EFFECT OF MELATONIN AND ELECTROACUPUNCTURE (EA) ON NK CELL ACTIVITY, INTERLEUKIN-2 PRODUCTION AND POMC-DERIVED PEPTIDES IN TRAUMATIC RATS

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ABSTRACT:
The present study was to evaluate the effect of melatonin(MT) and EA on the cytotoxic activity of natural killer(NK) cells, the dynamic changes of the induction of interleukin-2(IL-2) and the content of POMC-derived peptides, β-endorphins (βE) and ACTH in spleen lymphocytes and in plasma of traumatic rats. The results showed that intraperitoneal (i.p.) injection of MT was able to recover the lower levels of NK cell activity and the induction of IL-2 production; MT could also decrease the higher βE and ACTH levels induced by trauma in spleen lymphocytes and plasma. EA needling of Zusanli(St.36) and Lanwei(Extra.37) points obviously improved the immunosuppression produced by trauma and antagonized the elevation of βE and ACTH contents induced by trauma stress in spleen lymphocytes and plasma. MT + EA could further modulate the depressed immune function, and there was a significant difference compared with MT (i.p.) or EA alone. MT + EA group further decreased the βE and ACTH contents in immune cells and plasma. Yet, the mechanisms of the attenuation of MT and EA on immunosuppression induced by trauma need further study.

KEY WORDS: Melatonin, Electroacupuncture, Surgical trauma, Natural killer cell activity, Interleukin-2, β-endorphin, ACTH

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INTRODUCTION

Previous studies indicated that surgical traumatic stress could inhibit the immune functions accompanied with the disturbance of neuroendocrine system. It is now evidence that the pineal neurohormone melatonin (MT) is able to exert modulating effects on immune function [1,2]. The administration of MT increases the antibody response, the lymphocyte proliferation capacity to ConA and antigen presentation by macrophage [3]. Conversely, surgical or pharmacological pinealectomy depresses the humoral and cell mediated immune response and decreases of IL-2 production [4]. Acupuncture therapy could effectively activate the function of host immune system in various degrees [5,6]. In order to determine the effect of MT and EA on immune function on traumatic rats, the NK cell activity and the induction of IL-2 production after surgical trauma were investigated.

β-endorphin(βE), adrenocorticotropin(ACTH) and related peptides are formed by enzymatic processing of the pro-opiomelanocortin (POMC) precursor molecule. Exposure to stress could release ACTH and opioid peptides from central and peripheral sites. It has been shown that βE and ACTH have a closely relationship with immune regulation [7,8]. For example, in vivo and vitro studies showed that βE and ACTH alter the reactivity of T cells to mitogenic stimulation. Additionally, ACTH and opioid receptors have been identified on various components of immune system such as granulocytes, monocytes and lymphocytes. Thus, it may be that βE and ACTH mediate some of the effects of MT and/or EA on immune system in traumatic stress rats. Therefore, we examine the βE and ACTH levels in spleen lymphocyte and plasma of traumatic rats to assess whether POMC-derived peptides play role in immunoregulatory effect of MT and/or EA.

MATERIALS AND METHODS

1. Animals and Drugs

Male Spargue-Dawley rats weighing 180-220g were supplied by the Experimental Animal Center, Fudan University. The animals were maintained in a temperature-controlled environment under 10h/14h light/dark cycle with free access to standard rat diet and tap water. MT was purchased from Department of Pharmcochemistry, Fudan University. It was dissolved in 5% ethanol saline (v/v) immediately before use. βE and ACTH Radioimmunoassay Kits were purchased from Second Military Medical University.

2. Model of Traumatized Rats

Under light Nembutal anesthesia, the rats were operated longitudinal incision on dorsal median line lengthening 6 cm and on abdominal median line lengthening 5cm. After surgical trauma the animals were kept warmly under standard housing conditions, and given food and water ad libitum [9]. The rats were divided into five groups: Trauma+MT vehicle group, Trauma+EA group, Trauma+MT (i.p.5mg/kg in the late evening) group,
Trauma+MT+EA group and control group. Immune functions and βE and ACTH contents were measured 24 hours after surgery.

3. Method of Electroacupuncture (EA)

Fig. 1 showed that the rat was in a special house where it can move freely. The EA stimulation was applied unilaterally on the right “Zusanli” (St.36) and “Lanwei” (Extra 37) points by using mode G6805 EA apparatus with a continuous wave type (2Hz, 1 mA) after trauma and lasted 60 minutes [9].

