Efficacy of Acupuncture on Pain Mechanisms, Inflammatory Responses, and Wound Healing in the Acute Phase of Major Burns: An Experimental Study on Rats

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ABSTRACT:

Objectives: We investigated acupuncture, a potential contributor for burn-care, on physiological and pathological pain mechanisms and systemic and local inflammatory responses in a rat experimental burn model. **Methods:** Forty male Sprague-Dawley rats were divided into 2 groups. One-hour groups(5 rats/group) were observed for 1 hour and included Sh1(sham/observation), ShA1(sham+acupuncture/observation), Brn1(burn/observation), and BrnA1(burn+acupuncture/observation). Seven-day groups(5 rats/group) were observed for 7 days and included Sh7(sham/observation), ShA7(sham+acupuncture/observation), Brn7(burn/observation), and BrnA7(burn+acupuncture/observation). “Pain-distress scores” were noted daily, acupuncture was repeated within every wound-dressing change on alternate days. After observation periods, blood samples for interleukin-6 and beta-endorphin and skin biopsies for inflammatory-changes and immunohistochemical-staining of interleukin-6 were collected for analysis($P < .05$). **Results:** In 1-hour groups, interleukin-6 accumulation in burn wounds of BrnA1 was less than Brn1, with Brn1 having the highest mean blood level($P < .05$). Mean beta-endorphin levels were higher in ShA1, Brn1, and BrnA1 than in Sh1($P < .05$). In all 7-day groups, the agonizing period was 48 to72 hours after burn, with Brn7 most affected($P < .05$). Microvessels were multiplied in Brn7group, with significantly higher numbers in burn wounds of BrnA7($P < .05$). Burn wounds of BrnA7 had less

accumulation of interleukin-6 than Brn7 with Brn7-group having the highest mean blood level and Sh7, ShA7, and BrnA7 having similarly low levels($P > .05$). Beta-endorphin levels in ShA7, Brn7, and BrnA7 were lower than in Sh7($P < .05$). **Conclusions:** Acupuncture contributed to management of physiological and pathological pain, modulation of inflammatory responses, and associated enhancement of angiogenesis in acute phase of burn injury in rats.

KEY WORDS: Beta-endorphin, Cytokines, Inflammatory pain, Interleukin-6, Nonpharmacological pain control

INTRODUCTION

Both physiological and pathological pain mechanisms provoke the pain experience of burn victims. Although physiological pain is triggered in response to direct severe tissue damage, pathological pain mechanisms are activated by cytokines, which also play pivotal roles in the systemic inflammatory response to burn injury.[1-3]

Interleukin 6 (IL-6) is a cardinal proinflammatory cytokine in major burn trauma events. This cytokine continuously increases in systemic circulation and various tissues, including burn wounds[2,4-7] . It is mostly produced by active immune cells, such as macrophages; it is also produced by glial cells and even neurons.[8,9] Interleukin 6 exerts its biological effects on various target cells by interacting with the nonsignaling membrane-bound IL-6 receptors (m-IL-6R). By "trans-signaling mechanisms," IL-6 can stimulate cells that do not contain endogenous m-IL-6R.[10,11] Recent studies have demonstrated that IL-6 plays a pivotal role in pathological pain induced by inflammatory mechanisms; IL-6 sensitizes peripheral nociceptors to various stimuli, including mechanical and thermal stimuli.[1,3,12,13]

Beta-endorphins are primarily synthesized and stored in the anterior pituitary gland; they are released into mouse and human plasma in response to stressors such as pain and stress.[14-16] Beta-endorphins relieve pain by binding to opioid receptors, and they exert their effects via presynaptic and postsynaptic binding. However, their primary effect is presynaptic binding.[17] In addition to the pituitary gland, T lymphocytes, B lymphocytes, monocytes, and macrophages are thought to be capable of beta-endorphin synthesis because they contain endorphins during inflammation.[18] In burn trauma, beta-endorphin levels have been shown to increase in response to direct tissue damage, playing an essential role in physiological pain of burn patients.[19-21]

These complex relations affect the wound healing process, with the healing process impaired via hypothalamic-pituitary-adrenal and sympathetic-adrenal medullary axes.[22,23]

