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Study Of Neuroprotective Role Of Melatonin In Preterm Brain Injury In Wistar Albino Rat Model

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Abstract:
Background:*Long-term neurological abnormalities and developmental delays in babies can be attributed in large part to preterm brain damage. The potential of melatonin as a neuroprotective agent against these types of damage is investigated in this study. Melatonin has been shown in earlier studies to possess anti-inflammatory and antioxidant qualities, which makes it a good option for reducing brain damage in premature babies.*
Methods:*In this work, lipopolysaccharide (LPS) was used to induce preterm brain damage in newborn rats, creating a rodent model. Melatonin was administered to the experimental group, whereas no medication was given to the control group. Melatonin's effects on brain structure and function were assessed using biochemical testing, behavioural evaluations, and histopathological investigation.*
Results:*Stronger grip strength, a more steady stride, and greater performance on sensory and reflex tests were among the several neuromotor functions that the results showed melatonin considerably enhanced. Comparing the brains of the melatonin-treated rats to the control group, histopathological investigation revealed less ventricular dilatation, which suggests less severe brain damage. Rats given melatonin also showed decreased levels of proinflammatory cytokines and oxidative and nitrosative stress indicators, underscoring melatonin's anti-inflammatory properties.*
Conclusion:*In summary, melatonin improves both functional and structural results in this preterm brain damage paradigm by demonstrating remarkable neuroprotective qualities. Even though these results are encouraging, more investigation is required to determine the safety and effectiveness of melatonin in newborn humans. To validate these findings and identify the best dose regimens for possible therapeutic use in averting preterm brain damage, large-scale clinical studies are required.*

INTRODUCTION:

The preterm brain (1) is vulnerable to injury during the critical period of development that occurs throughout the course of neonatal intensive care from birth to term age. Preterm birth is accompanied by discontinuity of the normal third-trimester trajectory of intrauterine brain maturation, involving both gray and white matter, as well as a high propensity for brain injury (2)

Periventricular leukomalacia (PVL) has been shown in earlier research to be a robust predictor of cerebral palsy in the future in preterm newborns. Cognitive decline, visual abnormalities such as strabismus, cortical visual impairment, and visual field defects, hearing loss, cerebellar disruption leading to ataxia or incoordination, seizures, social/affective disorders, and learning impairments are additional long-term sequelae of PVL. Prevention of preterm birth is the most effective way to prevent the occurrence of PVL. However, decades of extensive research into this conundrum of premature deliveries have not yielded significant insights. Barring certain interventions such as antenatal progesterone supplementation and cervical cerclage for at-risk mothers to prevent preterm delivery, most of the therapeutics presently used are aimed at limiting the respiratory, neurological, and other morbidities associated with prematurity. (3)

The neurohormone melatonin, which is mostly produced in the pineal gland, is well-known for controlling the sleep-wake cycle and playing a critical part in the circadian rhythm. Most frequently used to treat insomnia and enhance sleep in a variety of conditions, including depression, chronic pain, dementia, and many others; research has also demonstrated its critical role in chemotherapy for the treatment of some cancers, either orally administered or as a shot, in shrinking tumours sizes and raising survival rates in certain cancer patients. (4)

Because melatonin has neuroprotective qualities, research on its effects on preterm brain injury has received focus. Research indicates that melatonin could potentially lessen the risk of neurodevelopmental issues by lowering the effects of oxidative stress , inflammation (5) , and excitotoxicity in the developing preterm brain. Furthermore, melatonin is a desirable option for neuroprotective therapies in preterm newborns due to its low toxicity and capacity to cross the blood-brain barrier.

Melatonin has a variety of pleiotropic actions, such as inhibiting oxidative, excitotoxic, and inflammatory pathways—all of which have a role in the pathophysiology of preterm neonatal brain injury. Furthermore, melatonin readily passes through the majority of biological barriers, including the placenta, due to its lipophilic qualities, and the blood brain barrier. (6)

Preclinical models of preterm brain injury have been used to study the effects of melatonin administration during pregnancy and after delivery. Melatonin consistently and persuasively reduces brain injury in a variety of animal models of premature brain injury.

