with 100 units / mL penicillin, and 100 µg/mL streptomycin, at 37 ºC in a humidified atmosphere of 5% carbon dioxide and 95 % air.

Inducing Agents

5 out of 11 studies have employed the hydrogen peroxide induced neuroinflammation model. H_2O_2 diffuses between the cellular environments. An elevated release of H_2O_2 into the extracellular space can affect oxidant-dependent intracellular processes of adjacent cells. Intracellular H_2O_2 has the ability to trigger the signal pathways controlling the inducible pro-inflammatory gene expressions, regulating macrophage-effector functions and cytokine signaling.14–16 H_2O_2

is reported to induce the production of pro-inflammatory cytokines *in vitro* **[49]**.

2 studies employed methamphetamine as the inducing agent in an act to evaluate the tendency of the methamphetamine's ability to cause inflammation, which was negated very effectively by MLT. It was found out through these studies that methamphetamine infact induces inflammation through a NF-κB dependent pathway.

1 study employed the use of cyfluthrin, an insecticide, which is known to easily cross blood brain barrier and modulate the various neurotransmitter levels, producing a loss of dopamine and metabolites, while upregulating several gene expressions like Gpx1, Cox2, Il-1β, NF- κB, TNFα, rD1 and MAOB **[50]**.

1 study used the glyphosate and AMPA to induce cyto- and neuro-toxicity through oxidative stress, neurite outgrowth, apoptosis, autophagy and necrosis pathways.

MLT's role

All 11 studies prove the neuro-protectant role of MLT against neuroinflammation. It was noticed that MLT could significantly decrease the elevated pro-inflammatory cytokines in the induced cell lines, such as TNFα, IL-1β, and IL-6. It also reduced all lipid peroxidations, by decreasing the levels of malondialdehyde. It enhances autophagy through SIRT1 pathway by increasing SIRT1, Beclin1, and LC3-II while decreasing the acetylated-Lys310. MLT also decreases the levels of phosphorylated NF-κB, decreases the TNFα overexpression, while also decreasing the IκB degradation, hence reducing the nuclear translocation of NF-κB. MLT induces Bcl2, but not Bax, hence suggesting an anti-apoptotic effect. Specifically, in case of Alzheimer's, MLT seems to promote the non- amyloidogenic pathway by enhancing the alpha secretase activity, while inhibiting beta and gamma secretases to keep amyloidogenic pathway at bay. The tabulated results strongly suggest the anti-inflammatory activity of MLT, which needs to be further explored, to unfold any other actions this molecule might hold.

CONCLUSION

This systematic review was drafted by examining the present evidences of anti-inflammatory activity of melatonin in human neuroblastoma SH-SY5Y cell line. At an outset, 11 studies were used for this review, all of them reporting the neuroprotective effects of melatonin against neuroinflammation. The reports of all of the studies on analysis, have given enough indication that melatonin alters the NF-B pathway to regulate its anti-inflammatory actions. This finding has given an open path

for researchers and scientists to further explore the potential of Melatonin against neuroinflammation-associated diseases and disorders. Mainly, we believe that the information gathered here systematically, will help scientists determine the gap or where melatonin lags from being used clinically more actively.

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