To break these cyclespain management and modulation of systemic and local inflammatory responses are indispensable in modern burn care. In this regard, long-lasting and sophisticated pain management strategies have been created for burn victims. Burn teams currently favor medications such as opioids, ketamine, nonsteroidal anti-inflammatory agents, and other sedoanalgesics. Because these agents, especially the opioids, have disturbing and/or life-threatening side effects, nonpharmacological methods for pain and distress management are receiving ongoing attention. For instance, virtual reality analgesia techniques are currently in use during acute and long-term burn care, including during physical rehabilitation.[24,25]

Acupuncture, a part of traditional Chinese medicine, has been used as a nonpharmacological therapy for pain control for thousands of years. The basic mechanism of acupuncture is microinjury, which increases local blood flow, thus facilitating healing and analgesic effects. An acupuncture needle stimulates the nerves of local tissue, releasing neuropeptides that play roles in vasodilation and increased local circulation.[26] Increased local blood flow in the affected area is followed by subsequent modulation of local and distant neuropeptides, cytokines, and other vasoactive substances.[27] Acupuncture is useful in immunomodulation and anti-inflammatory therapies in a range of disorders. For instance, scalp acupuncture can improve neurologic function, which is related to upregulation of interleukin 10 (IL-10) expression and then downregulation of IL-6 and tumor necrosis factor alpha (TNF-α; reducing inflammatory response) expression in the parahippocampal gyrus.[28-31]

Some recent studies have focused on the possible contribution of acupuncture in first aid of burn care, wound care, posttraumatic distress syndrome, pain and distress management,

and scar therapy.[32-34] In our recent study, we created an experimental model for the application of acupuncture in burn trauma. In that study, we observed that repeated and continuous acupuncture application to both Ashi points (acupuncture points around the affected area) and Back-Shu points (acupuncture points on the relevant dermatomes) resulted in lower pain and distress scores in rats. Concurrent signs of enhanced neovascularization at burn wounds was observed in association with these findings.[35]

In the present study, we investigated the effects of repeated and continuous acupuncture on blood levels of beta-endorphin and IL-6 with simultaneous observations on histopathological changes in rats. We also evaluated the immunohistochemical expression of IL-6 on rat burn wound tissues. Our aim was to analyze the influence of repeated and continuous acupuncture on physiological and pathological pain mechanisms, inflammatory responses, and wound healing in our rat experimental burn model.

MATERIALS AND METHODS

Our study included 40 male Sprague-Dawley rats weighing 300±15 gr. Rats were housed in the ‘’B……’’University Laboratory Animal Center under standard conditions with 12:12-h light-dark cycle and fed standard rat chow and water ad libitum for at least 1 week before start of experiments. Before the study, all protocols were approved by ‘’B…..’’ University’s animal welfare regulatory committee, and all protocols were in conformity with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health 86-23, revised in 1985.(DA:14/56-2).

Anesthesia and burn model

Rats were anesthetized with an intramuscular injection of 70 mg/kg ketamine hydrochloride 10% and 7 mg/kg xylazine hydrochloride 2%. Burns were induced at the right lower quadrants of the animals using our previously described model.[35] Ringer's solution with lactate (2 mL/100 g) was used intraperitoneally for prompt fluid replacement after burn induction/sham procedures.

The thickness of each burn wound was verified histologically in all samples. All were extending to the deepest one-third of the dermis in the Brn1 group, BrnA1 group, Brn7 group, and BrnA7 group (see group descriptions below).

Acupuncture procedure

For the present study, we used our previously described experimental acupuncture model. Ashi points (acupuncture points located around the imaginary burn injury sites for sham groups and around the burn wounds for burn groups) and Back-Shu points (acupuncture points on the related dermatomes) were the locations where acupuncture applied[35].

Steel temporary acupuncture needles (KINGLI 0.16 × 7 mm, Acupuncture needle, moxa, massage product; Jiangsu, China) were pinned to both Ashi points and Back-Shu points. After 20 minutes, temporary needles were replaced with long-acting ones on the same points.[35]

Groups

Rats were divided into 2 subgroups according to blood and skin sampling timings. The 1-hour groups (Sh1 group, ShA1 group, Brn1 group, and BrnA1group) were created to investigate the early effects of the procedure, and the 7-day groups (Sh7 group, ShA7, Brn7 group, and BrnA7 group) were created to observe initial week after injury induction. All animals were anesthetized before the procedures.