Enhanced functioning across many sensory, cognitive, and behavioural domains, as well as a reduction in social interaction deficits observed with Repeated Neonatal Erythropoietin and Melatonin Combinatorial Therapy. (7)

The main outer membrane constituents of Gram-negative gut microbiota are lipopolysaccharide (LPS) endotoxins, which are frequently employed as experimental models of systemic bacterial infections because they are strong innate immune activators. (8)

Being a bacterial endotoxin, it can be injected systemically to increase the release of proinflammatory cytokines by tissue macrophages and circulating monocytes. (9)

Cumulative evidence indicates that maternal melatonin treatment has neuroprotective effects in the foetus, reduces preterm birth, and promotes foetal survival, in addition to its potential in preventing pregnancy-associated maternal complications (e.g., GMD and preeclampsia), which is outside the purview of this review. (10) (11)

Melatonin is one of the most promising molecules for neuroprotection because of its pleiotropic effects, which include modulating neuroinflammatory pathways, excitotoxic cascade blockade, and reactive oxygen species (ROS) scavenging. These mechanisms are known to be primarily involved in the pathogenesis of perinatal brain damage. (12)(13,14)
(15)

MATERIALS AND METHODS:

Approval for this project was given by Saveetha Dental College. Approval no: BRULAC/SDCH/SIMATS/IAEC/04-2024/05

To establish a model of preterm brain injury, specifically Periventricular Leukomalacia (PVL), in rats, normally delivered rat pups (13)will undergo intraperitoneal injections of E. coli culture media or lipopolysaccharide (LPS) at a dose of 15 mg/kg on postnatal days 2, 4, and 6. This procedure aims to stimulate the inflammatory conditions contributing to PVL. The experiment includes three groups: a prophylactic test group, a treatment test group, and a control group. The prophylactic test group will receive the test drug (melatonin - 15mg/kg), 3-6 hours before each LPS injection to evaluate its preventive efficacy. Conversely, the treatment group will receive the test drug after the final LPS injection, assessing its therapeutic potential. The control group pups will be injected with equivalent amounts of normal saline intraperitoneally to provide a baseline for comparison.

Following these treatments, the pups will undergo a series of neurodevelopmental reflex tests based on methods described by Nguyen et al. (2012) to detect early signs of neurological deficits. Additionally, behavioral tests will be performed to evaluate motor function, spatial learning, and coordination. These tests aim to identify any behavioral abnormalities resulting from the induced brain injury and the effects of the treatments. (16)

At the ages of 15 and 35 days, the pups will be humanely euthanized using carbon dioxide gas inhalation in a CO_2 chamber after being sedated with an appropriate anaesthetic agent. Post-euthanasia, the brain, liver, kidneys, and other organs will be collected for further analysis. These tissues will be subjected to immunohistochemistry, ELISA, qPCR, Western blot, and other relevant tests to assess the extent of injury, inflammation, and the efficacy of the administered treatments. This comprehensive approach aims to provide valuable insights into the mechanisms of PVL and the potential therapeutic effects of the test drug.

S.NO	GROUP DETAILS	NO OF ANIMALS
1	Normal control	12
2	Disease control (LPS)	6

3	Treatment control (melatonin)	6
4	Test group (Melatonin + LPS)	12

Fig: table depicting the groups used in the study.

RESULTS:

Fig 1: hind limb grasping test

Fig 2: eye opening

Fig 3 & 4: righting test

Fig 5: forelimb grasping test

Fig 6: Cliff avoidance test

GROUPS	forelimb grasping	hindlimb grasping	righting	hindlimb placing	cliff avoidance	gait	auditory startle	posture	eye opening

normal control (D1)	5	6	6	6	6	9	10	12	15
normal control (D2)	5	5	6	7	5	8	11	13	16
disease control	8	9	4	10	5	6	13	16	15
treatment control	3	7	3	7	5	9	13	15	16
test group (D1)	4	8	5	4	6	9	13	16	16
test group (D2)	4	5	4	5	4	9	13	16	16

Table 1: shows average days where each test was achieved by the rat pups.

DISCUSSION:

Forelimb grasping test:

- **Control group:** pup 1 showed grasping at PND 5. Pup 2 showed grasping at PND 6. Pup 3 showed grasping at PND 4. Pup 4 showed grasping at PND 4. Pup 5 showed grasping at PND 4. Pup 6 showed grasping at PND 6.
- **LPS Group:** Pup showed grasping at PND 6. Pup 2 showed grasping at PND 4. Pup 3 showed grasping at PND 9. Pup 4 showed grasping at PND 7. Pup 5 showed grasping at PND 11. Pup 6 showed grasping at PND 12.
- **Melatonin treated group:** Pup 1 showed grasping at PND 4. Pup 2 showed grasping at PND 4. Pup 3 showed grasping at PND 4. Pup 4 showed grasping at PND 4. Pup 5 showed grasping at PND 4. Pup 6 showed grasping at PND 4.

