Articles

Modulation of proinflammatory cytokines and leukocyte mobilization by melatonin in response to sterile tissue injury in Wistar albino rats

Hussein F. Sakr, MBBS, PhD, *Bahjat Al-Ani,* DCA, PhD.

ABSTRACT

الأهداف: اختبار الفرضية والتي تنص على أن هرمون الميلاتونين العصبي يزيد من التهاب الخلية مثل الانترولين، وعامل النخر، وحركة الكريات البيضاء في الدم المحيطي وذلك نتيجة إصابة الأنسجة المعقمة.

الطريقة: أجريت هذه الدراسة خلال الفترة من نوفمبر 2011م حتى سبتمبر 2012م في قسم الفسيولوجيا، كلية الطب، جامعة الملك خالد، أبها، المملكة العربية السعودية. تم عمل اصابات الجلد أو الجهاز الهضمي لفئران ويستر والتي تتراوح أعمارهم من 8–7 أسابيع وتزن 200-150 غرام العدد=20 فأر. وتم تقسيمها إلى مجموعة تم علاجها بالميلاتونين والسواغ.

النتائج: ارتفع هرمون الميلاتونين والأنسجة المصابة المعقمة في البلازما بشكل إحصائي $p<0.05$ وذلك في بلازما الانترولين وعامل النخر بالمقارنة مع المستويات المعيارية. سجلت مستويات عالية من الانترولين بالمقارنة مع عامل النخر تم الحصول عليها مع علاج الميلاتونين. إضافة إلى ذلك، ارتفعت عدد كريات الدم البيضاء مع علاج الميلاتونين وذلك قبل إصابة الجلد والجهاز الهضمي واستمر في الارتفاع لمدة طويلة أكثر من السواغ. إضافة إلى ذلك، أدت طريقة إحداث إصابة في الجلد والجهاز الهضمي إلى ارتفاع مستويات كريات الدم البيضاء في الجهاز الدموي.

خاتمة: أن هرمون الميلاتونين يزيد عامل النخر والانتروكين ويرفع عدد كريات كريات الدم البيضاء قبل وبعد إصابة الأنسجة المعقمة. المزيد من الاختبارات لمعرفة الأثر العلاجي للميلاتونين والأمراض الالتهابية التي تشمل اختيار الكريات الدم البيضاء لتحديد مكان الإصابة.

Objectives: To test the hypothesis that the neurohormone, melatonin, differentially activates the release of the proinflammatory cytokines, such as, interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α), as well as inducing leukocyte mobilization into the peripheral blood in response to a sterile tissue injury.

Methods: This study was conducted between November 2011 and September 2012 at the Department of Physiology, College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia. Sterile tissue injury of either skin injury or gastric ulceration was induced in equal numbers in Wistar albino rats aged 7-8 weeks (150-200 g) (20 each), with each group being equally divided into melatonin treated or vehicle-treated.

Results: Melatonin treatment and sterile tissue injuries significantly ($p<0.05$) increased the plasma levels of IL-1β and TNF-α compared to baseline levels. However, higher levels of IL-1β compared with TNF-α were obtained only with melatonin treatment. Furthermore, melatonin treatment significantly increased ($p<0.05$) total leukocyte counts before the induction of skin injury and gastric ulceration, and remained elevated for a longer period than injured, but vehicle-treated rats. In addition, our methods of inducing skin injury or gastric ulceration caused an increase in leukocyte levels in the blood circulation ($p<0.05$).

Conclusion: Melatonin differentially stimulated plasma IL-1β and TNF-α, and increased blood leukocyte counts before and after sterile tissue injuries. It is worth pursuing further investigation into the therapeutic effect of melatonin in inflammatory disease that involves leukocyte recruitment to sites of injury.

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From the Medical Physiology Department (Sakr), College of Medicine, Mansoura University, Egypt, and the Department of Physiology (Al-Ani), College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia.

