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Melatonin protects mice infected with Venezuelan equine encephalomyelitis virus

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Abstract. We investigated whether the administration of melatonin (MLT) reduces the death rate and evolution of the disease in mice infected with Venezuelan equine encephalomyelitis (VEE) virus. Our results show that MLT protects mice infected with the virus. The mortality rate was reduced from 100% to 16% merely by increasing the dose from 0 to 1000 μg/MLT per kg body weight MLT significantly postponed the onset of the disease and death by several days. In surviving mice very high titres of VEE virus IgM antibodies were found seven weeks after virus inoculation. MLT significantly reduced VEE virus levels in blood and brain of infected mice and increased the survival rate when the length of pretreatment was augmented from 3 to 7 or 10 days before virus inoculation. Serum levels of interleukin-2 were not affected by MLT administration. In control mice receiving MLT as well as in infected mice treated or non-treated with MLT, interferon gamma levels in sera were increased. Interleukin-4 concentrations were found to be elevated in sera of non-infected mice receiving MLT, but did not differ from controls in infected mice treated or non-treated with the hormone. MLT reduced the degree of cell destruction produced by VEE virus in culture plates of chicken embryo fibroblasts. The protective effect of MLT warrants further investigation of the possibility of using this hormone for the treatment of humans and equines infected with VEE virus.
Key words. Melatonin; encephalomyelitis; Venezuelan; equine; virus.

The hormone melatonin (N-acetyl-5-methoxytryptamine) appears to be involved in synchronizing the circadian and seasonal timing of several physiological and behavioural processes [1–7]. Melatonin seems to be the most powerful and effective hydroxyl radical scavenger which provides on-site protection against oxidative damage to cell components [8]. Besides, melatonin plays an important role in the body's immune system by binding to T-helper cells and giving rise to a cascade of events leading to an increase in the immune response [9]. A protective effect of melatonin in mice infected with Semliki Forest virus (SFV) and in stressed mice injected with the attenuated non-invasive West Nile virus (WN-25) was reported [10]. SFV is an arbovirus of low pathogenicity in humans [9], but in mice it invades the central nervous system causing a fatal encephalitis [11]. WN-25 virus does not invade the brain but it can induce encephalitis in mice exposed to various stressful stimuli [12].

Venezuelan equine encephalomyelitis (VEE) virus is a mosquito-borne virus of the family Togaviridae, genus *Alphavirus* [13]. VEE is an important human and equine disease. Outbreaks have occurred in northern South America from the 1920s to the 1970s with thousands of people and horses, donkeys and related varieties affected [14–16]. In previous studies in our laboratory we have found that mice injected intraperitoneally with this virus showed excitation and hypermotility followed by hypomotility, paralysis, coma and death occurring by the sixth day after inoculation [17–19].

In the present work we demonstrate that the administration of melatonin protects mice against VEE virus infection.

Materials and methods

Male albino mice, NMRI- IVIC strain from the Venezuelan Institute for Scientific Research (IVIC), weighing 25–30 g and fed ad libitum with laboratory chow and tap water, were maintained in a room with controlled temperature (24 °C) under a 12 h light/dark cycle. The VEE virus stock used for experiments was prepared in Vero cells and contained 6.8×10^7 plaque-forming units per/ml (PFU/ml). The mice were inoculated intraperitoneally with 0.3 ml containing 10 LD_{50} of the Guajira strain of VEE virus suspended in 0.4 % bovine albumin borate-buffered saline solution (BABS) [20].

Melatonin (Research Biochemicals International, MA, USA) was diluted in PBS and injected daily subcutaneously, 2 h before darkness, starting 3 days before and continuing to 10 days after virus inoculation [10].

Virus content in the serum and brain was plaque assayed in chicken embryo fibroblasts [21]. Serial dilutions of VEE virus were added to confluent monolayers in

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Table 1. The effect of melatonin on survival of mice after inoculation with 10 LD_{50} of VEE virus.

Treatment	Dead/Total	% Dead	Days to death (mean)
VEE	25/25	100	6
VEE + 250 μg MLT/kg	9/20	45*	8
VEE + 500 μg MLT/kg	10/25	40*	10
VEE + 1000 μg MLT/kg	4/25	16*	10

Melatonin (MLT) was injected s.c. daily (5–6 pm) from 3 days before until 10 days after inoculation. *$p < 0.05$ as compared to control infected mice on the sixth day after the virus inoculation.

Table 2. The effect of melatonin on brain virus levels in mice infected with VEE virus.