![Figure 1. Schematic diagram showing the electroacupuncture procedure on conscious rats moving freely](image)

4. Assay of NK cell activity

Measurement of NK cell activity was conducted according to the methods of lactate dehydrogenase release [10]. The spleen was teased apart, and single-cell suspensions were prepared for culture. The NK sensitive cell line YAC-1 was used as target cell. Tumor cells
were washed twice with RPMI 1640 and adjusted to 1x10^5 cell/ml, the splenic cells were adjusted to 5x10^6 cell/ml. Effective cells were incubated with 100ul target cells in 96 well plates. The spontaneous release was determined by incubating the cells with complete medium, the maximum release was obtained by the treatment with 1% Triton-X-100. After 2h incubation in a humidified atmosphere with 5% CO_2 at 37°C, the plates were centrifuged for 5 min. 100ul supernatant was transferred to the corresponding wells of another flat-bottomed plate. 0.1ml of lactic acid dehydrogenase substrate mixture was added to each well. After 12 min intervals, the plate was read on the ELISA autoreader at a test wavelength of 570nm and a reference wavelength of 655nm. The NK cell activities were calculated according to the formula:

\[
\text{% Specific Lysis} = \left( \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \right) \times 100\%
\]

5. IL-2 activity assay [11]

Spleen lymphocytes were cultured in 24 well plates with 1ml per well of a solution containing 5x10^5 cell/ml at 37°C in humidified 5% CO_2 incubator and stimulated with ConA at a concentration of 5ug/ml. After 24 hours supernatant was collected and frozen at -20°C for later assay. The activity of IL-2 in cell supernatant was determined by measuring the proliferation of murine IL-2 dependent T cell line CTLL-2. Briefly, 1x10^5 CTLL-2 cells were cultured with 1:8 diluted cultured supernatants for 24 h. The cultures were pulsed with 20ul of MTT (5mg/ml). Solution was added to dissolve the dark blue crystals at 25°C. The plate was read on the ELISA autoreader at a test wavelength of 570nm and a reference wavelength of 655nm. The activity of IL-2 was expressed as OD values.

6. ACTH and βE Radioimmunoassay

ACTH and βE were measured by radioimmunoassay using an RIA kit (Department of Neurobiology, Second Military Medical University). Sample collection: The spleen lymphocyte suspension (2x10^5/ml) was incubated with 0.2M HCL, heated in a boiling water bath for 5 min, cooled on ice and homogenized by ultrasound. After centrifugation (10000rpm, 20min), supernatant was collected and stored at -20°C until further processing. Truck blood was collected on ice into tubes containing 1% heparin sodium. The blood was centrifuged and the plasma was stored at -20°C until assay.

7. Statistical Analysis

Results were expressed as mean ± SE. Statistical significance was determined by using analysis of variance (ANOVA). Statistical analysis was made by Student-Newman-Keuls test. P<0.05 was considered statistically significant.
RESULTS

1. Effect of EA and MT on nature killer cell activity of traumatic rats

Fig. 2 showed the effect of MT and/or EA treatment on NK cell cytotoxicity of spleen lymphocytes from traumatic rats. Surgical trauma led to a severe suppression of NK cell activity (16.5±3.26) (P<0.01 vs control). When EA or MT 5mg/kg (i.p.) was given alone after trauma, it produced an obvious antagonism on inhibition of NK cell activity (24.8±4.8, 26.5±5.1) (P<0.05 vs trauma). A significant increase of NK cell activity was found in MT+EA group (32.8±5.34) when compared with EA or MT group (P<0.05 vs Trauma + EA, Trauma + MT).

![Graph showing NK activity](image)

**Fig. 2 Effect of EA and MT on nature killer activity of Traumatic rats**

n=6  * P<0.05 vs trauma;  ** P<0.01 vs trauma;  * P<0.05 vs EA;  
* P<0.05 vs MT;  ** P<0.01 vs control