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Address correspondence and reprint request to: Dr. Hussein F. Sakr, Department of Physiology, College of Medicine, King Khalid University, Abha 61421, Kingdom of Saudi Arabia. Tel. +966 540273993. E-mail: sakr_doctor@yahoo.com

The physiology of leukocyte recruitment to the site of inflammation and its signaling pathways has been thoroughly investigated.[1,2] Neutrophils are recruited to the site of sterile and non-sterile injury, and involved in the inflammatory response and also in the healing process as neutrophil depletion markedly impairs tissue healing.[3,4] The inherent immune system consists of many cells, such as macrophages, dendritic cells, mast cells, neutrophils, eosinophils, and natural killer (NK) cells. These become activated during inflammation, which is virtually always a sign of infection with pathogenic microbes.[5] Melatonin is a hormone produced primarily by the pineal gland in the brain. It is derived from the neurotransmitter, serotonin,[6] and plays an important role in controlling the body's circadian rhythms, sleep-wake cycle,[7] and regulates other hormones, such as insulin and insulin growth factor receptor, and thyroid hormone. Melatonin is formed not only by the pineal gland, but also in the retina, kidneys, and digestive tract.[8] Melatonin exerts most of its physiological effects by binding to the melatonin receptors, MT1 and MT2, which are G protein-coupled receptors located in the brain and some peripheral organs.[9,10] However, the strong antioxidant effects of melatonin are produced via a receptor-independent method that may help strengthen the immune system.[11] The immune system could be affected by melatonin synthesized from different organs of the body. Additionally, it was found that human peripheral blood mononuclear cells synthesize biologically relevant amounts of melatonin,[12] and melatonin receptors have been identified in human lymphocytes and monocytes.[13] In recent years, much attention has been devoted to the possible interaction between melatonin and the immune system.[14-16] Melatonin has significant immunomodulatory roles in immunocompromised states. Inhibition of melatonin production was reported to suppress cellular and humoral responses in mice.[17] Interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) are acute phase proteins. Both of them stimulate the proliferation of neutrophils.[18,19] They participate in the induction of non-specific resistance to infection. Their effects are multiple involving immunomodulation, inflammation, wound healing, hematopoiesis and tumor necrosis.[20] Therefore, the aim of the present work is to investigate whether melatonin is able to differentially induce the release of IL-1β and TNF-α, as well as increase the total leukocyte count in response to sterile tissue injury; localized skin damage caused by a sterile cut in the feet followed by formaldehyde injection, or an acutely induced gastric ulceration caused by hypertonic solution of sodium chloride.

Disclosure. The authors have no conflict of interests, and the work was not supported or funded by any drug company.

Methods. This study was conducted between November 2011 and September 2012 at the Department of Physiology, College of Medicine, King Khalid University, Abha, Kingdom of Saudi Arabia.

Chemicals. All reagents and chemicals were obtained from Sigma Aldrich (St. Louis, MO, USA). A stock solution of melatonin was freshly prepared every 3 or 4 days containing 348 mg of melatonin dissolved in 10 ml of 96% ethanol, and stored at -20°C. The final concentration of melatonin (20 μg/ml) was administered orally in tap water for a period of 6 weeks. Water bottles were covered with aluminum foil to protect against light.

Experimental animals and experimental design. Wistar male albino rats 7-8 weeks of age (150-200 g) were obtained from the experimental animal care center of the College of Medicine of King Khalid University, Abha, Kingdom of Saudi Arabia (KSA). The rats were provided Purina chow diet ad libitum and were kept individually in well-ventilated cages under standard conditions of humidity (55±5%), temperature (25°C), and light (12/12 hour light-dark cycles). All experiments performed on laboratory animals in this study followed the "Principles of Laboratory Animal care" (NIH Publication No. 85, Rev, 1985).[21] This work has been approved by the Ethical Committee of the College of Medicine, King Khalid University, KSA. After acclimatization for 2 weeks, rats were randomly assigned to 4 groups (each containing 10 rats), such as: Group 1 - rats with skin injury treated with melatonin; Group 2 - rats with skin injury only; Group 3 - rats with gastric ulcer treated with melatonin; and Group 4 - rats with gastric ulcer only. Treatment with melatonin was initiated 4 weeks before the induction of injury and 2 weeks post-injury induction. Data collected before the induction of injury in Groups 2 and 4 were regarded as the baseline levels (control); whereas, in Groups 1 and 3, data collected before melatonin treatment were regarded as the baseline levels (control) for these groups. Baseline levels of white blood cells (WBC [total leucocyte, lymphocyte, monocyte, neutrophil, and eosinophil]) counts were recorded at the start of melatonin treatment. Counts were recorded again on

day one of the 5th week at 1-2 hour before induction of the injury. Post-injury counts were performed on days 4 and 7 of the 5th week, and finally on days 4 and 7 of the 6th week. Blood was drawn from the retro-orbital plexus of veins using heparinized capillary tubes. The samples were then immediately analyzed using an automated closed tube hematology system (ADVIA 60 [Bayer Corp, NY, USA]) that yields a report on levels of all WBC types present at 9:00 AM.

