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Stage-dependent analgesia of electro-acupuncture in a mouse model of cutaneous cancer pain

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Abstract

Acupuncture is one of the most effective alternative medical treatments in pain management with the advantages of simple application, low cost and minimal side effects. However its scientific evidence and laws of action are not very clear in cancer pain relieving. The aim of this study was to examine the immediate and therapeutic anti-hyperalgesic effect of electro-acupuncture (EA) on a mouse model of cutaneous cancer pain. B16-BL6 melanoma cells were inoculated into the plantar region of unilateral hind paw and the thermal hyperalgesia was measured by using radiant heat test and hot plate test. C57BL/6 mice showed moderate and marked hyperalgesia during days 8–12 and from day 14 after the orthotopic inoculation of B16-BL6 melanoma cells into the hind paw. Single EA on day 8 after inoculation showed significant analgesic effect immediately after the treatment, the analgesic effect reached its maximum within 15–30 min and declined to its minimum at 50 min after EA treatment. Single EA treatment on day 20 showed no significant analgesic effect; Repeated EA treatments (started from day 8, once every other day) showed therapeutic analgesic effect, while it showed no therapeutic effect when started from day 16, a relatively late stage of this cancer pain model. The results demonstrated that EA had anti-hyperalgesic effect on early stage of cutaneous cancer pain but not on late stage. These results indicated a tight correlation of EA anti-hyperalgesic effects with the time window of cancer pain.

Keywords: Electro-acupuncture; Melanoma; Cutaneous cancer pain; Analgesia; Mouse

Cancer pain, a kind of common clinical pathological pain syndrome, decreased the cancer patients' quality of life greatly. As improvements in cancer detection and treatment have extended the life expectancy of cancer patients, more attention must be focused on improving the patients' quality of life. Approximately 30–50% of all cancer patients would experience moderate to severe pain, and 75–95% of patients with advanced-stage or metastatic cancer would experience substantial, life-altering cancer-induced pain (Mercadante, 2001; Mantyh et al., 2002). According to the guidelines of the World Health Organization's 'analgesic ladder', treatment with non-steroidal anti-inflammatory drugs and/or opioids, great progress has been made in cancer pain relieving. But because of the relative ineffectiveness and the side effects of currently available treatments, it has been reported that 45% of cancer patients have inadequate and undermanaged

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pain control (De Wit et al., 2001; Meuser et al., 2001). Thus it is necessary to find new effective approaches for cancer pain relieving.

Acupuncture is an ancient form of medicine that originated in China, which has been practiced for over 2000 years. It is one of the most effective alternative medical treatments with the advantages of simple application, low cost and minimal side effects in pain management (Cao, 1997; Eshkevari, 2003). In particular, previous clinical and basic reports showed that acupuncture displayed notable analgesic effect in many kinds of chronic pain conditions (Carlsson and Sjölund, 1994), such as peripheral inflammatory and neuropathic pain (Rapson et al., 2003; Sekido et al., 2003).

Further more, previous reports also showed that acupuncture was one of the most important alternative medical treatments used in cancer patients for pain relieving (Pan et al., 2000; Alimi et al., 2003; Deng et al., 2004). However, very little literature has been reported on its related basic experimental studies. Therefore scientific evidence was still lacking and the mechanisms of acupuncture needed to be investigated. Recently, Sasamura et al. reported a mouse model of cancer pain by orthotopic inoculation of B16-BL6 melanoma to hind paw (Sasamura et al., 2002). The focus of the present study was to examine the immediate and therapeutic analgesic effect of EA in a mouse model of cancer pain while providing experimental evidence for clinical treatment using acupuncture.

Male C57BL/6 mice (6 weeks old) were provided by Shanghai Laboratory Animal Center, Chinese Academy Sciences, Shanghai. They were housed in plastic cages under controlled conditions (24 ± 0.5 °C, 6 a.m. to 6 p.m. alternate light–dark cycles, free access to food and water). All experiments were conducted in accordance with the IASP's guidelines for pain research to minimize the number of animals used and their suffering (Zimmermann, 1983).

