Immunomodulatory Potential of Melatonin in Immunosuppression: An *in-vivo* Study

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ABSTRACT

Objective: To assess the immunomodulatory effects of melatonin on the acquired immunity of immunosuppressed male Wistar rats by checking Interleukin 6 (IL-6) levels, total leucocyte counts (TLC), and differential leucocyte counts.

Study Design: Experimental study.

Place and Duration of the Study: Animal laboratory, CMH Lahore Medical College, from June to October 2023.

Methodology: Fifty male Wistar rats, weighing 180g to 200g, were included in this study with 10 rats in each of the five groups namely cyclophosphamide (CP), CP + melatonin, CP + immunomodulator, melatonin-only, and control. A calculated dose of CP was administered intraperitoneally for 30 days (from 13/06/23 to 13/07/23) to each group except the control groups. After this, the experimental group was given melatonin CP + Melatonin for 7 days (from 14/07/23 to 20/07/23). CP + Immunomodulatory group was kept for comparison of immunomodulatory effects. Blood samples were drawn from all 5 groups. IL-6 was estimated through ELISA. Other parameters assessed were TLC and absolute differential leucocyte counts.

Results: Melatonin increased IL-6 levels significantly ($p = 0.042$) as well as the TLC levels ($p < 0.001$) compared to the immunosuppressed CP group. Melatonin was seen to have an upregulation of IL-6 levels in immunosuppression compared to the administration of immunomodulator preparation ($p = 0.506$) which was not as effective. Administration of melatonin significantly increased the TLC, neutrophil, lymphocyte, monocyte, and eosinophil count compared to known immunomodulators.

Conclusion: Melatonin as a supplement may have some role in activating multiple immune response processes in immune-depressed states. It was also established that it allows quick recovery of cell components from immunosuppressed states.

Key Words: *Melatonin, Immunomodulation, Rats, Interleukin-6, Acquired immunity, Cyclophosphamide.*

How to cite this article: Qureshi AQ, Jafri SA, Tauseef A, Siddiqui WA, Irum A, Zia AE. Immunomodulatory Potential of Melatonin in Immunosuppression: An *in-vivo* Study. *J Coll Physicians Surg Pak* 2024; **34(11)**:1270-1274.

INTRODUCTION

There are multiple diseases associated with poor immune function that are very detrimental to the patient's living standards. Such immune-depressed states can be acquired states, autoimmune, or due to external interventions for example acquired immunodeficiency disorder, primary immunodeficiency disorder, or patients undergoing chemotherapy. These diseases need to be managed appropriately and timely to benefit both the patient and the healthcare system of the country, as the longer such diseases progress, the longer the burden on the country.[1] There is a room for better non-invasive treatments to assist in the prognosis of such diseases.

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Received: January 29, 2024; Revised: September 05, 2024; Accepted: October 19, 2024
DOI: https://doi.org/10.29271/jcpsp.2024.11.1270

Melatonin is an indoleamine primarily secreted by the pineal gland. Apart from its main known role of controlling the circadian cycle, it has been observed to have a significant role in releasing pro-inflammatory cytokines causing an upregulation of acquired immunity.[2] It has been associated with antioxidant activity, faster cell recovery, and combating cellular damage which may suggest its role as immune cell sparing.[3] Melatonin has been shown to influence various immune cells involved in acquired immunity. It can enhance the function of T lymphocytes, B lymphocytes, and natural killer cells.[4] melatonin can enhance the production of anti-inflammatory cytokines, such as interleukin-10, which further contributes to immune regulation. The anti-inflammatory activity and promotion of immunity in such diseases are unique. It has been seen to achieve this through several cell signalling pathways, such as extracellular signal-regulated kinase (ERK) and mitogen-activated protein kinase (MAPK).[5]

Interleukin-6 (IL-6) is a multifaceted cytokine regulating different functions in acquired immunity. IL-6 was observed to act in conjunction with other cytokines to drive the differentia-

tion of Helper T cells into specific subsets, such as Th17 and follicular helper T cells.[6] Moreover, IL-6 acts in synergy with other pro-inflammatory cytokines, such as IL-1β and TNF-α, to exacerbate inflammation and recruit immune cells to the site of infection or tissue damage and is also involved in the generation of immunological memory by promoting the survival and differentiation of memory B and T cells.[7]

Thus, there is evidence that supports melatonin has immunomodulatory effects. However, there are few conclusive studies on its effects in immunodeficiency states. The objective of this study was to establish the role of melatonin as an adjunct in therapy in patients suffering from immune-suppressed states by assessing the immune markers.