Treatment	Log_{10} PFU/g		
	day 3	day 4	day 5
VEE	9.19 ± 0.04	8.87 ± 0.07	8.48 ± 0.10
VEE + MLT	8.22 ± 0.07*	8.22 ± 0.09*	non-detected

Melatonin (500 μg/kg) was injected s.c. daily (5–6 pm) from 3 days before until 10 days after inoculation. Six mice of each group were killed daily for testing. Values are expressed as mean ± SEM. *$p < 0.05$ as compared to VEE group.

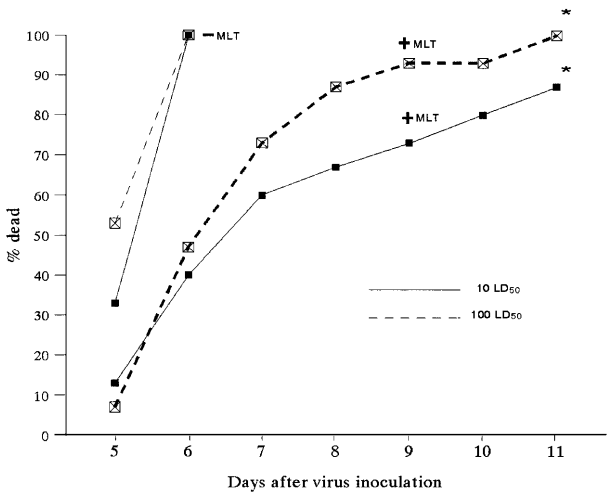

Figure 1. The effect of melatonin (MLT) on mortality rate of mice (n = 15) inoculated with 10 or 100 LD_{50} of VEE virus. MLT (500 μg/kg) was injected s.c. daily (5–6 pm) from 3 days before until 10 days after virus inoculation. *$p < 0.05$ as compared to control infected mice.

24-well culture plates which were incubated at 37 °C for 2 h for viral adsorption. The standard overlay (Eagle's medium 2× and 0.5% agarose) was added. Plates were incubated at 37 °C in 5% CO_2 for 48 h (until cytopathology was evident). Plaques were counted after staining monolayers with 0.2% crystal violet.

For detection of antibodies to VEE virus in infected mice an enzyme-linked immunosorbent assay (ELISA) [22] was used. Plates were coated overnight at 4 °C with goat anti-mouse IgM (μ chain specific) (Sigma Chemical Co., St. Louis, Mo, USA). After a blocking step, plates were washed five times. Samples for titration were added in duplicate, with serial dilutions from 1:20 to 1:10,240, then incubated for 2 h at room temperature. VEE TC-83 antigen was added and incubated for 1 h at 37 °C. VEE polyclonal antibody 1:200 followed by peroxidase-labelled goat anti-mouse IgG conjugate (Sigma) diluted 1:200 were added. After incubation ABTS substrate (azinodiethylbenzthiazoline sulfate) was added and the reaction stopped with 3M NaOH after 30 min incubation. Plates were read at 405 nm in an automatic micro ELISA spectrophotometer. Positive and negative controls were included in each test.

The quantitative determination of mouse interleukin-4 (IL-4), interleukin-2 (IL-2), and interferon gamma (IFN-γ) in sera were based on a solid phase ELISA system developed by Amersham International plc (Biotrak™).

To study the protective effect of melatonin in vitro, concentrations of 1, 10, and 100 μg/ml were added to culture plates of chicken embryo fibroblasts (1×10^5 cells/ml) to which serial dilutions of VEE virus were added. Cell destruction was determined microscopically by counting at least 200 cells from each well, giving a reproductibility to within 5%.

Data are expressed as mean ± SEM and were analysed by means of the analysis of variance and the Bonferroni's multiple comparison test where appropriate. The significance between specific means was determined by Student's t-test. Differences were considered statistically significant when $p < 0.05$.

Results

At day 6 after inoculation of VEE virus (10 LD_{50}/mouse) the mortality rates of mice treated with 250, 500 and 1000 μg melatonin/kg body weight were 45%, 40% and 16%, respectively, as compared to 100% in control infected mice (table 1). In addition, melatonin treatment delayed the onset of the disease and prolonged the time to death in the treated mice (6 days vs. 8 to 10 days) (table 1 and fig. 1).

In five surviving mice treated with melatonin (500 μg/kg) the VEE virus IgM antibody titres were very high, ranging from 1:2560 to 1:5120 and from 1:640 to 1:1280, three and seven weeks after virus inoculation, respectively.

