Effect of melatonin on proliferation of neonatal cord blood mononuclear cells

Wei Zhou, Ping Wang, Li Tao

Guangzhou, China

Background: **Pineal melatonin (MLT) is a neuroendocrine hormone that possesses a wide variety of biological effects. MLT regulation of the immune system has been studied in recent years. But very little is known about MLT interaction with neonatal cord blood mononuclear cells (CBMCs) and the lymphocyte immune system in neonates. This study was designed to investigate the proliferative effects of MLT on CBMCs and peripheral blood mononuclear cells (PBMCs).**

Methods: **Cord blood samples were collected from 10 normal full-term infants at the Guangzhou Maternal and Infant Hospital, China. Ten samples of adult peripheral blood were also collected from healthy volunteers. ^{3}H-thymidine (^{3}H-TdR) incorporation was used to analyze the influence of MLT on proliferation of CBMCs. The effects of MLT on proliferation of CBMCs and PBMCs were compared.**

Results: **^{3}H-TdR incorporation increased in a dose-dependent manner with varying MLT concentrations (50 pg/ml-50 ng/ml), but peaked at 5 ng/ml. After incubation with MLT (5 ng/ml), interleukin-2 (IL-2, 50 ng/ml), MLT+phytohemagglutinin (PHA, 5 μg/ml), and MLT+IL-2, respectively in CBMCs media, ^{3}H-TdR incorporation rates were 114 327±52 863, 16 087±9006, 118 360±59 207, and 17 682±7391. Compared to the control cell suspension (14 133±8688), ^{3}H-TdR incorporation rates of the MLT and MLT+PHA groups were significantly increased (t=5.9143, P<0.001; t=5.5078, P<0.001). ^{3}H-TdR incorporation was not different between the IL-2 and MLT+IL-2 groups (t=0.4983, P>0.05; t=0.9839, P>0.05). PHA treatment (110 397±48 663) presented no difference in ^{3}H-TdR incorporation compared to the MLT or MLT+PHA groups (t=0.1730, P>0.05; t=0.3286, P>0.05). ^{3}H-TdR incorporation was significantly greater in CBMCs than in PBMCs cultures after addition of various stimulators to the culture media.**

Conclusions: **MLT promoted proliferation of PBMCs and also enhanced proliferation of CBMCs. The proliferative effects of MLT were greater on CBMCs than on PBMCs.**

World J Pediatr 2009;5(4):300-303

Key words: **cell proliferation**
cord blood;
melatonin;
mononuclear cell;
neonate

Introduction

The immature neonatal immune system predisposes infants, especially premature infants, to the development of infections. These infections can be life-threatening and are often one of the main causes of neonatal mortality. Therefore, analysis of neonatal immune mechanisms is crucial to understanding how to modulate neonatal immune function to reduce infection incidence and neonatal fatality. The neonatal immune system is still developing, and the function of neonatal lymphocyte phenotype is different from that of adults and children. The levels of interleukin-2 (IL-2), IL-4, IFN-γ, and IL-12 are lower in neonates than in adults. Neonatal B cells secrete mainly IgM, but produce low levels of IgG and IgA stimulated by various factors. Interaction of T and B cells as well as cytokines secreted by activated T cells are two major aspects of immunoglobulin isotype switching of B cells.[1]

Pineal melatonin (MLT) is a neuroendocrine hormone that possesses a wide range of biological effects. MLT regulation of the immune system has been studied in recent years.[2-4] Pineal MLT levels correlate to age, and the neonatal pineal gland does not exhibit MLT diurnal rhythms. The circadian rhythm of MLT does not appear until the end of the first 3 months of

Author Affiliations: Department of Neonatology, Guangzhou Children's Hospital, Guangzhou Medical College, Guangzhou 510120, China (Zhou W, Wang P, Tao L)