2. Effect of electroacupuncture and melatonin on the IL-2 activity of traumatic rats

The results showed that the induction of IL-2 production of the rat spleen lymphocytes was significantly decreased at dilution 1:8 after trauma (0.715±0.034) (P<0.01 vs control). An enhancement of IL-2 production was appeared, when i.p. injection of MT or EA was given alone after trauma (0.857±0.047, 0.868±0.044) (P<0.05 vs trauma). In addition, the increase in IL-2 production of spleen lymphocytes was significantly greater in MT+EA group (0.935±0.059) than in trauma, EA or MT group (P<0.01 vs trauma, P<0.05 vs Trauma + EA, Trauma + MT) (Fig 3).
Fig. 3 Effect of EA and MT on the IL-2 activity of traumatic rats

\[ n=6 \quad ^* P<0.05 \text{ vs trauma}; \quad ^{**} P<0.01 \text{ vs trauma}; \]
\[ ^a P<0.05 \text{ vs EA}; \quad ^b P<0.05 \text{ vs MT}; \quad ^{cc} P<0.01 \text{ vs con} \]

3. βE and ACTH contents in spleen lymphocytes

From the Tab. 1 it was shown that the contents of βE and ACTH were significantly elevated (P<0.001 vs control) in lymphocytes after trauma. After MT treatment, the higher βE and ACTH level induced by trauma was decreased (P<0.001 vs trauma). EA treatment antagonized the elevation of βE and ACTH contents in spleen lymphocytes (P<0.001 vs trauma). MT + EA further decreased the βE and ACTH contents in immune cells (P<0.05 vs EA or MT, P<0.001 vs trauma).

Tab. 1 Effect of EA and MT on βE and ACTH levels in spleen lymphocyte on traumatic rats

<table>
<thead>
<tr>
<th>Spleen Lymphocyte</th>
<th>βE (pg/mg)</th>
<th>ACTH (pg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.91 ± 1.67</td>
<td>131.92 ± 31.68</td>
</tr>
<tr>
<td>Trauma</td>
<td>24.53 ± 2.20</td>
<td>337.76 ± 27.60</td>
</tr>
<tr>
<td>EA</td>
<td>16.93 ± 1.71</td>
<td>209.12 ± 29.68</td>
</tr>
<tr>
<td>MT</td>
<td>17.30 ± 2.10</td>
<td>185.92 ± 28.72</td>
</tr>
<tr>
<td>EA + MT</td>
<td>13.49 ± 1.95</td>
<td>140.40 ± 28.60</td>
</tr>
</tbody>
</table>

\[ n=5-6 \quad ^{***} P<0.001 \text{ vs Trau}; \quad ^{cccc} P<0.001 \text{ vs Con}; \quad ^* P<0.05 \text{ vs EA}; \quad ^P<0.05 \text{ vs MT} \]
4. **βE and ACTH contents of plasma**

Plasma βE and ACTH levels in traumatic rats were considerably higher compared with the control group (P<0.001). MT or EA alone could decrease the higher βE and ACTH levels induced by trauma (P<0.05 vs trauma). The βE and ACTH contents in MT+ EA group had a significant difference compared with EA group or MT group (P<0.05 vs EA or MT, P<0.01 vs trauma) (Tab. 2).

<table>
<thead>
<tr>
<th>Plasma</th>
<th>βE (pg/ml)</th>
<th>ACTH (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>394.35±45.90</td>
<td>187.50±31.83</td>
</tr>
<tr>
<td>Trauma</td>
<td>680.00±90.00</td>
<td>306.77±42.00</td>
</tr>
<tr>
<td>EA</td>
<td>529.00±58.95</td>
<td>258.63±8.07</td>
</tr>
<tr>
<td>MT</td>
<td>517.50±59.70</td>
<td>258.43±24.33</td>
</tr>
<tr>
<td>EA+MT</td>
<td>428.35±45.65</td>
<td>214.53±27.5</td>
</tr>
</tbody>
</table>

n=5-6  *p<0.05 vs trau; **p<0.01 vs trau; ***p<0.001 vs con;  
  a<p<0.05 vs EA; b<p<0.05 vs MT