Induction of gastric lesions and skin injury. Protocols used to induce gastric ulcers were the same as described before.[22] Briefly, following an overnight fast, rats were anesthetized and then gavaged with 10 ml/kg of 25% (w/v) aqueous sodium chloride solution. To assure that there was successful induction of an ulcer, a set of 3 (fasted) naïve rats were gavaged in parallel. Two hours later, these rats were dissected under anesthesia by a single intraperitoneal (IP) administration of pentobarbital sodium (150 mg/kg), and their stomachs excised and opened through the greater curvature. After washing with saline, the extent of the gastric lesion(s) that was induced was quantified using a binocular magnifier; degeneration of the gastric mucosa was qualified by direct examination. Previously utilized protocols[23] were applied to induce skin injury in the designated rats. Briefly, each rat had the skin of the planter aspect of their feet cut superficially with a sterile scalpel, followed by injection into the site with 0.2 ml of formaldehyde (36%) solution.

Measurement of IL-1β and TNF-α. The serum levels of IL-1β and TNF-α and were assayed by enzyme linked immunosorbent assay (ELISA) as described by the manufacturer (Biosource, USA, and R & D Systems, Minneapolis, USA) before and after injury.

Statistical analysis. All of the data are expressed as the mean ± standard deviation. Statistical analysis was performed using the 2-tailed Student t-test and ANOVA to evaluate the main effects and interactions of different groups, and time on WBCs simultaneously. Post-hoc least significance difference multiple comparisons procedures were conducted simultaneously to examine comparisons between all possible pairs of group means at their corresponding times. All statistical analyses were conducted using Statistical Package for Social Sciences version 10.0 (SPSS Inc, Chicago, IL, USA). A $p<0.05$ was considered statistically significant.

Results. Tissue injuries are known to cause up-regulation of proinflammatory cytokines.[24] To test that a hypertonic solution of sodium chloride-induces gastric ulceration and a sterile skin cut infused with