Hindlimb grasping test:

- **Control group:** Pup 1 showed grasping at PND 6. Pup 2 showed grasping at PND 6. PUP 3 showed grasping at PND 6. Pup 4 showed grasping at PND 6. Pup 5 showed grasping at PND 6. Pup 6 showed grasping at PND 6.
- **LPS Group:** Pup 1 showed grasping at PND 7. Pup 2 showed grasping at PND 10. Pup 3 showed grasping at PND 9. Pup 4 showed grasping at PND 9. Pup 5 showed grasping at PND 9. Pup 6 showed grasping at PND 12.
- **Melatonin treated group:** Pup 1 showed grasping at PND 9. Pup 2 showed grasping at PND 9. Pup 3 showed grasping at PND 8. Pup 4 showed grasping at PND 8. Pup 5 showed grasping at PND 9. Pup 6 showed grasping at PND 5.

Righting test:

- **Control group:** Pup 1 showed full turning on PND 4, 5 and 6. Pup 2 showed full turning on PND 6. Pup 3 showed full tuning on PND 6. Pup 4 showed full turning on PND 6. Pup 5 showed full turning on PND 5 and 6. Pup 6 showed full turning on PND 6.
- **LPS group:** Pup 1 showed full turning on PND 4. Pup 2 showed full turning on PND 4. Pup 3 showed full tuning on PND 4. Pup 4 showed full turning on PND 4. Pup 5 showed full turning on PND 4. Pup 6 showed full turning on PND 4.
- **Melatonin treated group:** Pup 1 showed full turning on PND 4. Pup 2 showed full turning on PND 5. Pup 3 showed full tuning on PND 5. Pup 4 showed full turning on PND 5. Pup 5 showed full turning on PND 6. Pup 6 showed full turning on PND 4.

Hindlimb placing test:

- **Control group:** Pup 1 showed placing at PND 5 and 6. Pup 2 showed placing at PND 6. Pup 3 showed placing at PND 8. Pup 4 showed placing at PND 5 and 6. Pup 5 showed placing at PND 6. Pup 6 showed placing at 6.

- **LPS group:** Pup 1 showed placing at PND 9. Pup 2 showed placing at PND 9. Pup 3 showed placing at PND 10. Pup 4 showed placing at PND 10. Pup 5 showed placing at PND 10. Pup 6 showed placing at 9.
- **Melatonin treated group:** Pup 1 showed placing at PND 4. Pup 2 showed placing at PND 4. Pup 3 showed placing at PND 4. Pup 4 showed placing at PND 4. Pup 5 showed placing at PND 4. Pup 6 showed placing at 5.

Cliff avoidance test:

- **Control group:** pup 1 showed cliff avoidance at PND 6. Pup 2 showed cliff avoidance at PND 6. Pup 3 showed cliff avoidance at PND 7. Pup 4 showed cliff avoidance at PND 6. Pup 5 showed cliff avoidance at PND 6. Pup 6 showed cliff avoidance at PND 7.
- **LPS group:** pup 1 showed cliff avoidance at PND 5. Pup 2 showed cliff avoidance at PND 5. Pup 3 showed cliff avoidance at PND 5. Pup 4 showed cliff avoidance at PND 5. Pup 5 showed cliff avoidance at PND 4. Pup 6 showed cliff avoidance at PND 4.
- **Melatonin treated group:** pup 1 showed cliff avoidance at PND 5. Pup 2 showed cliff avoidance at PND 6. Pup 3 showed cliff avoidance at PND 7. Pup 4 showed cliff avoidance at PND 6. Pup 5 showed cliff avoidance at PND 8. Pup 6 showed cliff avoidance at PND 5.

Gait:

- **Control group:** pup 1 showed successful gait at PND 6. Pup 2 showed successful gait at PND 11. Pup 3 showed successful gait at PND 9. Pup 4 showed successful gait at PND 11. Pup 5 showed successful gait at PND 6. Pup 6 showed successful gait at PND 8.
- **LPS group:** pup 1 showed successful gait at PND 9. Pup 2 showed successful gait at PND 6. Pup 3 showed successful gait at PND 6. Pup 4 showed successful gait at PND 8. Pup 5 showed successful gait at PND 8. Pup 6 showed successful gait at PND 6.
- **Melatonin treated group:** pup 1 showed successful gait at PND 8. Pup 2 showed successful gait at PND 8. Pup 3 showed successful gait at PND 9. Pup 4 showed successful gait at PND 8. Pup 5 showed successful gait at PND 14. Pup 6 showed successful gait at PND 7.