One-hour groups

Each of the 1-hour groups included 5 rats The Sh1 group was the 1-hour sham/observation group. After application of anesthesia, dorsa were shaved. After 1 hour of waiting, blood and skin samplings were completed and animals were killed.

The ShA1 group was the 1-hour sham-acupuncture/observation group. After the anesthesia procedure and shaving, a 4 × 4-cm area was drawn on the right lower quadrant of dorsum for each animal. Temporary needles were pinned to this imaginary wound's Ashi points and to Back-Shu points. After a 20-minute wait, these needles were replaced with long-acting ones. One hour after sham-acupuncture procedures, ShA1 animals were killed after the samples were collected.

The Brn1 group was the 1-hour burn/observation group. After the anesthesia procedure and shaving, burns were induced on right lower quadrant of dorsa (4x4 cm^2). One hour after burn induction, the samplings were completed and Brn1 animals were killed.

The BrnA1 group was the burn/acupuncture group with observation during one hour after burn induction. After anesthesia induction and shaving, burn injuries were induced as described before. Temporary needles were pinned to Ashi points and to Back-Shu points. After a 20-minute wait, these needles were replaced with long-acting ones. One hour after the acupuncture procedure, the samples were collected, and the animals were killed.

Seven-day groups

Each of the 7-day groups included 5 rats. The Sh7 group was the sham/7-day observation group. In this group, rats were anesthetized, and dorsa were shaved. After a 1-hour wait, wound dressings were put on. Wound dressings were renewed every other day. Pain-distress scores were noted daily for 7 days. On postinjury day 7, after blood skin samplins were completed and animals were killed.

The ShA7 group was the sham-acupuncture/7-day observation group. Rats were anesthetized, dorsa were shaved, Temporary needles were pinned to Ashi points of the imaginary burn injury sites and to Back-Shu points. After a 20-minute wait, these needles were replaced with long-acting needles. Wound dressings were put on. Acupuncture procedure+wound dressings were repeated every other day, and pain- distress scores were noted daily during 7 days. Animals were killed after the samples were obtained, at 7 days after sham-acupuncture procedures.

The Brn7 group underwent burn with 7-day observation after injury induction. Rats were anesthetized, their dorsa were shaved, and burns were induced as described above. One hour after injury induction, wound dressings were put on. The procedure, including wound dressing changes, was repeated every other day. Pain-distress scores were noted daily for 7 days. On postinjury day 7, animals were killed after the samples were collected.

The BrnA7 group underwent burn-acupuncture with observation for 7 days after injury induction. After rats were anesthetized, dorsa were shaved, and burns were induced. Temporary needles were pinned to Ashi points and Back-Shu points. After a 20-minute wait, these needles were replaced with long-acting ones. Acupuncture procedures and wound dressings were renewed every other day; pain-distress scores were noted daily during 7 days. Animals were killed following collection of the samples on postinjury day 7.

Pain and distress evaluation, pain control

For optimum pain and distress management without affecting the experimental design, evaluation of pain and distress was based on our pain-distress scale, which had been modified from ‘pain and distress rating scale and long-term postsurgery monitoring for non-USDA-regulated rodent studies’ (BIACUC protocol 2008.06) (Table 1).[35,36] Two veterinary technicians were tasked with observing and noting the scores of the animals’ behaviors every 24 hours. They consulted the veterinarian for pain relief, warming and/or fluid replacement of

affected animals, if the score was higher than +2 in any category and/or if the total score was higher than +3. If pharmacological analgesia methods were indicated, fentanyl hydrochloride (0.02 mg/kg) was preferred. The technicians also noted the frequency of needs for warming and frequencies and amounts of fentanyl hydrochloride injections and fluid resuscitations.

Each animal was inhabited in separate cages to protect the wound dressings from opening until they were changed on scheduled dates.

