Effect of Zinc and Melatonin Supplementation on Cellular Immunity in Rats with Toxoplasmosis

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ABSTRACT

The effects of zinc (Zn) and/or melatonin supplementation on cellular immunity were investigated in rats infested with *Toxoplasma gondii.* Fifty Sprague–Dawley male rats were used for this study. All animals were fed a normal diet, *ad libitum*, containing 97 mg Zn/kg. They were divided into five experimental groups, as follows. Group I ($n = 10$) received intraperitoneal injections of zinc sulfate at a dose of 3 mg/kg/d for 3 wk. Group II ($n = 10$) received intraperitoneal injections of melatonin at a dose of 3 mg/kg/d for 3 wk. Group III ($n = 10$) received intraperitoneal injections of zinc sulfate (3 mg/kg/d) and melatonin (3 mg/kg/d) for 3 wk. Group IV ($n = 10$) was infested controls. Group V ($n = 10$) was healthy controls. There were no differences in the percentage of CD3+ lymphocytes among all groups. For groups I–III, the CD4+ and CD8+ ratios were higher than those of the groups IV and V controls ($p<0.01$). Similarly, the total lymphocyte ratios in groups I–III were higher than those of infested and healthy controls ($p<0.01$). The total lymphocyte ratios in group III were significantly higher than those of groups I and II ($p<0.01$). The plasma Zn levels in the supplemented groups were significantly higher than those of control groups IV and V ($p<0.01$). These results suggest that melatonin and/or Zn supplementation may activate cellular immunity by stimulating CD4+ and CD8+ production in infected rats with *T. gondii.*

Index Entries: Cellular immunity; melatonin; toxoplasmosis; *Toxoplasma gondii;* zinc supplementation.

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INTRODUCTION

Zinc (Zn) deficiency is a common nutritional cause of immune deficiency *(1)*. Immunity is directly related to the amount of Zn present in cells and tissues, and in the case of deficiency, infections occur more often *(2)*. Zinc promotes an increase of lymphocyte count and activation and is accepted as a natural T-lymphocyte mitogen *(3,4)*. It has been shown that the levels of interferon-γ (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-α (TNF-α), which are products of Th1 lymphocyte subgroup of CD4+ Th-lymphocytes, depend on Zn and a Zn-dependent hormone called thymulin decrease in Zn-deficient adults *(5)*.

Melatonin, which is a hormone primarily secreted by the pineal gland, has both direct and indirect effects on the immune system and especially on cellular immunity *(6)*. It is believed that melatonin increases the absorption of Zn, and through this, it adjusts body Zn levels *(7)*. Decreased levels of Zn, thymulin, and IL-2 in pinealectomized mice support this relation *(8)*. There is increasing interest in the use of parasite models for the investigation of T-lymphocyte subgroups, cytokine regulation, and their functions *(9)*. Cellular immunity is a primary control mechanism in *Toxoplasma gondii* infestations *(10)*. It was shown in both humans and laboratory animals that T-lymphocytes play a major role against resistance to this parasite *(11)*. Assuming that there is strong relation between melatonin and Zn in the immune system, this study was designed to investigate how supplementation with melatonin, Zn, and in combination influence cellular immunity in rats infested with *T. gondii*.

MATERIALS AND METHODS

The study was carried out in the Experimental Medicine and Research Center at Selçuk University, using rats grown at the same center and in strict compliance with the guidelines of its ethical committee. Fifty adult male Sprague–Dawley rats were selected and divided into five experimental groups. All animals received a standard diet that contained 97 mg Zn/kg in their feed.

Group I ($n = 10$) received Zn-supplemented by intraperitoneal injections of zinc sulfate at a dose of 3 mg/kg/d for 3 wk. Group II ($n = 10$) received intraperitoneal injections of melatonin at a dose of 3 mg/kg/d for 3 wk. Group III ($n = 10$) received intraperitoneal injections of zinc sulfate (3 mg/kg/d) and melatonin (3 mg/kg/d) for 3 wk. Group IV ($n = 10$) was infested controls. Group V ($n = 10$) was healthy controls.

The weight of the animals was recorded before and at the end of the experimental period. After 3 wk supplementation, all of the animals were killed by decapitation and blood samples were obtained in 5-mL tubes containing ACD (anticoagulant diluted) and processed for immunological and biochemical analysis.

Toxoplasma gondii *Infestation*

All animals used in the study, except those of group V (healthy controls), were infested by intraperitoneal injection of 0.5 mL serum containing 10–12 *T. gondii* parasites per area, as determined under an optical microscope *(12)*.

T-Lymphocyte Determination

Different monoclonal antibodies (CD3+, CD4+, CD8+) were obtained from Pharmingen (San Diego, CA, USA) and were used for the determination of the lymphocyte subgroups by flow cytometry at the Immunology Laboratory of the Gülhane Military School of Medicine (GATA). The blood samples were mixed with 20 μL monoclonal antibody and were incubated in darkness at room temperature (20–25°C). After 15–30 min, the erythrocytes were removed by adding a lysing solution (FACS, fluorescence activated cell sorter; Becton Dickinson, San Jose, CA, USA) and continuing incubation in the dark for 10 min. The samples were then washed with the lysing solution, washed with saline phosphate buffer (PBS), and added with 500 μL of 1% paraformaldehyde in PBS, setting it aside until analysis. The samples were studied by means of a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) using the Cellquest Program. A count of 10,000 cells was used to determine the percentages of T-lymphocytes and their subgroups.