Corresponding Author: Wei Zhou, MD, Department of Neonatology, Guangzhou Children's Hospital, Guangzhou Medical College, Guangzhou 510120, China (Tel: +86-20-81330577; Fax: +86-20-81861650; Email: zhouwei_pu002@126.com)

doi:10.1007/s12519-009-0057-7

life, and the highest MLT values are reached between 3 and 6 years of age with a steady decline thereafter.[5] By 6-8 years, the levels are similar to adult levels; however, MLT expression decreases once again between 8-13 years of age, and increases again between 13-15 years of age. MLT receptor expression has been verified in many tissues and organs, and studies[2,6] have demonstrated the various biological effects of MLT: day-night rhythm regulation, sexual maturation, reproduction, immune reaction, tumor and aging, modulation of arterial blood pressure and heart rate, free radical clearance and anti-oxidation, as well as inhibition of platelet aggregation and lipid peroxidation. MLT can enhance immune function, increase proliferation of spleen lymphocytes induced by concanavaline A, stimulate the generation of T cells, and activate NK cells.[7,8] MLT can also enhance antibody response, induce T cell activation, and increase the Th/Ts cell ratio, as well as increase IL-1 levels, elevate the ratio of CD4 to CD5, and regulate tumor necrosis factor alpha and IFN-γ levels.[9] MLT results in proliferation of peripheral blood mononuclear cells (PBMCs) and an increase in levels of IL-2, IL-4, IL-6, IL-12, and IFN-γ.[10-12] Nevertheless, to understand the physiological function and mechanisms of MLT, further studies are required.[6] In particular, very little is known about MLT interaction with neonatal cord blood mononuclear cells (CBMCs) and the lymphocyte immune system in neonates.

Phytohemagglutinin (PHA) is a lectin found in plants, especially in beans. With a number of physiological effects it is used in medical research. For example, it induces mitosis and is used for the stimulation of cell proliferation in lymphocyte cultures. IL-2 is a kind of cytokine that can stimulate proliferation of T lymphocytes. MLT activates $CD3^+$/$CD4^+$ lymphocytes by increasing IL-2 production, and it can increase the level of IL-2 secreted by Jurkat cell. In this study, we observed the effects of MLT, PHA, PHA+MLT, IL-2 and MLT+IL-2 on proliferation of CBMCs and PBMCs.

Methods

Cord blood samples were collected from 10 normal full-term infants at the Guangzhou Maternal and Infant Hospital, China. Ten samples of adult peripheral blood were also collected from healthy volunteers. Neither pregnant women nor healthy adults had histories (four weeks before blood collection) of infection, treatment with blood products, or with immune-stimulating or -supressing medication. The study was approved by the Institutional Ethics Committee and informed consent was obtained from all the volunteers before collection of blood samples.

Blood (15 ml each) was collected separately in heparin anticoagulation tubes, and mixed with lymphocyte separation media to isolate CBMCs and PBMCs by density gradient centrifugation. PBMCs and CBMCs were washed twice with phosphate buffered solution (PBS), and counted with a cell counting plate in a high-power light microscopic field. Suspensions were adjusted to 10^6 cells/ml with RPMI 1640 medium (GIBCO, USA). Stimulators were added to 96-well plates in the following treatment groups: 5 μg/ml PHA (Sigma, USA); 50 ng/ml MLT (Sigma, USA); 5 ng/ml MLT; 500 pg/ml MLT; 50 pg/ml MLT; 50 ng/ml IL-2 (Sigma, USA); 5 ng/ml MLT + 5 μg/ml PHA; and 5 ng/ml MLT + 50 ng/ml IL-2. Each group was triplicated. CBMCs or PBMCs (200 μl/well) were incubated with the various stimulators for 72 hours with 5% CO_2. Cell suspension of CBMCs or PBMCs (with no stimulant) was used as the control.

^{3}H-thymidine (^{3}H-TdR), 1 μl Ci (3.7×10^4 Bq)/well, (Shanghai Institute of Nuclear Research of Chinese Academy of Sciences) was added to the cultures and 24 hours later the cells were harvested. Cycles per minute (CPM) were measured with a YJS-80 Liquid Scintillation Counter (Shanghai Institute of Nuclear Research of Chinese Academy of Sciences).

Statistical analysis

Data were analyzed with SPSS 11.0 software. All results were presented as means ± SD. LSD *t* test was used to determine the mean difference. A value of $P<0.05$ was considered statistically significant.