**DISCUSSION**

Clinical and experimental studies have demonstrated the role of MT in increasing immune response and recovering immunosuppression induced by stress or other diseases [12,13]. Wichmann et al [14] reported that 10 mg/kg MT subcutaneously administration after trauma-hemorrhage could significantly improve the splenocyte IL-2 release and the splenocyte proliferative capacity in mice. Pineallectomy led to an impairment of lymphocyte proliferation, which was restored by ip MT 10μg/kg at 16:00 for 7 d [15]. We also found in this study that MT ip 5mg/kg in late evening was able to recover the low levels of natural killer activity and the dynamic changes of the induction of interleukin-2 (IL-2) in rats under traumatic stress. But the mechanisms by which MT induced immunoregulatory effect remains unclear. The presence of specific binding sites or MT receptors on the immune cells suggested that MT might have a direct action on the immune system [16]. On the other hand, the emerging evidences showed that the neuroendocrine system might be an indirect pathway for MT's immunoenhancing effect. T-helper cell-derived opioid peptides as well as by lymphokines and by pituitary hormones seemed to be able to mediate the immunoenhancing action of melatonin [17,18]. Our present result showed that βE and ACTH levels in spleen lymphocytes and plasma parallel the changes of NK cell activity in traumatic stress rats. βE and ACTH are derived from POMC, which are synthesized and processed within various types of immunocytes and central nervous
system, particularly under pathological conditions. Immune cell-derived opioid peptides could interact with opioid receptors. In vivo, injection of naloxone could enhance the spleen NK cell activity and ConA induced lymphocyte proliferation, which suggested that the endogenous opioid peptides might interact with opioid receptor on lymphocyte, then immune function was depressed [19,20]. ACTH was able to bind with ACTH receptor on lymphocyte or induced the production of glucocorticoid, which led to the inhibition of immune function [21,22]. Therefore, it might have a relationship between the improvement of immune function and the decline of POMC-derived peptides contents in spleen lymphocyte after MT treatment in traumatic stress rats. The βE and ACTH in the plasma was known to originate mainly from hypothalamus and pituitary and their levels were regulated predominantly by hypothalamus factors such as corticotropin-releasing hormone (CRH), vasopressin and dopamine, and were influenced by the glucocorticoid feedback acting on the hypothalamus [23,24]. In traumatic rats, plasma βE and ACTH levels were significantly higher than normal group. This was most probably due to the enhancement of the release in pituitary. The decline of βE and ACTH levels in plasma with MT treatment reported in this study could be related either to a decrease in the release of the hormones from the pituitary or to an increase in their metabolism or excretion. Although the significance of the changes of βE and ACTH levels in plasma was not very clear, the previous studies had shown that the endogenous opioid peptides and ACTH may mediate the suppressive effect of certain forms of stress on immune function. The decline of βE and ACTH levels in plasma after MT treatment might contribute to the recovery of nature killer cell and IL-2 activities in traumatic rats.

Acupuncture is an ancient therapeutic technique, which is used in the treatment and prevention of diseases. A considerable amount of evidence has demonstrated that acupuncture could enhance immune function either in man or rodents [25]. Our previous [9,26] and present work observed that EA of “Zusanli” (St. 36) point effectively improved the immunosuppression induced by trauma. When EA+MT was applied after trauma, the low levels of natural killer cell activity and the induction of interleukin-2 on traumatic rats were further augmented as compared to using MT or EA alone. To investigate whether the POMC derived peptides involved in the modulatory effect of the EA in traumatic stress, we have examined the changes of βE and ACTH levels in spleen lymphocytes and plasma. As shown in Table 1 and 2, the traumatic stress itself could cause a significant enhancement of βE and ACTH contents both in spleen lymphocytes and in plasma. When EA was administrated after trauma, the enhanced βE and ACTH levels were recovered significantly in comparison with traumatic group. Our previous work [27] have found that EA could potentiate the function of splenic lymphocyte proliferative response to ConA and the induction of IL-2 production and this effect of EA was blocked by pretreated with opioid antagonist naloxone. These results were similar with Jiu's [28] work. Jiu DH has reported that EA was able to modulate the βE and ACTH contents in normal rats accompanied by the enhancement of the lymphocyte proliferative capacity. These findings, taken together, indicated that βE and ACTH might play a role in the modulation of EA on traumatic stress, the improvement of the suppression of NK cell activity and dynamic changes of the induction of IL-2 production of spleen lymphocytes might be partially mediated by POMC
derived peptides. MT+ EA further depressed the βE and ACTH levels compared with MT group or EA group, further suggesting that MT and EA have a cooperative regulation effect on traumatic rats. Yet, the complex relationship between POMC-derived peptides and immune function need further investigate.

Anecdotal evidence suggested that very large doses (up to 6g) of melatonin could be safely given to human subjects without any apparent side-effects [29]. Sugden [30] has reported that MT had a very low acute toxicity administrated to mice or rats by various routes (i.e., p.o., s.c., i.v.). Our results showed that MT+ EA was an effective curative method to modulate the immune function, and MT+EA could be considered as a safe and effective therapeutic agent for restoring the depressed immunological function after trauma.

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