The individual blood samples were Wright stained and the total lymphocyte percentages were also determined by optical microscopy.

Melatonin Supplementation

A stock solution of melatonin was prepared by dissolving 40 mg of melatonin (Sigma, M-5250) in 3 mL ethanol. This solution was then sterilized and transferred to tubes that were covered by aluminum foil to store in the dark at –20°C until needed. At time of usage, 0.1 mL of this solution was added to 0.9 mL saline and the resulting solution was injected intraperitoneally at 10 PM at a dose of 3 mg/kg to the animals in group II *(13)*. This regime was continued daily for 3 wk.

Zinc Supplementation

The appropriate amount of zinc sulfate was dissolved in distilled water and intraperitoneally injected to the animals in group III. The dose was 3 mg Zn/kg and was given daily at 10 PM for 3 wk *(12)*.

Determination of Zn and Melatonin

The plasma Zn levels were determined by measured flame atomization technique at 213.9 nm by means of a Shimatsu ASC-600 atomic

Table 1
Weight* and Weight Increase of the Animals in the Study Groups

Study Groups**	Weight Before Supplementation	Weight At End of Experiments	Weight Increase
I Zinc Supplemented Infected Group	265.00 ± 8.80	269.62 ± 8.64	4.61 ± 0.44
II Melatonin Supplemented Infected Group	258.46 ± 12.97	261.15 ± 13.28	2.69 ± 1.23
III Zinc plus Melatonin Supplemented Infected Group	262.73 ± 12.38	266.82 ± 12.41	4.09 ± 1.55
IV Infected Control Group	266.00 ± 10.38	270.50 ± 10.65	4.50 ± 1.57
V Normal Control Group	263.50 ± 13.96	269.50 ± 13.53	6.00 ± 1.00

* Means ± SE.
** $n = 10$ in each group.

absorption spectrometer. The results were expressed in micrograms per deciliter.

Plasma melatonin was determined by radioimmunoassay (RIA) by means of a Melatonin I-25 RIA DDV Biochemie GmbH (Germany). The values were reported in picograms per milliliter.

Statistics

The statistical analysis was performed with the SPSS statistical program. The results were expressed as means ± standard error (SE). The Kruskal–Wallis analysis of variance was used for comparison between groups and Mann–Whitney's *U*-test was applied to those with $p<0.05$. The level of statistical significance was set at $p<0.01$.

RESULTS

There were no significant weight differences between and within the experimental and control groups (Table 1). There were no differences of CD3+ lymphocyte percentages among the groups, but the CD4+ and CD8+ lymphocyte values of the infested groups were significantly higher than those of the two control groups ($p<0.01$). The corresponding values of the infested controls were higher than those of the healthy control group.

Table 2
Percentages of the CD3+, CD4+, CD8+ T-Lymphocyte Subgroups
and Total Lymphocyte Percent in the Supplemented Groups and Controls

Groups	CD3+ (%)	CD4+ (%)	CD8+ (%)	Lymphocytes (%)
I	59.73±1.16	50.96±0.80 **a**	48.03±1.20 **a**	75.61±3.94 **b**
II	58.72±2.22	50.77±1.59 **a**	47.19±0.76 **a**	75.76±2.71 **b**
III	59.89±2.43	52.28±1.31 **a**	48.29±1.10 **a**	84.72±3.15 **a**
IV	55.41±2.37	45.88±2.50 **b**	42.13±2.92 **b**	64.50±2.37 **c**
V	53.11±2.83	41.29±1.97 **c**	37.55±2.12 **c**	53.80±1.02 **d**

Note: The letters on each column indicate statistically significant differences ($p<0.01$):
CD4+: a>b, c; b>c
CD8+: a>b, c; b>c
Total lymphocytes: a>b, c, d; b>c, d; c>d

Table 3
Plasma Zinc and Melatonin Levels of Study Groups

Groups	Zinc (µg/dl)	Melatonin (pg/ml)
I	209.15±8.02 **a, b**	74.10±3.70 **c**
II	198.38±9.17 **b**	98.85±3.83 **b**
III	217.36±4.00 **a**	132.23±10.66 **a**
IV	121.30±3.14 **c**	17.47±1.24 **d**
V	124.70±4.08 **c**	18.73±2.53 **d**

Note: The letters on each column indicate statistically significant differences ($p<0.01$):
Zinc: ab>c, a>b, c; b>c
Melatonin: a>b, c, d; b>c, d; c>d

Similarly, the total lymphocyte percentage of all of the infested groups, including controls, was higher than those of the normal controls ($p<0.01$). The total lymphocyte percentage of group III was significantly higher than that of groups I and II ($p<0.01$; Table 2). The plasma Zn and melatonin levels were higher in the infested groups, relative to the infested and healthy control groups ($p<0.01$). The plasma Zn and melatonin levels of group III and the melatonin level of group II were significantly higher than those of group II, the other experimental groups and group I ($p<0.01$; Table 3).