B16-BL6 melanoma cells were provided by Institute of Development Aging and Cancer, Tohoku University of Japan. Cells were cultured in Eagle's minimum essential medium containing 5% fetal bovine serum, 2% penicillin/streptomycin. Cells were released from plastic by exposure to mixture of 0.25% trypsin and 0.02% EDTA and then collected by centrifugation for 3 min at 1200 rpm. The resulting pellet was washed twice with 10 ml PBS and re-centrifuged for 3 min at 1200 rpm. After that, the pellet was suspended in PBS, and then counted using a haemocytometer. Finally it was diluted to final concentration for inoculation and kept on ice until injected to mice. Cells (2×10^5) or heat-killed cells were injected subcutaneously into the plantar region of the unilateral hind paw of the mouse in a volume of 20 μl and equi-volume of PBS into the contralateral hind paw. In order to determine the volume of the melanoma growth, the varicose perimeter of the paw in situ was examined by wrapping silk thread three times around the perimeter of the paw in this study.

Mice with melanoma cells inoculation were randomly divided into EA, sham EA (treatment without electrical current) and model (non-treatment) groups. During EA treatment, the trunk of the mice was kept motionless while the head and four limbs kept freedom of movement in a specially designed holder. EA was administered by two stainless steel needles (0.18 mm in diameter) inserted into unilateral acupoint Zu-San-Li (ST36, posterolateral of the knee joint, below the fibular head, interosseous space between tibia and fibula; between anterior tibial muscle and extensor digitorum longus muscle; the anterior tibial artery and vein; superficially, the lateral sural cutaneous nerve and the cutaneous branch of the saphenous nerve; deeper, the deep peroneal nerve) and Kun-Lun (BL60, in the depression between the tip of the external malleoulus and the Achilles tendon; subcutaneously, the peroneus brevis muscle, the small saphenous vein, the posteroexternal malleolar artery and vein, the sural nerve) at a depth of 3 mm and 2 mm, respectively. When restraining the mice into the holders and inserting the acupuncture needles to the acupoint, the mice were underlying brief anesthesia by inhaling ether. The two needles (one in ST36 and the other in BL60) were connected with the output terminals of an EA apparatus (Model G-6805-1A, Shanghai Huayi Medical Electronic Apparatus Company, China). Alternating trains of dense-sparse frequencies (100 Hz for 1.05 s and 4 Hz for 2.85 s alternately, bidirectional asymmetric pulse, 0.6 ms pulse width) were selected. The intensity of stimulation was approximately 1 mA. The stimulation lasted for 30 min each time. The immediate effect of EA was tested for 60 min instantly after the end of EA treatment. To observe the possible therapeutic effects of EA, EA was applied every other day repeatedly, and the analgesic effect was tested the day after EA treatment. In order to exclude the possibility of analgesia induced by stress such as animal fixation, sham EA group animal was accepted the same manipulation as the EA group except without electrical current during sham EA treatment.

In order to examine thermal hyperalgesia in cancer pain mice, the paw withdrawal latency and paw licking response latency were measured using an IITC Model 390 Paw Stimulator Analgesia Meter (Life Science Instruments, USA) and hot plate (Institute of Biomedical Engineering, Chinese Academy of Medical Science, Tianjin, China). In radiant heat test, mice were placed into an inverted, clear plastic cage upon an elevated floor of window glass. After an accommodation period of 30 min, radiant heat was applied to the plantar surface of the mice hind paw until the mice lifted its paw. The intensity of radiant heat was adjusted to elicit the response around 12 s in normal mice. The time from onset of radiant heat application to paw withdrawal

was defined as the paw withdrawal latency. The hyperalgesic threshold was presented as hyperalgesia score (ipsilateral withdrawal latency – contralateral withdrawal latency). In hot plate test, mice were placed into an open-ended cylindrical space with a floor consisting of a metallic plate that was heated by a thermode. The hot plate was maintained at $50 \pm 0.2\,^{\circ}C$ and the response latency of hind paw licking was measured as hyperalgesic threshold, noted as % of control latency ((response latency of experimental mouse/response latency of normal mice) × 100%).