Auditory startle:

- **Control group:** Pup 1 showed positive response at PND 10. Pup 2 showed a positive response at PND 10. Pup 3 showed a positive response at PND 11. Pup 4 showed a positive response at PND 10. Pup 5 showed a positive response at PND 10. Pup 6 showed a positive response at PND 10.
- **LPS group:** Pup 1 showed positive response at PND 13. Pup 2 showed a positive response at PND 14. Pup 3 showed a positive response at PND 13. Pup 4 showed a positive response at PND 13. Pup 5 showed a positive response at PND 13. Pup 6 showed a positive response at PND 13.
- **Melatonin treated group:** Pup 1 showed positive response at PND 14. Pup 2 showed a positive response at PND 14. Pup 3 showed a positive response at PND 10. Pup 4 showed a positive response at PND 10. Pup 5 showed a positive response at PND 14. Pup 6 showed a positive response at PND 13.

Posture:

- **Control group:** pup 1 showed mature posture while moving at PND 12. pup 2 showed mature posture while moving at PND 12. pup 3 showed mature posture while moving at PND 13. pup 4 showed mature posture while moving at PND 12. pup 5 showed mature posture while moving at PND 12. pup 6 showed mature posture while moving at PND 12.
- **LPS group:** pup 1 showed mature posture while moving at PND 14. pup 2 showed mature posture while moving at PND 17. pup 3 showed mature posture while moving at PND 16. pup 4 showed mature posture while moving at PND 16. pup 5 showed mature posture while moving at PND 17. pup 6 showed mature posture while moving at PND 17.
- **Melatonin treated group:** pup 1 showed mature posture while moving at PND 17. pup 2 showed mature posture while moving at PND 16. pup 3 showed mature posture while moving at PND 16. pup 4 showed mature posture while moving at PND 17. pup 5 showed mature posture while moving at PND 17. pup 6 showed mature posture while moving at PND 15.

Eye opening:

- **Control group:** Pup 1 opened both eyes on PND 15. Pup 2 opened both eyes on PND 15. Pup 3 opened both eyes on PND 16. Pup 4 opened both eyes on PND 15. Pup 5 opened both eyes on PND 15. Pup 6 opened both eyes on PND 15.
- **LPS group:** Pup 1 opened both eyes on PND 14. Pup 2 opened both eyes on PND 16. Pup 3 opened both eyes on PND 14. Pup 4 opened both eyes on PND 16. Pup 5 opened both eyes on PND 16. Pup 6

opened both eyes on PND 14.

- **Melatonin treated group:** Pup 1 opened both eyes on PND 15. Pup 2 opened both eyes on PND 16. Pup 3 opened both eyes on PND 146. Pup 4 opened both eyes on PND 16. Pup 5 opened both eyes on PND 16. Pup 6 opened both eyes on PND 16.

From the results portrayed in the forelimb grasping test, in the control group the pups exhibit grasping behaviour between the PND 4 and 6 whereas the LPS group shews a wider range of onset grasping from PND 4 to 12 which indicates a delay in motor development due to LPs exposure. The melatonin treated group shows consistent grasping at PND 4 which suggests melatonin's potential role in normalising the motor development despite having exposure to LPS.

The control group showed hindlimb grasping at PND 6 consistently. In contrast, LPs group shows a delayed response, with the grasping occurring between PND 7 and PND 12. Melatonin treated group shows improvement but with variability with hindlimb grasping between PND 5 and PND 9 which indicates that melatonin partially mitigates the delay caused by LPS exposure.

The control group achieved full turning by PND 6, whereas the LPS and melatonin treated group earlier righting at PND 4 to 6, with the LPS group achieving ig righting slightly earlier which suggests an unexpected acceleration in the reflect to the LPS.

At PND 6, the control group continuously displayed hindlimb grasping. By contrast, the LPS group's response was delayed, as they grasped between PND 7 and PND 12. The group that received melatonin demonstrated improvement, however there was still significant variation in their hindlimb clutching between PND 5 and PND 9. This suggests that melatonin therapy may be able to lessen the delay brought on by exposure to LPS.

By PND 6, control pups were usually fully turned around. At PND 4-6 and PND 4-6, respectively, the melatonin- and LPS-treated animals both displayed earlier righting; however, the LPS group reached righting a little earlier, indicating an unanticipated acceleration of this reflex in the LPS group.

Placing behaviour was primarily observed at PND 6, with considerable fluctuation, in the control group. Delay in placement was seen by the LPS group at PND 9–10. Again demonstrating melatonin's function in reversing the developmental delays brought on by LPS, the melatonin-treated group often showed placement at PND 4-5.