METHODOLOGY

The investigation was conducted through experimental design to assess the immunomodulatory effects of melatonin in rats within an immunosuppressed context. The study was conducted in the CMH research centre, animal house of CMH Medical College, Lahore, from June to October 2023. Ethical approval was obtained from the ORIC of CMH Lahore Medical College. Healthy adult male Wistar (Rattus norvegicus) rats weighing 180-200 g were included in the study. They were procured from the educational research centre at the college animal experimental laboratory and were kept for acclimatisation for 2 weeks in cages (5 per cage).[8] The initial weighing was done and recorded. The laboratory was kept at room temperature (temperature maintained by a thermostat at 24°C) under a 12hour light and dark cycle. The rats were fed regular rodent chow and water *ad libitum.* Standard animal care and experimental protocols were followed.[9]

A total of 50 rats were included in the study and were divided into 5 groups of 10. First group was labelled as the cyclophosphamide (CP) group (diseased group), second group as CP and Melatonin group (treatment group), third group as CP and Immunomodulator group (positive control group), fourth group as Melatonin only group, and fifth group as control group. Weighing was done every week, with maintenance of a gain or loss table, with dose adjustments done accordingly. CP injection was diluted using normal saline to get the required dose of 10mg/kg body weight once daily for 30 days, before the administration of melatonin to induce a marked non-lethal state of immunosuppression in the rats.[10,11] All groups were administered CP at 0th day of the experiment until the 30th day except for melatonin-only group and the control group. A prepared solution of melatonin was used and diluted in normal saline.[12] A dose of 200 micrograms/kg/day of melatonin as an active agent was calculated and given orally for 7 days, starting from the 30th day of the experiment, to the melatonin-treated diseased group and melatonin-only group, leaving the immunosuppressed diseased group and the control group. The immunomodulator preparation consisted of 1 g of sodium selenate, 15 g of vitamin E, 9 g of sodium chloride, and distilled water, with the volume adjusted to 1000 ml.[13] All ingredients were sourced from the CMH pharmacology laboratory and a local pharmacy. The immunomodulatory drug preparation was given at a dose of 0.1ml/kg/day orally for 7 days on the 30th day of experimentation to the immunomodulator-treated immunosuppressed group as a reference for the immune-stimulating effects of melatonin. The control group was given normal rat chow for 37 days without any interventions.

The animals were anaesthetised by ketamine (200mg/kg of body weight) intraperitoneally and 2 ml blood was drawn with the help of a 3 ml disposable syringe through cardiac puncture. Two vials were filled with the sample drawn, 1 ml each in EDTA vacutainer tube and serum separator tube. The serum was separated by centrifugation (at 3000rpm for 20 minutes) with the help of a micropipette extracted and stored in serum vials at 2-8 degrees till tested. IL-6 was estimated using E0135Ra Rat interleukin 6 ELISA Kit which was manufactured by BT Laboratories China. All preparation of reagents, samples, and standards were done according to the kit requirement.[14] Total leucocyte count (TCL) and absolute differential leucocyte count (DLC) were done using the automated haematology analyzer Sysmex XS-1000i by SAMSUNG. Some of the non-conclusive results were reassessed manually by an expert haematologist.

All data obtained were entered into a spreadsheet digitally on MS Excel. Manual proformas were also filled in for the record. Data obtained after the experimentation were subjected to statistical analysis using SPSS version 26. Normalcy of data was checked using the Shapiro–Wilk's test. One-way ANOVA and Post-hoc Tukey's test were performed to compare the mean ± SD of all groups.[15] A value $p < 0.05$ was considered statistically significant.