Virus levels in the brains of infected mice are shown in table 2. The administration of melatonin significantly decreased the virus levels in brain as compared to infected control mice on day 3 (9.19 vs. 8.22 log_{10} PFU/g), on day 4 (8.87 vs. 8.22) and on day 5 (8.48 vs. 0).

Table 3 shows that melatonin administration also reduced the virus levels in blood on day 3 (5.00 vs. 2.77 log_{10} PFU/ml). On day 5 no virus was detected in control and melatonin treated mice.

Table 3. The effect of melatonin on blood virus levels in mice infected with VEE virus.

Treatment	Log_{10} PFU/ml		
	day 3	day 4	day 5
VEE	5.00 ± 0.14	3.50 ± 1.03	non-detected
VEE + MLT	2.77 ± 0.10*	1.96 ± 0.69	non-detected

Melatonin (500 μg/kg) was injected s.c. daily (5–6 pm) from 3 days before until 10 days after inoculation of the virus. Six mice of each group were tested daily. Values are expressed as mean ± SEM. *$p < 0.05$ as compared to VEE group.

Table 4. The effect of length of pretreatment with melatonin on survival of mice inoculated with 10 LD_{50} of VEE virus.

Days of pretreatment with melatonin	Dead/Total	% Dead
3	6/15	40%
7	5/20	25%*
10	4/15	27%*

Melatonin (500 μg/kg) was injected s.c. daily (5–6 pm) from 3, 7 or 10 days before until 10 days after inoculation of the virus. On the sixth day after the virus inoculation a 100% mortality rate was observed in mice not treated with melatonin. *$p < 0.05$ as compared to 3 days of pretreatment.

When mice were treated with melatonin for 7 or 10 days before virus inoculation, a significant increase in the survival rate was observed when compared with that obtained after 3 days of pretreatment (table 4).

As shown in figure 2 the degree of cell destruction in culture plates of chicken embryo fibroblasts was significantly reduced by melatonin in the presence of viral dilutions ranging from 10^{-7} to 10^{-4}. Melatonin did not stimulate the synthesis of IL-2. However, it increased the serum content of IFN-γ. In infected mice treated or non-treated with melatonin, IFN-γ levels in sera were also augmented. IL-4 concentrations were found to be elevated in sera of non-infected mice receiving melatonin, but did not differ from control in infected mice treated or non-treated with the hormone (table 5).

Discussion

The present study shows that melatonin administration protects mice infected with the VEE virus. When evaluated 6 days after virus inoculation, the mortality rate was reduced from 100% to 16% merely by increasing the dose from 0 to 1000 μg melatonin per kg body weight. Melatonin significantly postponed the onset of the disease and death by several days. Maestroni et al. [23] were the first to report that melatonin prevented paralysis and death in mice infected with encephalomyocarditis virus after acute stress. Ben-Nathan et al. [10] evaluated the antiviral activity of melatonin using SFV, a classic encephalitis arbovirus, which invades the central nervous system. Unlike VEE virus, SFV is generally considered to be of low pathogenicity in humans

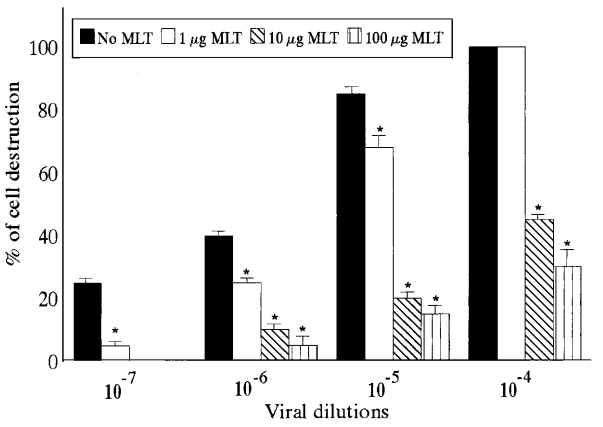

Figure 2. The effect of melatonin on cell destruction in cultures of chicken embryo fibroblasts to which serial dilutions of VEE virus were added. *$p < 0.001$ when compared to its corresponding control (no MLT).

[24] but its replication in the mouse brain leads to death. However, melatonin injection (500 μg/kg) reduced the mortality of SFV (10 PFU)-inoculated mice from 100% to 60%. In those inoculated with a higher dose of SFV (100 PFU), melatonin reduced mortality by only 20%. In mice inoculated with VEE virus we found that, after injecting the same dose of melatonin, mortality decreased from 100% to 40% (10 PFU) and 47% (100 PFU), respectively.