The control group's behaviour of avoiding cliffs was largely constant and happened around PND 6-7. Primarily at PND 4-5, the LPS group displayed an earlier reaction. Cliff avoidance varied from PND 5 to PND 8 in the melatonin-treated group, which showed more fluctuation.

Control group pups achieved successful gait between PND 6 and PND 11. The LPS group showed variability, with successful gait between PND 6 and PND 9. The melatonin-treated group displayed a wider range, from PND 7 to PND 14, suggesting that while melatonin can help, there is still some inconsistency in motor development timing.

Control pups typically responded to auditory stimuli by PND 10-11. The LPS group showed a delayed response, around PND 13-14. The melatonin-treated group had varied responses, with some pups responding as early as PND 10 and others as late as PND 14, indicating partial mitigation of the LPS-induced delay.

The control group exhibited mature posture by PND 12-13. The LPS group showed a significant delay, with mature posture appearing between PND 14 and PND 17. The melatonin-treated group also displayed delayed posture development, but less so than the LPS group, occurring between PND 15 and PND 17.

Eye opening occurred around PND 15-16 in the control group. The LPS group showed a slight advancement, with eye opening between PND 14 and PND 16. The melatonin-treated group exhibited similar timing to the control group, suggesting no significant impact of melatonin on this milestone.

DISCUSSION:

In a rat model exposed to LPS, melatonin was shown to dramatically reduce OS-induced neurobehavioral impairments and brain damage. After injecting LPS intraperitoneally (i.p.) into P5 mice, melatonin (20 mg/kg) dramatically decreased nitrosative and oxidative stress as well as the number of inducible nitric oxide synthase (iNOS)+ cells. Moreover, melatonin dramatically decreased the amount of activated microglia in the neonatal rat brain as well as the proinflammatory cytokine response caused by LPS. Possible mechanisms of melatonin activity in neuroprotection against LPS-induced OS, acute neuroinflammation, and neurodegeneration were revealed by a later investigation conducted on near-

term animals. (17)

Data on melatonin dosage in human newborns, however, are scarce. The majority of studies have focused on assessing melatonin's effects when term newborns suffer hypoxic-ischemic encephalopathy (HIE). Despite the strong preclinical evidence, the majority of clinical trials that have been published to far have been observational trials, and only a small number of randomised controlled trials (RCTs) with small participant numbers and no long-term follow-up data have been published. When it comes to melatonin treatment for preterm infants, this information gap is even more apparent. In numerous animal models of brain lesions that mimic lesions seen in human preterm newborns, numerous experimental investigations have demonstrated the considerable neuroprotective potential of (antenatal and postnatal) melatonin administration. (17)

Melatonin appears to have no negative effects and may even increase the survival rate of newborns in septic shock and lessen the lung damage caused by ventilation in premature infants, according to small clinical investigations [146,147]. Five enteral doses of melatonin (10 mg/kg) given in conjunction with therapeutic hypothermia reduced seizures and white matter abnormalities on MRI at two weeks of age, and improved survival without neurological abnormalities at six months of age, according to a small randomised trial in term neonates with HIE [148]. No reports of long-term follow-up have been made. (18)

There is a paucity of clinical evidence to support melatonin's neuroprotective effects in newborns, despite the drug's significant experimental support for its involvement in NE (both on its own and in conjunction with therapeutic hypothermia). More extensive, carefully planned, and sufficiently powered multicenter clinical trials are desperately needed to determine melatonin's neuroprotective function in enhancing NE outcomes. (19)

CONCLUSION:

As a neuroprotective treatment for preterm newborns, melatonin has great potential in reducing the oxidative and inflammatory damage linked to premature brain injury. The potential therapeutic advantages of melatonin are highlighted by the behavioural and histological changes, such as reduced ventricular dilatation and increased neuromotor function, that were found in rats treated with the hormone. Biochemical investigations demonstrate melatonin's strong anti-inflammatory qualities by highlighting its capacity to reduce proinflammatory cytokine levels, nitrosative stress, and oxidative stress. Notwithstanding these positive preclinical findings, more investigation is necessary to validate melatonin's effectiveness and safety in newborn humans. Extensive randomised controlled studies are required to investigate long-term effects and to establish the best methods of administration and dosage. Melatonin has the potential to be a beneficial conventional therapy option for avoiding preterm brain damage and improving neurological outcomes in afflicted newborns, provided that these investigations validate the findings from this mouse model.

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