Results

Effect of different MLT concentrations on ^{3}H-TdR incorporation rates of CBMCs

^{3}H-TdR incorporation rates were 18 901±7589, 23 355 ±11 010, 114 327±59 106, and 93 806±51 750 in 50 pg/ml, 500 pg/ml, 5 ng/ml, and 50 ng/ml MLT treatment groups, respectively. There was a significant difference of ^{3}H-TdR incorporation between the 5 ng/ml and 50 ng/ml MLT treatment groups (t=5.3036, $P<0.001$; t=4.8014, $P<0.001$) and the cell suspension group (14 133±8688). However, there were no significant differences between the 50 pg/ml and 500 pg/ml MLT treatment groups and the cell suspension group (t=1.3070, $P>0.05$; t=2.0793, $P>0.05$) (Fig. 1).

^{3}H-TdR incorporation rates of CBMCs and PBMCs after 72-hour incubation with various mitogens

^{3}H-TdR incorporation rates after incubation with MLT, IL-2, MLT+PHA, and MLT+IL-2 in CBMCs medium are shown in Table. ^{3}H-TdR incorporation in the

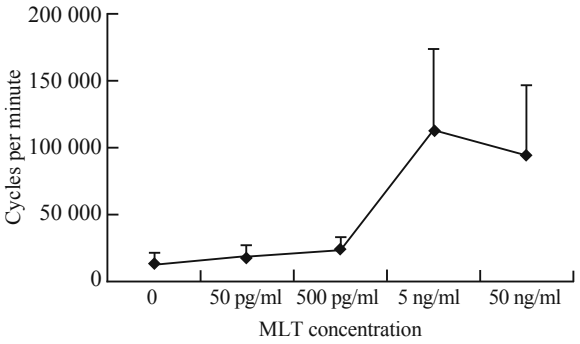

Fig. 1. Effect of different MLT concentration on ^{3}H-TdR incorporation rates of CBMCs.

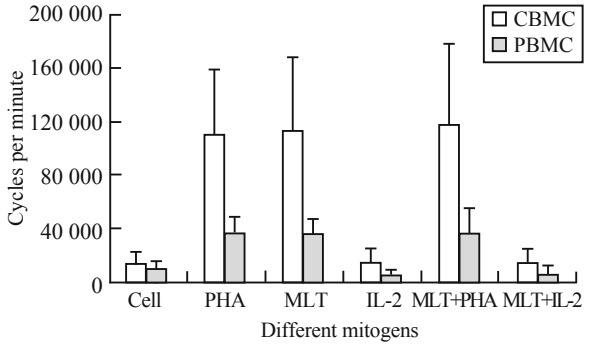

Fig. 2. ^{3}H-TdR incoporation rates of CBMCs and PBMCs with various mitogens.

Table. ^{3}H-TdR incorporation rates of CBMCs and PBMCs after 72-hour incubation with various mitogens (n=10)

Group	Cell suspension	PHA (5 μg/ml)	MLT (5 ng/ml)	IL-2 (50 ng/ml)	MLT+PHA	MLT+IL-2
CBMCs	14 133±8688*	110 397±48 663†	114 327±52 863‡	16 087±9006§	118 360±59 207‖	17 682±7391¶
PBMCs	8192±1597	36 274±12 229	36 550±11 907	5820±3196	37 015±18 877	7602±4385
t	2.1268	4.6715	4.5389	3.3975	4.1394	3.7091
P	0.0475	0.0002	0.0003	0.0032	0.0006	0.0016

‡ vs *: t=5.9143, P<0.001; ‖ vs *: t=5.5078, P<0.001; § vs *: t=0.4938, P>0.05; ¶ vs *: t=0.9839, P>0.05; ‡ vs †: t=0.1730, P>0.05; ‖ vs †: t=0.3286, P>0.05. CBMCs: cord blood mononuclear cells; PBMCs: peripheral blood mononuclear cells; 3H-TdR: 3H-thymidine; PHA: phytohemagglutinin; MLT: melatonin; IL-2: interleukin-2.

MLT and MLT+PHA groups was significantly higher (t=5.9143, P<0.001; t=5.5078, P<0.001) than that in the cell suspension group; however, there was no significant difference between the IL-2 and MLT+IL-2 groups (t=0.4983, P>0.05; t=0.9839, P>0.05). Comparison of the MLT and MLT+PHA groups with the PHA group showed no significant difference in ^{3}H-TdR incorporation (t=0.1730, P>0.05; t=0.3286, P>0.05). After incubation with various mitogens, ^{3}H-TdR incorporation rate of CBMCs was significantly greater than that of PBMCs (Table, Fig. 2).