The mean values and the corresponding errors were calculated for nociceptive tests. All data obtained in this study were analyzed to confirm intergroup differences at each of the times studied by one-way analysis of variance (ANOVA), using statistical software SPSS 10.0. Criteria for significance in all analysis was considered as $P < 0.05$.

There were no apparent changes in the size of melanoma tissue until 7 days after inoculation. The melanoma became apparent as a black nodule around 12 days after inoculation (Fig. 1A). Since the volume of mice paws was too small to measure accurately by volumeter, the varicose perimeter of the paw in situ was considered as the relative tumor size and which was examined by using silk thread in this study. The varicose perimeter of inoculated paw was increased markedly 12 days after inoculation (Fig. 1B). As for the test of thermal hyperalgesia, hyperalgesia score was markedly decreased during day 8–12 after live cells inoculation and moderately decreased during day 12–20 after inoculation in comparison to heat-killed group, which indicated an augmented hyperalgesia (Fig. 1C). Similarly in the test of hot plate, response latency was decreased since day 8 after inoculation in live cell injected group in comparison to heat-killed injected group (Fig. 1D).

The immediate effect of EA was examined on day 8 and day 20 after inoculation. On day 8, response latency in melanoma cells injected group was decreased to approximately 80% of normal latency (Fig. 2A). Response latency was increased markedly immediate after the end of EA treatment, reached its maximum within 15–30 min and declined to its minimum at 50 min after the end of EA treatment (Fig. 2B). In contrast, on day 20, response latency in melanoma cells injected group was decreased to approximately 57% of normal latency (Fig. 2A). Response latency showed no significant change before and after EA treatment on day 20 after inoculation (Fig. 2C).

To observe the therapeutic effect of EA analgesia, EA was applied to cancer pain model mice for 30 min every other day. When repeated EA started from day 8 after inoculation, hyperalgesia score was markedly increased after EA treatment twice and peaked after 3 times of