RESULTS

Comparison of serum IL-6 between the control group and the CP group showed significant immunosuppression established ($p = 0.01$). On comparing the means of the immunosuppressed CP group and the treated group with melatonin it was observed that there was a significant increase in IL-6 ($p = 0.042$) represented as * in Figure 1. Compared to the control group the IL-6 rise in the melatonin-only group was however not significant. There was no significance in comparison between the group treated with melatonin as compared to the group treated with immunomodulator solution as well. However, the immunomodulatory effect was not significant.

Comparison of mean ± SD between the CP group and the control group for total leucocytes, was significant immunosuppression established in all groups ($p < 0.001$). Comparison between the CP group and the treated group with melatonin showed a significant increase in TLC ($p < 0.001$). There was also a significant increase in TLC with melatonin as compared to the group treated with immunomodulator solution ($p < 0.001$). The TLC comparison between both the melatonin-only group and the control group was not statistically significant.

Table I: Immunomodulatory effects of melatonin.

	Cyclophosphamide (CP) Group (Mean ± SD)	Cyclophosphamide (CP) + Melatonin Group (Mean ± SD)	Cyclophosphamide (CP) + Immunomodulator Group (Mean ± SD)	Melatonin only Group (Mean ± SD)	Control Group (Mean ± SD)
IL-6 (pg/ml)	0.08 ± 0.047	0.17 ± 0.04	0.13 ± 0.06	0.24 ± 0.08	0.24 ± 0.09
TLC (x 10^3 cell/µl)	5.98 ± 2.85	14.56 ± 4.05	7.34 ± 2.99	13.87 ± 4.22	15.8 ± 3.25
Neutrophil Count (x 10^3 cell/µl)	2.95 ± 2.45	7.13 ± 3.48	2.86 ± 1.67	5.06 ± 2.45	5.93 ± 2.40
Lymphocyte Count (x 10^3 cell/µl)	1.97 ± 0.85	5.3 ± 1.94	3.39 ± 2.19	7.73 ± 3.09	7.84 ± 1.8
Monocyte Count (x 10^3 cell/µl)	0.83 ± 0.1	1.78 ± 0.79	1.00 ± 1.02	0.82 ± 0.52	0.8 ± 0.32
Eosinophil Count (x 10^3 cell/µl)	0.22 ± 0.23	0.58 ± 0.37	0.24 ± 0.1	0.26 ± 0.11	0.32 ± 0.08

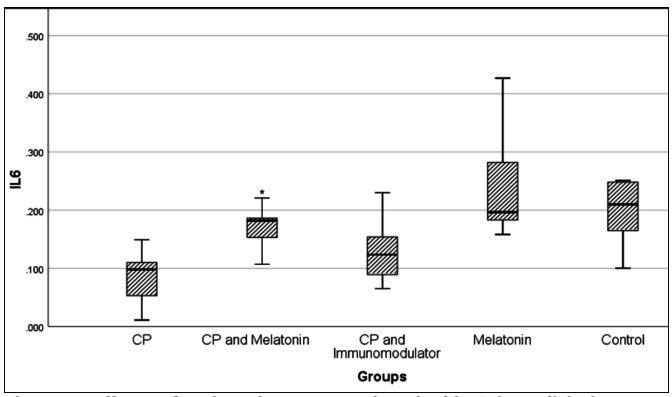

Figure 1: Effects of melatonin on serum interleukin-6 (pg/ml) in immunosuppressed rats.

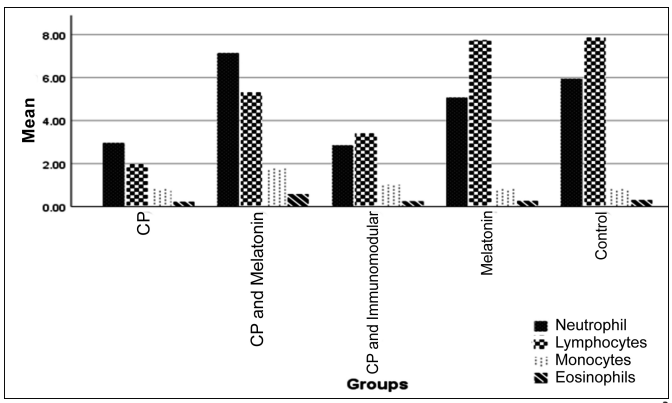

Figure 2: Effects of melatonin on differential leucocyte counts (x10^3 cell/µl) of the immunosuppressed rats.