Tissue processing

Skin biopsies were collected from intact skin in the Sh1 and Sh7 groups. In the ShA1 group and ShA7 group, samples were collected from the drawn 4 × 4-cm area. In the Brn1 group, BrnA1 group, Brn7 group, and BrnA7 group, samples were collected from burn wounds. All skin samples were embedded in paraffin blocks after fixing in formaldehyde. Several 4-μm thick sections were prepared. By grid at a magnification of ×200 using an eyepiece screen with an examination area of 0.25 mm², all representative sections were examined under a light microscope for confirmation of burn depth and for evaluation of angiogenesis and inflammatory changes (hematoxylin and eosin).

For evaluation of inflammation, amounts of mononuclear inflammatory cells and neutrophil leukocytes in tissue samples were graded in a semiquantitative manner[35]:

No mononuclear inflammatory cell infiltration was expressed by ‘grade 0’, mononuclear inflammatory cell accumulation lower than 30% of the inspection area of 0.25 mm^2 was expressed by ‘grade 1’. ‘Grade 2’ expressed 30% or more mononuclear inflammatory cell infiltration in the inspection area.

For neutrophil leukocytes, ‘grade 0’ indicated no neutrophil infiltration; ‘grade 1’indicated that neutrophil occupation lower than 10% of the inspection area (0.25 mm^2), and ‘grade 2’ indicated that neutrophil occupation 10% or more of the inspection area.

Numbers of microvessels per unit of area were evaluated with a grid. Microvessels were determined by counting all vessels using an eyepiece screen with an inspection area of 0.25 mm^2 (×200).

Briefly, 3-μm-thick sections were embedded on poly-L-lysine-coated slides for immunohistochemical staining, after deparaffinization. Interleukin 6 monoclonal antibodies (Dako) were stained in all tissues automatically by a Dako autoimmunostainer as described by the manufacturer. Tissues that immunostained with IL-6 antibody were graded in a semiquantitative manner (the 0 to 2+ grading scale): 'Grade 0' expressed no IL-6 staining, 'grade 1' expressed that stained cells were less than 20% of total cells. 'Grade 2' indicated that stained cells were denser than 20% of total cells (Figure 1).

Measurement of blood levels of interleukin 6 and beta-endorphin

Serum samples were separated from whole blood by centrifugation. Samples were stored in aliquots at -70 ºC. Beta-endorphin levels and IL-6 levels were measured by means of sandwich enzyme immunoassay kits (Cloud-Clone Corp) according to the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed using SPSS software (Statistical Package for the Social Sciences, version 16.0, SSPS Inc). Results of semiquantitative measurements are presented as medians. Results of quantitative measures are presented as means ± SE. For nonnormal distributions of variations, the Kruskal-Wallis test was used. The Mann-Whitney U test with Bonferroni correction was performed when the Kruskal-Wallis test indicated significant differences. The chi-squared test was used for investigations on categorical data ($P < .05$)

RESULTS

No skin infections were observed in the Sh1 group, ShA1 group, Sh7 group, and ShA7 group. No burn wound infections were observed in the Brn1 group, BrnA1 group, Brn7 group, and BrnA7 group.

One-Hour Groups:

Histopathological examination of 1-hour groups

No neutrophil accumulation (grade 0) was observed in the Sh1 group, ShA1 group, and BrnA1 group. In the Brn1 group, neutrophil amounts were higher than 10% (grade 2). Mononuclear cell accumulation was lower than 30% (grade 1) in the Sh1 group, ShA1 group, Brn1 group, and BrnA1 group. (Figure 2A).

Quantitative measurements of vascular structures revealed that mean numbers of vascular structures in the Sh1 group (5.0 ± 1.58) and ShA1 group (6.4 ± 1.95) were similar ($P > .05$). Mean numbers of vascular structures in the Brn1 group (22.8 ± 2.94) and BrnA1 group (19.4 ± 2.41) were identical ($P > .05$), but they were significantly higher than mean vascular numbers in the Sh1 and ShA1 groups ($P < .05$) (Figure2B).

Immunohistochemical evaluation of burned skin samples in the 1-hour groups

Our immunohistochemical investigation indicated that, in the initial 1 hour, the Brn1 group had high IL-6 accumulation (1.0 ± 0.13). However, the BrnA1 group had more trace amounts of IL-6 (0.5 ± 0.19) ($P < .05$) (Figure 3).