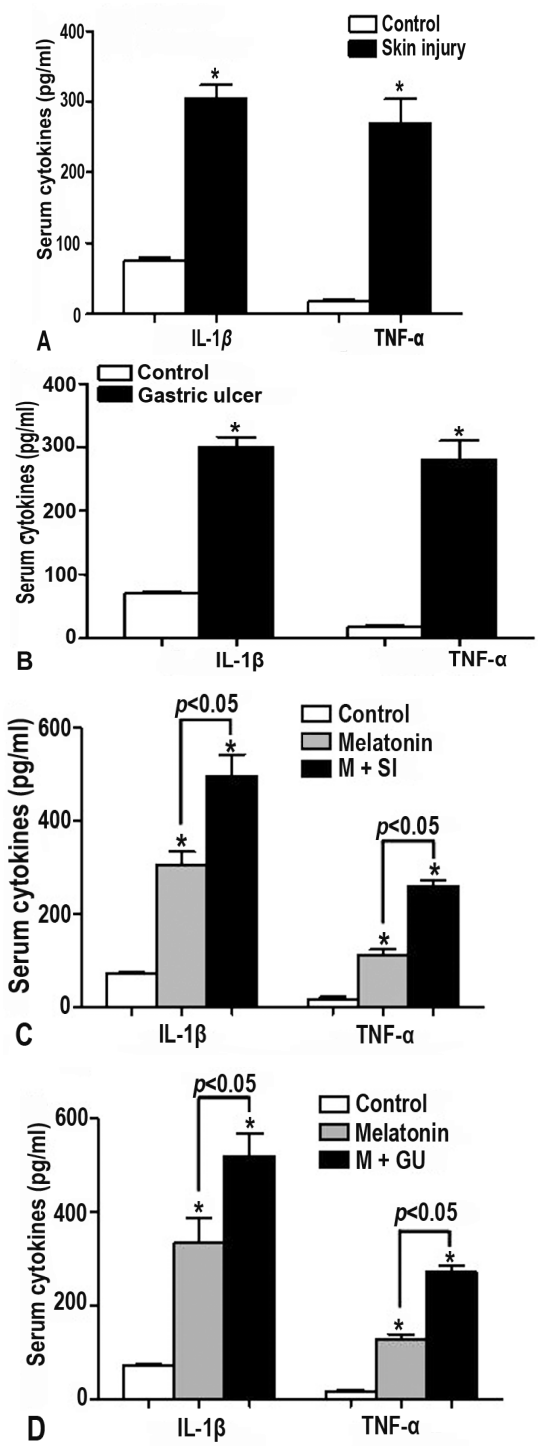

Figure 1 - Serum levels of interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) in response to sterile skin injury (**A**) or gastric ulcer were measured (**B**). The effect of melatonin treatment on the serum levels of IL-1β and TNF-α in the presence or absence of skin injury (**C**) or gastric ulceration (**D**) were also measured. *$p<0.05$ versus control. The time of these measurements were at days 4, 7, 11. M+SI - melatonin + skin injury, M+GU - melatonin + gastric ulcer.