DISCUSSION

Despite an increased tendency to gain weight, there were no significant differences in the animals' weight before and after the experiment (Table 1). Although, as shown in Table 2, there was an apparent increase in the CD3+ percentages of the experimental groups, the differences relative to controls did not have statistical significance.

The catalytic, structural, and regulatory functions of zinc make it an essential trace element that plays an important factor in immunity *(14,15).* It is accepted that Zn deficiency has a stronger effect on immune response than that of any other metallic element *(1,2).* Dietary Zn deficiency has a direct effect on the Th1 function of T-lymphocytes, resulting in increased production of IFN-γ and IL-2. This has a direct negative impact on cell-mediated immunity and lytic activity of natural killer (NK) cells *(3,5).*

Melatonin is a primary neural hormone secreted by the pineal gland. It affects cellular immunity by direct and indirect mechanisms *(7,8).* Melatonin controls secretion of IL-2 by Th1 lymphocytes and INF-γ, thus playing a central role in the activity of immune system *(16).* Following removal of the pineal gland, the plasma levels of zinc, a Zn-dependent hormone called thymulin, IL-2, INF-γ, number of T-lymphocytes, and cytotoxic activity of NK cells decrease significantly. This abnormality is corrected by application of pharmacological doses of melatonin *(16–19).* It appears that Zn is an important mediator of the effect that melatonin exerts on the immune system. In this study, we found that supplementation of Zn, melatonin, or Zn plus melatonin to rats infested with *T. gondii* significantly increases the production of CD4+ and CD8+ lymphocytes. This increase in the T-lymphocyte subpopulation is significantly higher than that of the healthy nonsupplemented group. Cellular immunity is the major control mechanism in toxoplasmosis *(10).* It was proved that the most important mechanism of resistance against this parasitic infestation is mediated by T-lymphocytes *(11,20,21).* Gazzinelli et al. reported that IL-2, a cytokine secreted by the Th2 subgroup of CD4+ lymphocytes, inhibits both IFN-γ syntheses by NK cells, preventing macrophage activation against *T. gondii* through IFN-γ blockage *(22).* It is thought that Th2-lymphocytes may be a negative triggering factor in *T. gondii* infestations because IFN-γ has been established as the most important mediator in the development of resistance in toxoplasmosis *(23,24).* Transfer of IFN-γ to *T. gondii*-infested pregnant rats decreases mortality and development of organ hypertrophy *(25).* In studies on microglial activation by *T. gondii*, in addition to production and regulation of cytokines by CD8+ lymphocytes, the major histocompatibility complex (MHC), class 1, class 2, and CD4+ lymphocytes have been found to be involved *(26,27).* The high CD4+ and CD8+ lymphocyte values reported here are comparable to those previously reported by other investigators. These results show that separate and combined supplementation with Zn and melatonin activates cellular immunity.

The total lymphocyte percentages of the three supplemented groups, I–III, were higher than those of the healthy controls, but similar values of the Zn plus melatonin-supplemented group were higher than both control groups. This suggests that the combined administration of Zn and melatonin is more effective to boost cellular immune functions. It is interesting that the plasma Zn level of group III is higher than that of groups I and II. Similar results are valid for plasma melatonin levels, which are highest in group III, followed by groups II and I, in that order. Also, the plasma melatonin levels of the three supplemented groups are higher than both control groups.

It has previously been shown that pinealectomy decreases plasma Zn levels and that this condition can be reversed by supplementation with melatonin *(7,8,28)*. Our results indicate not only that melatonin does increase the Zn level but also that Zn supplementation elevated the plasma melatonin levels. If pinealectomy and indirectly melatonin has a role in the modulation of the immune system, there must be some feedback involving the pineal gland. How Zn supplementation and/or Zn deficiency affects plasma melatonin levels under conditions of experimental disease will provide some insight about the effects of the immune system on the pineal gland.

CONCLUSIONS

1. Supplementation with Zn, melatonin, and Zn plus melatonin to *T. gondii*-infested rats significantly increases CD4+ and CD8+ lymphocyte percentages significantly. IFN-γ acts as an important defense mechanism in *T. gondii* infestations *(23–25,29)*. The targets of pineal gland-secreted melatonin are IFN-γ and IL-2 *(19,30)*. Similarly, Zn, an important trace element, increases secretion of Th1 products (viz. IFN-γ and IL-2), but it does not increase Th2 cells, which adversely affect cellular immunity *(3,5,14)*.
2. The higher total lymphocyte levels of the group supplemented with Zn and melatonin suggest that combined supplementation would have the greater effect in the activation of cellular immunity. Thus, supplementation with melatonin and Zn would be an important addition to standard therapy in toxoplasmosis.
3. Supplementation with melatonin significantly increases plasma Zn levels and the converse, suggesting an intimate interrelationship between these two factors. New and more detailed studies are needed to explain the Zn–melatonin interactions.

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