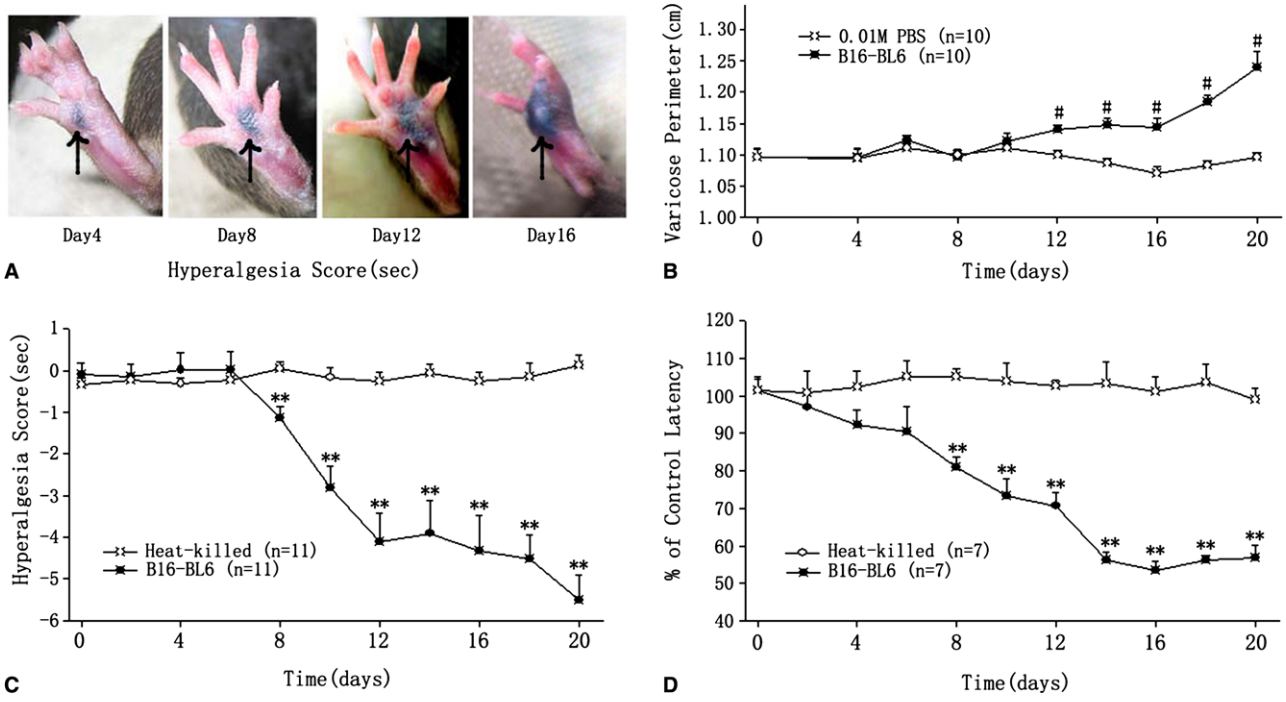

Fig. 1. Tumor growth and nociceptive behaviors of mice after inoculating B16-BL6 melanoma cells. B16-BL6 cells (2×10^5 in 0.01 M PBS) were subcutaneously inoculated into the plantar region of unilateral hind paw. (A) Visual aspect of hind paws on day 8, 12, 16 and 20 after inoculation. ↑, tumor growth site. (B) Changes of the varicose perimeter of the paws inoculated with melanoma cells. (C) Development of thermal hyperalgesia in mice inoculated with 2×10^5 alive or heat-killed melanoma cells (radiant heat test). (D) Development of thermal hyperalgesia in mice inoculated with melanoma cells (hot plate). $^{\#}P < 0.05$ vs. 0.01 M PBS group; $^{**}P < 0.01$ vs. heat-killed group.