A direct and/or an immune-based effect of melatonin on VEE viral replication within the brain is suggested by the fact that on day 5 after inoculation we could not detect the virus in the brain. Our findings of a significant reduction in VEE virus growth in tissue cultures treated with melatonin contrast with those obtained by Ben-Nathan et al. [10], who found no effect of the hormone on SFV growth in tissue culture. However, our results do not eliminate the possibility that melatonin affects the host resistance to the virus rather than viral replication via a peripheral inmmunostimulating effect, since fibroblasts are capable of producing tumor necrosis factor-alpha whose antiviral activity is known [25].

The immunoenhancing effects of melatonin are becoming increasingly clear. In fact, abrogation of cyclic melatonin secretion by evening administration of β-blockers or by permanent illumination leads to impairment of cellular and humoral immune responses in rodents [23, 24, 26], whereas exogenous administration of this neurohormone enhances antibody production [27]. When chronically injected into young mice or mice immunodepressed by aging or by cyclophosphamide treatment, melatonin was able to enhance the antibody response to a T-dependent antigen [28]. This effect was associated with increased induction of T-helper cell activity and IL-2 production. In the present work we could not detect an increase of IL-2 production in treated and non-treated mice three days after the infection with VEE

Table 5. Serum levels of IL-2, IL-4, and IFN-γ in mice infected with VEE virus.

	PBS	MLT	VEE	VEE + 250 (μg MLT/kg)	VEE + 500 (μg MLT/kg)
IL-2	32.0 ± 1.9	27.6 ± 1.8	27.3 ± 4.8	22.6 ± 5.0	22.6 ± 3.6
IFN-γ	185.0 ± 39.9	1600 ± 141.8*	1966 ± 108.3*	1443 ± 26.8*	1563 ± 194.5*
IL-4	83.0 ± 7.4	150 ± 24.5**	77 ± 12.4	71 ± 0.8	43 ± 14.5

Melatonin (250 or 500 μg/kg) was injected s.c. daily (5–6 pm) from 3 days before until 10 days after inoculation of the virus. Determinations of interleukin-2 (IL-2), interleukin-4 (IL-4) and interferon gamma (IFN-γ) were done 3 days after infection. Three mice of each group were tested. Values are expressed as pg/ml and as mean ± SEM. *$p < 0.001$ when compared to controls (PBS), **$p < 0.05$ when compared to control and the other groups.

virus. However, we cannot discount the possibility of an increase in IL-2 synthesis during the initial stage of the viral infection and immediately after the administration of melatonin. Nevertheless, the significant augmentation in IFN-γ levels detected in serum should have been associated with an inhibition of the IL-2-dependent proliferative response.

The protective effect of melatonin in mice infected with VEE virus could be explained by its stimulatory effect on the synthesis of IFN-γ. This effect would occur just before the mice are infected with the virus and, as a consequence, the probability of reducing viral replication would increase. The decrease in viral titres in serum and brain of infected mice treated with melatonin supports this assumption. Our observations seem to indicate that this viral infection activates the production of certain cytokines. In fact, Martz and Howell [29] have postulated the existence of a cytokine-dependent intracellular inactivation of certain viruses like herpes simplex virus. This mechanism should be thoroughly studied and included in the analysis of the activation by melatonin of other soluble compounds (tumor necrosis factor-alpha, interferons, neutralizing antibodies, etc.) that are considered to be important for the control of infections produced by cytopathic viruses [30].

It has been proposed that, in humans, melatonin represents a neuroendocrine regulator of IL-4 production in bone marrow T-helper cells. Such a neuroendocrine-cytokine mechanism might explain the hematopoietic rescue of melatonin as well as its antitumoral and immunoenhancing properties [31]. However, our results showed that, although serum levels of IL-4 were increased by melatonin, no changes were detected in VEE virus-infected mice treated with this neurohormone. This effect could be explained if a predominance of a type-1 cellular response occurred during the VEE viral infection [32]. The immunoenhancing role of melatonin could then be responsible for the increase in the survival rate we have observed after a longer pretreatment with this neurohormone.

During the 1995 VEE epidemic, people of all ages were affected. Patients usually presented with fever, chills, severe headache, myalgia, prostration, vomiting and sometimes diarrhoea [33]. Convulsions, disorientation, drowsiness and mental depression were also often seen. More than 20 VEE-associated deaths were reported among adults and children in Venezuela and Colombia. For these reasons the protective effect of melatonin on mice infected with VEE virus warrants further investigation, especially for ways of administering melatonin to reduce the final mortality rate of mice infected with the virus and the possibility of using this hormone for the treatment of humans and horses infected with or susceptible to infection by this virus.

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