Blood levels of IL-6 and beta-endorphin in 1-hour groups

In the initial 1 hour, Brn1 group had the highest mean blood level of IL-6 (238.10 ± 78.86 pg/mL). Levels of IL-6 in BrnA1 group (184.90 ± 54.37 pg/mL) and ShA1 group (128.1 ± 38.8 pg/ml) were higher than in the Sh1 group (80.4 ± 40.8 pg/mL); all were lower than in the Brn1 group ($P < .05$) (Figure 4A). Mean beta-endorphin level was 95.9 ± 14.8 pg/mL in

Sh1 group. Beta-endorphin levels similarly increased in the ShA1, Brn1, and BrnA1 groups(131.10 ± 40.6, 214.60 ± 42.50, and 237.30 ± 75.96 pg/mL, respectively) ($P < .05$). (Figure 4B)

Seven-day Groups:

Daily pain and distress observations in 7-day groups

The Sh7 group, ShA7 group, Brn7 group, and BrnA7 group were observed throughout 7 days. Alerting pain-distress scores were frequently observed in the Brn7 group. Thus, animals in the Brn7 group required more frequent veterinarian consultation than the Sh7, ShA7, and BrnA7 groups. All animals in the Brn7 group needed extra fentanyl and/or lactate Ringer injections and warming.

On day 1, median pain-distress scores were +3 or more for animals in the Brn7 group, and animals required veterinarian consultation. None of the animals in the other study groups needed consultation of veterinarian, with median pain-distress scores under +2.

On day 2, all Brn7 group animals experienced high pain and distress scores; one animal required veterinarian consultation (median pain and distress score was +3). Animals in the S7, SA7, and BA7 groups stayed at lower pain and distress scores.

On day 3, pain-distress scores were similar among the study groups except 2 animals in the Brn7 group, which were still experiencing severe pain and distress and required veterinarian care.

On days 4, 5, 6, and 7, none of the animals in any group required veterinarian consultation

(Table 2).

Histopathological examination in the 7-day groups

Neutrophils comprised lower than 10% of examination areas (grade 1) in 2 (40%) animals in the Brn7 group and 3(60%) animals in the BrnA7 group. Three animals (60%) in the Brn7 group and 2 animals (40%) in the BrnA7 group had neutrophil accumulation denser than 10% (grade 2). No neutrophil accumulations (grade 0) were detected in the Sh7 and ShA7 groups.Mononuclear cell accumulation was lower than 30% (grade 1) in the Sh7 group and ShA7 group, but it was denser than 30% (grade 2) in the Brn7 and BrnA7 groups (Figure 5A).

At the end of 7 days, the mean numbers of vasculary structures in the ShA7 group (8.6 ± 0.55) were higher than in the Sh7 group (6.0 ± 0.89; $P < .05$). In both the Brn7 group (24.8 ± 1.77) and BrnA7 group (31.0 ± 1.18) numbers of new microvasculary structures were higher than the Sh7 group and ShA7 group($P < .05$). The BrnA7 group had the highest mean number of microvessels ($P < .05$) (Figure 5B).

Immunohistochemical evaluation of burned skin samples in the 7-day groups

At the end of 7 days, the Brn7 group still had a high amount of IL-6 accumulation (1.5 ± 0.19) (Figure 6). The BrnA7 group had trace amounts of IL-6 (1.0 ± 0.18; $P < .05$) .

Blood levels of IL-6 and beta-endorphin in the 7-day groups

Seven days after burn injury, the Brn7 group had the highest mean blood level of IL-6 (176.20 ± 20.43 pg/mL; $P < 0.05$). The Sh7, ShA7, and BrnA7 groups had similar blood levels of IL-6 (64.3 ± 5 2.01, 31.14 ± 12.85, 31.14 ± 12.85, and 25.9 ± 15.79 pg/mL respectively) (Figure 7A). Seven days after burn injury, the mean beta-endorphin level in the Sh7 group was 129.2 ± 32.63 pg/mL. When compared with the Sh7 group, beta-endorphin levels were lower in the ShA7, the Brn7, and the BrnA7 groups (100.4 ± 49.8, 104.6 ± 41.67, and 99.09 ± 10.96 pg/mL, respectively; $P < .05$); however, the BA7 group had significantly lower beta-endorphin levels than the Brn7 group ($P < .05$) (Figure 7B).