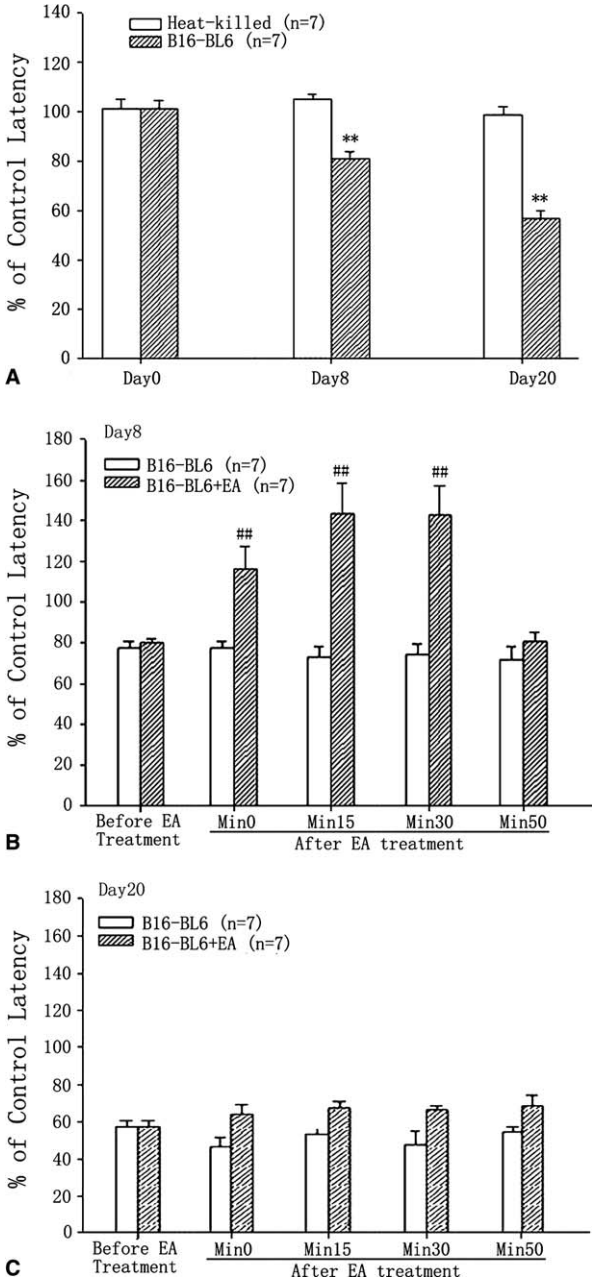

Fig. 2. Analgesic effect of single electro-acupuncture treatment on thermal hyperalgesia of mice inoculated with B16-BL6 melanoma cells (hot plate test). (A) Decrease of response latency of mice on day 8 and 20 after inoculating melanoma cells. (B) Immediate effect of single electro-acupuncture on the response latency of mice on day 8 after inoculating melanoma cells. (C) Immediate effect of single electro-acupuncture on the response latency of mice on day 20 after inoculating melanoma cells. Response latency was tested before and after electro-acupuncture (EA) treatment or sham EA. Data are expressed as means ± SEM. $^{**}P < 0.01$ vs. heat-killed group; $^{\#\#}P < 0.01$ vs. sham EA group.

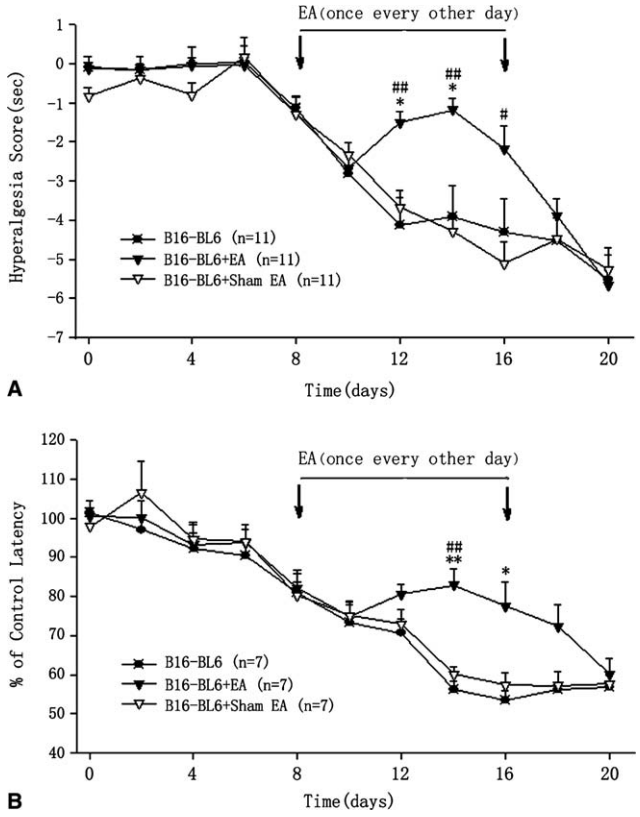

Fig. 3. Therapeutic effect of repeated electro-acupuncture (EA) on thermal hyperalgesia of mice inoculated with B16-BL6 melanoma cells, tested by radiant heat (A) and hot plate (B), respectively. Sham EA is a control treatment without electrical current on the needles. Hyperalgesia score was calculated as the difference of the bilateral paw withdrawal latency (ipsilateral-contralateral) to radiant heat stimulation. The percentage of control latency was calculated as 100% × response latency of experimental mice/response latency of normal mice (B). To avoid the disturbing effect of immediate EA analgesia on the observation of the therapeutic effect of repeated EA, EA and sham EA (as a control) were administered after the pain tests. ↓ symbol indicates the beginning (day 8) and ending (day 16) of the EA treatments, which were given once every other day, each treatment lasted for 30 min. Data are expressed as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. B16-BL6 group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. sham EA group.

treatments, indicating a therapeutic analgesic effect (Fig. 3A). Then the hyperalgesia score was gradually decreased though the EA treatments continued. In contrast, sham EA without electrical current stimulation showed no significant analgesic effect in comparison to model group (Fig. 3A). A similar effect was observed in the test of hot plate (Fig. 3B). However, repeated EA treatment showed no therapeutic effect when started from day 16, a relatively late stage of this cancer pain model (data not shown).