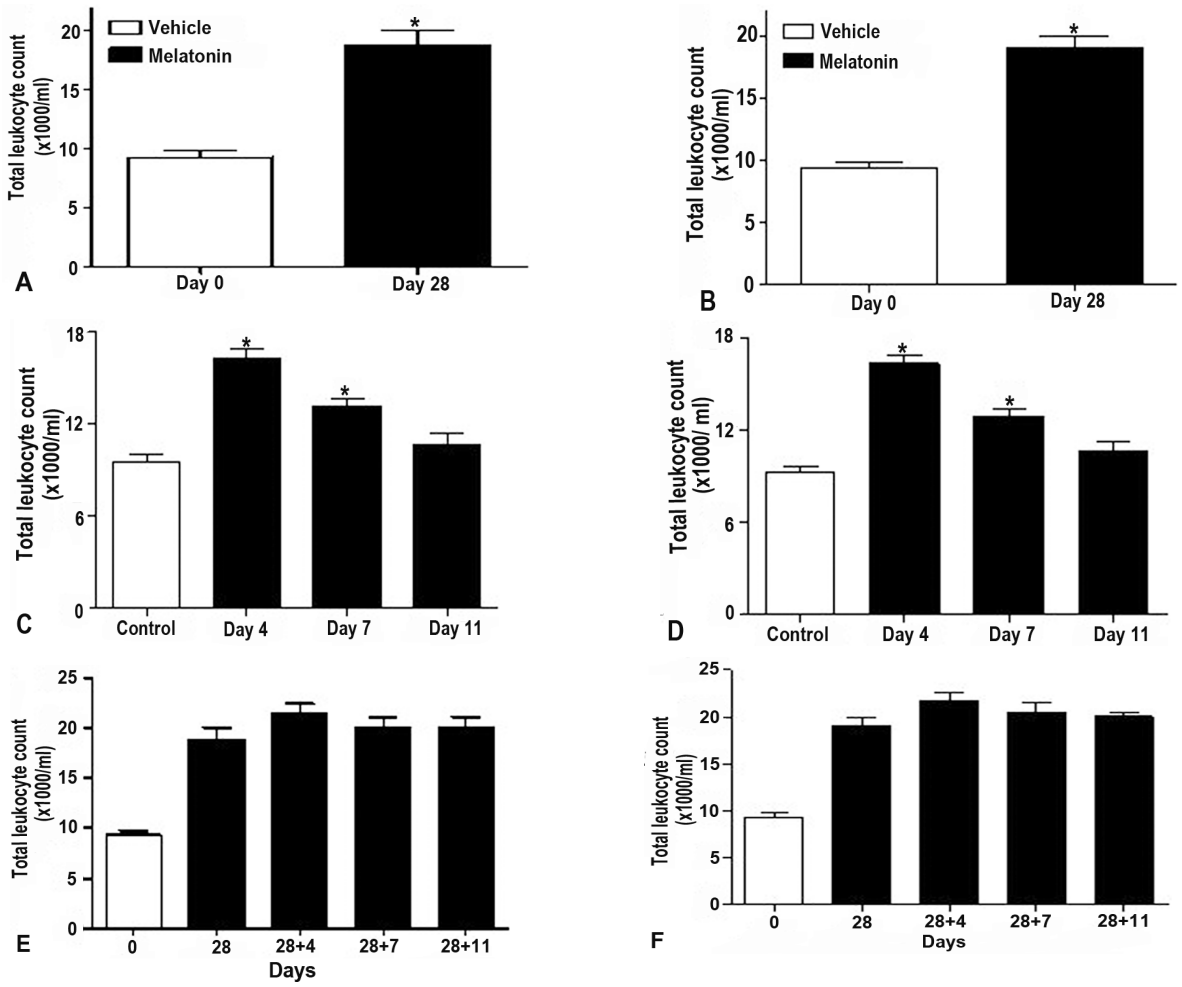

Figure 2 - Total leukocyte count in response to 28 days melatonin treatment before the induction of the sterile skin injury (**A**) or gastric ulcer (**B**) were measured (*p<0.05 versus day 0). Total leukocyte count at 4, 7, and 11 days post sterile skin injury (**C**), and post gastric ulcer (**D**) (*p<0.05 versus control). **E**) Total leukocyte count in response to sterile skin injury, or (**F**) gastric ulcer with melatonin pre-treatment.

formaldehyde are able to induce proinflammatory cytokines in albino rats, IL-1β and TNF-α were measured in the blood obtained from injured rats. As shown in **Figures 1A** and **1B**, both types of tissue injuries caused a comparable increase in IL-1β and TNF-α levels; more than a 3-fold increase compared to baseline levels (p<0.0001). We then measured the effect of melatonin on IL-1β and TNF-α levels before and after the induction of tissue injury. Melatonin alone was able to induce the secretion of both cytokines, but a higher level of IL-1β was produced compared to TNF-α (**Figures 1C** and **1D**). In addition, tissue injuries, whether skin injury (**Figure 1C**) or gastric ulceration (**Figure 1D**) significantly (p<0.0001) augmented melatonin-induced cytokine up-regulation. To test the effects of melatonin on leukocyte mobilization into the bloodstream in pre- and post-injured rats, we first measured total leukocyte counts in the pre-injured rats that received 20 μg/ml melatonin for 4 weeks. Melatonin significantly (p<0.0001) induced leukocytosis compared to vehicle-treated animals (**Figures 2A and 2B**). We then measured leukocyte count in response to injury in control animals. As expected, sterile injuries alone caused leukocytosis, which peaked 4 days post-injury and then declined in number until returning to the basal level after 11 days post-injury time in both, skin injured group (**Figure 2C**) and gastric ulceration group (**Figure 2D**). Whereas, in animals that received melatonin, the total leukocyte number did not decline and remained elevated after 11 days post-skin injury (**Figure 2E**) or gastric ulceration injury (**Figure 2F**). To discern the effect of melatonin from tissue injuries on leukocyte number, total

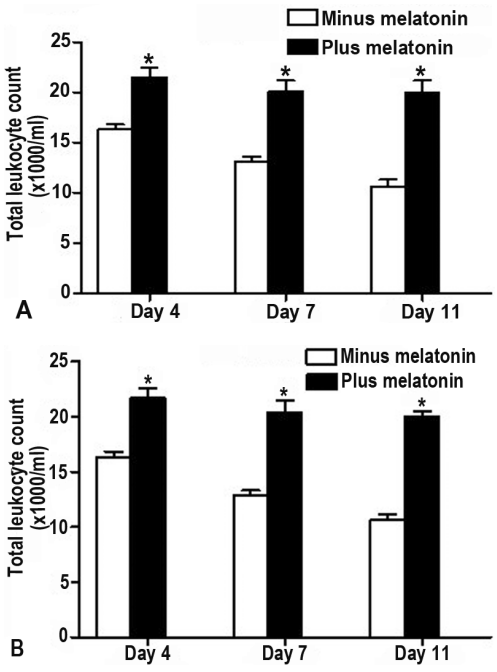

Figure 3 - Total leukocyte count in response to sterile skin injury (**A**) and gastric ulcer (**B**) in both melatonin pre-treated and untreated rats on days 4, 7, and 11. *p<0.05 compared with untreated rats.

leukocyte count was measured at days 4, 7, and 11 post skin injury (**Figure 3A**) and gastric ulceration (**Figure 3B**) in the presence or absence of melatonin. The presence of melatonin caused significant (p<0.0001) increase in leukocyte counts in all measured days compared to tissue injuries only, and the biggest gap was observed at day 11 in both injuries.