Discussion

This study demonstrated that MLT promoted CBMCs proliferation in a dose-dependent manner. MLT acts directly on cell proliferation probably by binding to high affinity receptors and by stimulating reactions between growth factors and their receptors.[13] The results also showed that MLT (5 ng/ml) and PHA (5 μg/ml) promoted similar proliferative effects. But there was no combined proliferative effect of MLT and PHA on CBMCs. The effect of MLT was greater on CBMCs proliferation than on PBMCs proliferation. T cell levels of $CD3^{+}$T, $CD3^{+}CD4^{+}$T, and $CD3^{+}CD8^{+}$T cells secreted by CBMCs were different from those secreted by PBMCs.[14] Hematopoietic stem cells and progenitor cells in CBMCs were responsible for significant differences of proliferation (^{3}H-TdR incorporation) between CBMCs and PBMCs (2×10^{5} before culture) in the control group after 72 hours in culture. Purified T cells and their subsets in cord blood should be used to examine cell proliferation in the future.

MLT has been shown to induce PBMCs to release more IL-2.[15] Following inhibition of MLT, IL-2 levels were reduced in lymphocyte culture supernatant; whereas the stimulation of MLT resulted in increased IL-2 levels.[16] IL-2 is a self-proliferating factor released by T lymphocytes, which stimulates activated T lymphocyte proliferation.[17] In this study, there was no significant difference of proliferation when comparing the IL-2 and MLT+IL-2 treatment groups to the control group. Although 3BP2 is the key receptor protein that mediates IL-2 gene transcription in T lymphocytes, the growth, proliferation, and signal transduction of T lymphocytes did not decrease with reduced IL-2 levels in 3BP2 knockout mice.[18] Taken together, the effect of IL-2 on lymphocytes requires further study.

MLT levels are low in the immature immune system of neonates.[5] In addition, lymphocyte phenotype and function are different between adults and children. In this study MLT enhanced the proliferation of neonatal mononuclear cells. Therefore, exogenous MLT could stimulate proliferation of neonatal T and B cells, resulting in increased cytokine release, promotion of neonatal immunoglobulin isotype switching, and an elevated neonatal immune system.

In this study, a low dose of ethanol was used in

MLT solvent. Many other studies showed that ethanol has a negative effect on cell growth. Ethanol exposure resulted in a concentration-dependent apoptosis in rat-pancreatic acinar AR42J cells.[19] Torricelli and coworkers[20] found a decrease in the cell viability and synthetic activity of osteoblasts exposed to ethanol. However, the inhibitory effect of ethanol in the solvent on CBMCs or PBMCs was not obvious in our study. Because the effect of ethanol on cells is concentration-dependent, and the dose of ethanol in our study was too small. 5% ethanol showed an inhibitory effect on the Mucor rouxii mycelial growth at all stages, whereas Mucor rouxii was tolerant to low ethanol concentrations (about 1%-3%).[21] The final concentration of ethanol in 50 ng/ml MLT cultures was just 0.5‰, which was much lower in the other groups. The exact effect of ethanol on lymphocytes awaits further study.

Funding: This study was supported by grants from the Tackle Key Program in Science and Technology for Social Development of Guangdong Province (No. 2002C31204) and the Medical Science Research Foundation of Guangdong Province (No. AZ2002595).

Ethical approval: The study was approved by the hospital's Ethics Committee and informed consent was obtained from all the volunteers before collection of blood samples.

Competing interest: No benefits in any form have been received or will be received from any commercial party related directly or indirectly to the subject of this article.

Contributors: Zhou W proposed the study and wrote the first draft. All authors contributed to the design and interpretation of the study and to further drafts. Zhou W is the guarantor.