DISCUSSION

Observations in the 1 hour after burn injury and during the subsequent 7 days demonstrated that repeated and continuous acupuncture had some effects on physiological and pathological pain mechanisms, inflammatory responses, and wound healing in the rat experimental burn model.

Decreased blood levels of IL-6 in the acupuncture-applied BrnA1 group suggested that, with severe burns, acupuncture may have a contributory role by decreasing blood levels of IL-6 even in the initial 1 hour after burn induction. Immunohistochemical findings supported this suggestion, with IL-6 accumulation in the BrnA1 group's burn wounds lower than in the Brn1 group. With consideration of the primary role of IL-6 in inflammatory responses, the observed decreased IL-6 levels in blood and the trace accumulation of IL-6 in burn wounds of the BrnA1 group indicated a suppressed inflammatory response in these animals. No neutrophil accumulation in burn wounds of the BrnA1 animals, in contrast to that shown in Brn1 animals, seems to be another clue for the contribution of acupuncture in modulation of inflammatory responses in the early phase of burn injury. However, the efficacy of early modulation of the inflammatory response remains a subject of debate. Some recent studies have asserted that inflammatory responses in the very early phase of burns were advantageous for the wound healing process[37]. In this regard, further experimental and clinical studies concerning the effects of early anti-inflammatory modulation, including timing of acupuncture in burn care, are needed. On the other hand, in several previous studies, IL-6 knockout mice showed reduced mechanical and thermal hyperalgesia in response to noxious inflammatory stimuli, indicating the cardinal role of this cytokine in

pathological pain mechanisms.[38, 39] In the present study, the contribution of acupuncture may be related to its efficacy on control of pathological pain rather than modulation of inflammation in the early phase of burn injury.

As expected, blood beta-endorphin levels were significantly high 1 hour after severe tissue damage from burn induction in the Brn1 group. Increased blood beta-endorphin levels in the ShA1 group and in the BrnA1 group indicated that acupuncture using single stimulation of Ashi points and Back-Shu points with dry needles also triggered beta-endorphin responses. This finding was supported by previous studies that reported that the acupuncture needle's mechanical movements are important for systemic release of neural signaling molecules, such as opioid peptides (particularly beta-endorphin), glutamate, and adenosine calcium.[26,40-42]

Daily "pain and distress scoring" observations revealed that animals in the Brn7 group experienced the most severe discomfort and fatigue, with the most painful and distressful period 48 to 72 hours after burn injury. However, pain experience in the acupuncture-applied burn group (BrnA7) was as mild as that shown in the sham groups at the end of 7 days. To explain the mild pain experience and low blood levels of beta-endorphin in the BrnA7 group, decreased blood levels of IL-6 and trace amounts of IL-6 in the group's burn wounds must be considered. Repeated and continuous acupuncture may have interrupted the ongoing pathological pain as well as the severe systemic inflammatory response in the initial 7 days,[6,8]hence controlling the pain experience and indirectly lowering beta-endorphin response in blood. This argument is compatible with a previous study from Qing and associates in which the investigators suggested that acupuncture could effectively downregulate serum levels of relative inflammatory factors in patients with cancer-related fatigue.[43] Another

eventual component is the simultaneous acupuncture treatment with both Ashi and Back-Shu points. The application of acupuncture to Ashi points, which was repeatedly and continuously applied throughout 7 days, may have triggered the consumption of endogenous opiates via local mu-opioid receptors in the burned area, whereas Back-Shu points triggered the systemic changes. These opioid receptors have been identified on numerous immune cells, including B lymphocytes, T lymphocytes, natural killer cells, neutrophils, and monocytes.[18,44,45] Further investigations concerning the effects of acupuncture on components of the proinflammatory phase and the counter anti-inflammatory phase of the immune response, local expression of endogenous opioids and their receptors in these cells, and interactions with pathways of pain mechanisms and acupuncture in and around the burn wounds will help to unveil the potential efficacy of acupuncture.