Discussion. This study demonstrates an incomparable induction of the proinflammatory cytokines IL-1β and TNF-α by the neurohormone, melatonin, where the increase in IL-1B is more than TNF-α increase that was further induced with sterile tissue injuries. Furthermore, this study shows that melatonin augmented leukocytosis induced by sterile tissue injuries in rats. This conclusion was supported by the data indicating that melatonin-induced higher concentrations of IL-1β than TNF-α, and that the maximum levels of these proinflammatory cytokines were obtained in injured animals receiving melatonin (**Figure 1**). On the other hand, the maximum levels of leukocytosis were achieved when melatonin was given to injured rats compared to melatonin or injuries alone (**Figure 2 and Figure 3**).

Conflicting data were reported on the effect of melatonin on proinflammatory cytokines. In an in vitro model that mimics inflammatory bowel disease, melatonin was found to suppress the induced TNF-α as an oxidant biomarker associated with the disease,[25] the authors attributed their findings to the antioxidant effects of melatonin.[26] Furthermore, the antioxidant and anti-inflammatory effects of melatonin causing a reduction in the production of proinflammatory cytokines was proposed by Konturek et al[27]to be the cause of their observation that melatonin provides gastro protection against stress induced acute gastric lesions, and in the healing of chronic gastric ulcers. In contrast, melatonin was reported to up-regulate cytokine production and immune function that favors T-helper 1 (Th1) response, such as TNF-α and IL1-β production.[28-31] Serum levels of melatonin were found to be higher in patients with rheumatoid arthritis, with maximum levels of IL-1β and TNF-α at evening when melatonin levels are highest.[32] Another study on the correlation between melatonin and asthma reported that melatonin acts as a proinflammatory agent that induced the secretion of IL-1β and TNF-α by peripheral blood mononuclear cells,[33] which may indicate an adverse effect of melatonin in asthma as suggested by the authors. In addition, it can be clearly concluded from their work that the levels of IL-1β are much higher than that of TNF-α secreted by these cells at early morning in response to melatonin stimulation, which are in agreement with our findings shown in **Figure 1** of melatonin inducing both IL-1β and TNF-α with a much higher IL-1β concentration. On the other hand, recent study results indicated that melatonin suppresses proinflammatory mediators by simultaneously targeting the multiple signaling, such as ERK/p38 MAPK, c/EBPβ, NF-κB, and p300, in LPS-stimulated VSM cell line CRL1999, and suggest that melatonin is a potential candidate compound for the treatment of proinflammatory disorders.[34] In conclusion, melatonin worked as an activator rather than a suppressor of the proinflammatory cytokine in this work.

Leukocyte mobilization into the blood stream in response to proinflammatory cytokines is well documented.[12,13] Our data suggesting the induction of leukocytosis by melatonin using sterile tissue injury models (**Figure 2** and **Figure 3**) can be compared with data obtained by others with animal and human experiments. For example, removal of the pineal gland, which produces melatonin or exposure to continuous illumination caused leucopenia, and depressed both cellular and humoral immunity that were restored by the addition of exogenous melatonin.[15,16,35] In addition, mice which received exogenous melatonin for 2 weeks were shown to have an increase in the number of monocytes

lineage in the bone marrow.[36] Also melatonin exerted anti-apoptotic effects on lymphocytes and neutrophils that was reported on rats injected with HL-60 leukemia cells,[37] that increased animals' immunity. These reports are consistent with our findings of an increase in rat blood monocyte, lymphocytes, and neutrophils (data not shown).

One limitation of the present study is that we did not measure the serum level of the macrophage activating protein. Further research is needed to explore the mechanism of action of melatonin on the leucocytes.

In conclusion, using sterile tissue injury models, our data point to the importance of melatonin as an inducer of leukocytosis and Th1 cytokines (TNF-α). These findings further support a role for melatonin in inflammatory disease that involves leukocyte recruitment to the injury sites.

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