Cancer pain is a difficult problem for clinicians because analgesic drugs do not always procure complete relief and it often remains the dominant symptom affecting the patient's physical and psychological state. It was considered that inflammatory and neuropathic factors contributed to cancer-induced pain (Wacnik et al., 2001; Mantyh et al., 2002). However, previous work indicated that cancer induced a unique pain condition though it shared many features with inflammatory and neuropathic pain conditions (Honore et al., 2000).

Therefore, pain relieving effect by currently available treatments on caner-induced pain was not as perfect as other types of pain conditions, partly due to the complicated mechanisms of cancer pain and partly to the relative ineffectiveness and the side effects of current treatments.

Both clinical practice and experimental studies had proved the significant analgesic effect of acupuncture on chronic pain conditions, such as peripheral inflammatory and neuropathic pain (Rapson et al., 2003; Sekido et al., 2003). In this study, with the persistent tumor growth, thermal hyperalgesia of the mice became apparent between day 8 and day 12 post-inoculation (early phase) and marked around day 14 to day 20 (late phase). These results indicated that there were two phases, with at least two distinct mechanisms, in pain responses to tumor inoculation which is consistent to the report of Sasamura et al. (2002). Single EA treatment on day 8 after inoculation showed significant analgesic effect immediately after the end of EA treatment. The analgesic effect reached its maximum within 15–30 min and disappeared at 50 min after EA treatment. However, EA treatment showed no significant analgesic effect on day 20 after inoculation.

Therapeutic effect of EA on cancer pain was also observed in our study. Repeated EA started from day 8 after inoculation showed significant analgesic effect. However, it showed no therapeutic effect when started from day 16, a relatively late stage of this cancer pain model. The results indicated that EA produced a therapeutic analgesic effect during early stage of cancer pain. This was coincident with the results of immediate effect. The therapeutic effect of repeated application of EA on early stage of cancer pain was similar to those produced in other disease (Chang et al., 1993; Han, 1999; Carlsson, 2002). All of these suggested that the effect of EA correlated with the time window of the cancer pain.

As described in previous studies, cancer-induced pain was a unique chronic pain condition with complicated mechanisms, which might include both inflammatory and/or neuropathic factors (Wacnik et al., 2001; Mantyh et al., 2002). Cancer pain progressively became moderate to severe with increased tumor growth and invasion. Hence, the effect of cancer pain relieving by EA treatment was limited as well as other current therapeutics. Despite the limitation, EA displays many advantages in cancer pain control. It was known to be effective in certain painful conditions with low cost and minimal adverse side effects (Cao, 1997; Eshkevari, 2003). Moreover, previous work showed that acupuncture played an important role in immune modulation (Zhang et al., 1996; Hahm et al., 2004) as well as analgesic effect (Pan et al., 2000; Alimi et al., 2003; Deng et al., 2004).

In general, EA displayed analgesic effect on early stage and no effect on late stage of cutaneous cancer pain. Our study indicated that the analgesic effect of EA was tightly correlated with the time window of cancer pain.

Although the result is novel and inspiring, it is considerably inadequate to start with electroacupuncture treatment instead of a detailed behavioral analysis and characterization of this cancer pain model. More detailed behavioral analysis (such as mechanical allodynia, spontaneous pain) as well as underlying mechanisms, such as electrophysiological recordings from primary afferent fibers and neurochemical changes in spinal dorsal horn, are spontaneously carried out now. These further study might help provide new informations for understanding cancer pain and give scientific evidence for clinical cancer pain relieving by using acupuncture.

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