References

1 Clark EA, Ledbetter JA. How B and T cells talk to each other. Nature 1994;367:425-428.
2 Claustrat B, Brun J, Chazot G. The basic physiology and pathophysiology of melatonin. Sleep Med Rev 2005;9:11-24.
3 Kühlwein E, Irwin M. Melatonin modulation of lymphocyte proliferation and Th1/Th2 cytokine expression. J Neuroimmunol 2001;117:51-57.
4 Silva SO, Rodrigues MR, Ximenes VF, Bueno-da-Silva AE, Amarante-Mendes GP, Campa A. Neutrophils as a specific target for melatonin and kynuramines: effects on cytokine release. J Neuroimmunol 2004;156:146-152.
5 Commentz JC, Uhlig H, Henke A, Hellwege HH, Willig RP. Melatonin and 6-hydroxymelatonin sulfate excretion is inversely correlated with gonadal development in children. Horm Res 1997;47:97-101.
6 Pandi-Perumal SR, Srinivasan V, Maestroni GJ, Cardinali DP, Poeggeler B, Hardeland R. Melatonin: Nature's most versatile biological signal? FEBS J 2006;273:2813-2838.
7 Drazen DL, Bilu D, Bilbo SD, Nelson RJ. Melatonin enhancement of splenocyte proliferation is attenuated by luzindole, a melatonin receptor antagonist. Am J Physiol Regul Integr Comp Physiol 2001;280:R1476-1482.
8 Currier NL, Sun LZ, Miller SC. Exogenous melatonin: quantitative enhancement *in vivo* of cells mediating non-specific immunity. J Neuroimmunol 2000;104:101-108.
9 Poon AM, Liu ZM, Pang CS, Brown GM, Pang SF. Evidence for a direct action of melatonin on the immune system. Biol Signals 1994;3:107-117.
10 García-Mauriño S, Pozo D, Carrillo-Vico A, Calvo JR, Guerrero JM. Melatonin activates Th1 lymphocytes by increasing IL-12 production. Life Sci 1999;65:2143-2150.
11 Garcia-Mauriño S, Gonzalez-Haba MG, Calvo JR, Rafii-El-Idrissi M, Sanchez-Margalet V, Goberna R, et al. Melatonin enhances IL-2, IL-6, and IFN-gamma production by human circulating $CD4^+$ cells: a possible nuclear receptor-mediated mechanism involving T helper type 1 lymphocytes and monocytes. J Immunol 1997;159:574-581.
12 Kuhlwein E, Irwin M. Melatonin modulation of lymphocyte proliferation and Th1/Th2 cytokine expression. J Neuroimmunol 2001;117:51-57.
13 Arias J, Melean E, Valero N, Pons H, Chacín-Bonilla L, Larreal Y, et al. Effect of melatonin on lymphocyte proliferation and production of interleukin-2 (IL-2) and interleukin-1 beta (IL-1 beta) in mice splenocytes. Invest Clin 2003;44:41-50.
14 Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997;130:388-393.
15 García-Mauriño S, Pozo D, Calvo JR, Guerrero JM. Correlation between nuclear melatonin receptor expression and enhanced cytokine production in human lymphocytic and monocytic cell lines. J Pineal Res 2000;29:129-137.
16 Carrillo-Vico A, Calvo JR, Abreu P, Lardone PJ, García-Mauriño S, Reiter RJ, et al. Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance. FASEB J 2004;18:537-539.
17 Saloga J, Schwinzer R, Renz H, Terada N, Or R, Gelfand EW. Characterization of the progression signal for human T-cell proliferation provided by monoclonal antibodies to the CD3-TCR complex. Clin Immunol Immunopathol 1993;67(3 Pt 1):232-239.
18 de la Fuente MA, Kumar L, Lu B, Geha RS. 3BP2 deficiency impairs the response of B cells, but not T cells, to antigen receptor ligation. Mol Cell Biol 2006;26:5214-5225.
19 Wu H, Bhopale KK, Ansari GA, Kaphalia BS. Ethanol-induced cytotoxicity in rat pancreatic acinar AR42J cells: role of fatty acid ethyl esters. Alcohol Alcohol 2008;43:1-8.
20 Torricelli P, Fini M, Giavaresi G, Rimondini L, Tschon M, Rimondini R, et al. Chronic alcohol abuse and endosseous implants: linkage of *in vitro* osteoblast dysfunction to titanium osseointegration rate. Toxicology 2008;243:138-144.
21 Laoteng K, Jitsue S, Dandusitapunth Y, Cheevadhanarak S. Ethanol-induced changes in expression profiles of cell growth, fatty acid and desaturase genes of Mucor rouxii. Fungal Genet Biol 2008;45:61-67.

Received March 27, 2008
Accepted after revision October 15, 2008