The relatively high blood level of beta-endorphins in the sham group (Sh7) can be explained with distress triggered by the sham interventions and long and lonely stays of these uninjured rats in their cages.[46] In the acupuncture-applied uninjured ShA7 group, the low systemic beta-endorphin response may be accepted as evidence for the efficacy of repeated and continuous acupuncture in lowering levels of distress in uninjured animals. Another unexpected finding was the relatively low levels of beta-endorphins in the Brn7 group at the end of 7 days. Fentanyl injections, which had to be administered to the Brn7 group for analgesia throughout the study period, may have effected the blood beta-endorphin response in Brn7 animals. Acute administration of exogenous opioids is known to inhibit the production of endogenous opiates, and chronic administration of exogenous opioids is known to inhibit the production of both endogenous opiates and mu-opioid receptors.[47-49]

Angiogenesis seemed to be accelerated with the application of acupuncture. Increased neovascularization in the BrnA7 group's burn wounds supported this suggestion, a finding also reported in previous experimental studies on rodents.[33,35] One of these studies suggested that acupuncture increased basic fibroblast growth factors and platelet-derived growth factors via an "unknown mechanism".[33] Repeated and continuous acupuncture application in the present study may have improved these wound healing accelerators and enhanced neovascularization by easing pain and distress and lowering the systemic and local inflammatory responses in rats. This chain of events may be a component of the "unknown mechanism" that accelerates the wound healing process via acupuncture; however, further investigations are needed to support this suggestion.

CONCLUSIONS

Our results revealed that acupuncture's contribution in the very early phase of burn trauma remained limited to modulation of systemic and local inflammatory responses and pathological pain pathways. Application of repeated and continuous acupuncture triggered multiple analyzable and effective changes in physiological and pathological pain mechanisms, systemic and local inflammatory responses, and wound healing. Acupuncture may become a preferred nonpharmacological method for pain and distress control and for modulation of systemic and local inflammatory reactions and the wound healing process in burn trauma. However, further experimental studies using the present model are needed as well as confirmation by clinical trials.

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FIGURE LEGENDS:

Figure 1: Semiquantitative grading of IL-6 antibody staining in the burn wounds.

Grade 0: No IL-6 staining , Grade I: Stained cells < 20% of total cells, Grade II: Stained cells > 20% of total cells

Figure 2: (**A)** Semi-quantitative evaluation for neutrophils and mononuclear cell accumulation in 1-hour groups (N= number of animals). **(B)** Distribution of mean numbers of microvasculary structures in 1-hour groups (mean ± SE). Mean numbers of microvasculary structures were increased in burn wounds of both burn groups (Brn1 and BrnA1; $P > .05$), but they were significantly higher than mean vascular numbers in the Sh1 and ShA1 groups (*$P < .05$)

Figure 3: Semiquantitative Evaluation for Interleukin-6 Expression Levels in BrnA1. Trace accumulation of Interleukin 6 was observed in BrnA1 Group (x400).

Figure 4: Comparison of Blood Levels of Interleukin 6 and Beta-Endorphins in the 1-Hour Groups (mean ± SE) (*$P < .05$). **A-**Burn injury induced a significant systemic IL-6 response in the Brn1 group; acupuncture reduced the systemic IL-6 response in the BrnA1 group. **B-**Beta-endorphine response was triggered by both acupuncture application and burn injury in the initial 1 hour.

Figure 5: (A) Semi-quantitative evaluation for neutrophils and mononuclear cell accumulation in 7-day groups (N= number of animals). **B-** Distribution of mean numbers of microvasculary structures in 7-day groups (mean ± SE). The BrnA7 group had the highest mean number of microvessels (*P < .05)

Figure 6: Semiquantitative Evaluation for Interleukin-6 Expression Levels in Brn7. Dense accumulation of Interleukin 6 was observed in Brn7 Group at the end of seven days (x200).