In comparison of the differential leucocyte count of the treated group with the diseased group, each component showed increase in cell counts such as neutrophils (p = 0.06), lymphocytes (p = 0.008), monocytes (p = 0.012), and eosinophils (p = 0.003). Some of the mean ± SD values of the cellular components showed increased cell counts compared to the immunomodulator group, especially neutrophils (p = 0.05) and eosinophils (p = 0.007) as shown in Table I and Figure 2.

DISCUSSION

The present study delves into the effects of melatonin on acquired immunity using quantitative analysis of IL-6, TLC, and DLC in immunosuppressed rat models. The goal was to identify melatonin's role in different immunocompromised states and its potential in reducing recovery time. Overall, the study suggests that melatonin has a positive impact on immunosuppressed animal models, showcasing its potential immune-boosting capabilities. The research aligns with the theory that melatonin is cell-sparing and increases cytokine expression, which is consistent with previous findings.[3]

Notably, the study indicates a substantial increase in IL-6 levels in the group treated with melatonin compared to the CP group. This finding supports melatonin's potential immunomodulatory properties,[15] differing from some studies that reported anti-inflammatory effects.[16,17] The study suggests that melatonin's effects on IL-6 levels may be influenced by external factors and that its multifaceted function requires consideration in experimental design. It also agrees with the role of melatonin to equally affect IL-6 compared to the immunomodulators.[18] The group administered melatonin only (without immunosuppression) showed a significant rise in IL-6 levels, challenging the expected decrease in immune expression as previously reported.[17] The study implies that external factors may modulate melatonin's effects on IL-6, urging future researchers to consider these factors in study design.

Examining TLC, the study found a significant increase in the melatonin-treated group during immunosuppression, supporting the idea that melatonin enhances immune function in such states, as observed in previous studies.[4,19] This contrasts with the decrease in leucocyte counts reported in other research.[20] However, melatonin alone, without immunosuppression, did not significantly affect TLC, emphasising its potential role as an immune booster only during compromised states. Further analysis of DLC revealed melatonin's significant role in raising neutrophil counts in immunosuppressed rats, suggesting its involvement in acute responses. Additionally, melatonin combined with immunosuppression led to increased lymphocyte, monocyte, and eosinophil counts, indicating its potential to enhance leucocyte formation during immunosuppression, as noted in previous research.[21] The study suggests that melatonin's effects on immune parameters may vary based on external factors and the presence of immunosuppression. The observed increase in immune cell counts during immunosuppression aligns with melatonin's immune-regulatory role, as explained in earlier studies,[22] proposing it as a potential natural intervention in patients with compromised immune systems. While the study offers valuable insights, it also emphasises the need for further research to understand the broader effects of melatonin on immune responses. The unexpected increase in IL-6 levels in healthy rats in this study, as opposed to findings from previous studies,[17] calls for a comprehensive exploration of the mechanisms and external factors influencing melatonin's actions.

Overall, the findings lay the groundwork for potential clinical applications of melatonin in optimising immunosuppression therapies and supporting patients during recovery. However, further research, especially in human subjects, is necessary to validate and refine these observations.

CONCLUSION

The results provide evidence for the significant immunomodulatory effect of melatonin in an immunosuppressed animal model. Melatonin administration led to a substantial increase in IL-6 levels in the treated group, indicating its potential role in enhancing immune responses in immunosuppressed conditions. Melatonin itself induced a significant rise in IL-6 levels even in the absence of immunosuppression, suggesting its direct impact on immune system regulation.

DISCLOSURE:
This study was done as part of partial completion of MPhil Physiology.

ETHICAL APPROVAL:
Ethical approval was taken from the Ethical Review Board of CMH Lahore Medical College (Approval No.: Case# 694/ER-C/CMH/LMC).

COMPETING INTEREST:
The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:
AQQ: Main contribution, animal experimentation, sample collection and analysis, writing, editing, and statistical analysis.
SAJ, AT: Supervision, writing, editing, and corrections.
WAS: Animal handling and sampling, writing, corrections, editing and analysis.
AI: Writing, editing, and corrections.
AEZ: Editing, data analysis, and corrections.
All authors approved the final version of the manuscript to be published.

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