Figure 7: Comparison of Blood Levels of IL-6 and Beta-Endorphins in 7-Day Groups (mean ± SE) (*P < .05). **A**-Systemic interleukin 6 (IL-6) response was still significant on postburn day 7 (Brn7 group). Repeated and continuous application of acupuncture significantly reduced blood IL-6 response in BrnA7 group down to levels of uninjured ShA7 and Sh7 groups in the same period. **B**-Continuous acupuncture application reduced systemic beta-endorphin response similarly in both unburned and burned animals

TABLES:

Table 1. Modified "Pain-Distress Scorring" Method for the Present Experimental Model

Assessment Score	**0**	**1**	**2**	**3**
Attitude and posture	Alert and not hunched	Not alert or hunched	Not alert and hunched	Not responsive to stimuli
Gait and movement	Active	Somewhat inactive	Completely inactive	Lying on side
Burn wound site	Clean and not swollen	Exudates or swelling	Exudates and swelling	Deepened burn wound
Appetite	Eating and drinking	Reduced eating or drinking	Not eating or drinking	Not eating or drinking for >3 days
Elimination	Normal	Softer than normal	Diarrhea	Diarrhea for >3 days

Strategies during treatment: Score+3 in any category: immediate euthanization; Score+2 in any category: evaluation and consultation with veterinarian; Total score >3: consultation with veterinarian **;** Score >1 in appetite/ easy access to food and water

Table 2. Distribution of Daily Pain-Distress Scores in 7-Day Groups (median ± SE) (*P < .05).All animals experienced pain-distress to some degree. However, the agonizing period was 48 to 72 hours postburn, and the Brn7 group was the most affected group, pain experience in the acupuncture-applied burn group (BrnA7) was as mild as that shown in the sham groups (Sh7, ShA7) at the end of 7 days

	Pain-Distress Scores (median ± SE)							
	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**	**7 days** ***medians of overall evaluation***
Sh7	1 ± 0,37	2 ± 0.5	2 ± 0.4	1 ± 0.4	1 ± 0.6	0 ± 0.4	1 ± 0.5	7 ± 1.3
ShA7	2 ± 0.4	0 ± 0.2	2 ± 0.2	1 ± 0.4	0 ± 0.3	1 ± 0.5	0 ± 0.3	7 ± 0.5
Brn7	**4 ± 0.9***	**3 ± 0.2***	2 ± 0.4	1 ± 0.9	**2 ± 0.4***	2 ± 0.4	1 ± 0.2	**14 ± 1.2***
BrnA7	0 ± 0.4	0 ± 0.4	1 ± 0.4	0 ± 0.6	0 ± 0.3	1 ± 0.2	0 ± 0.3	4 ± 0.7

Figure 1

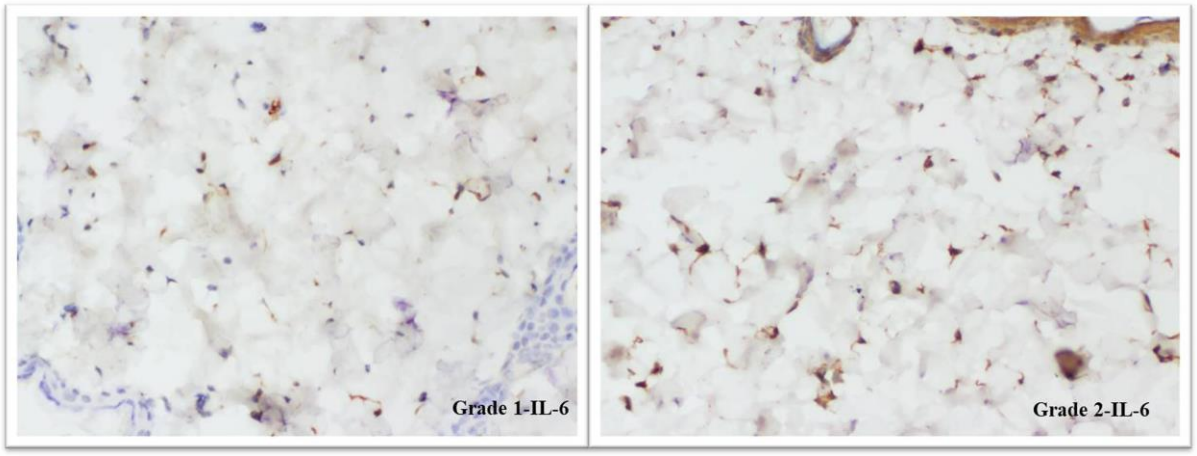

Figure 2A

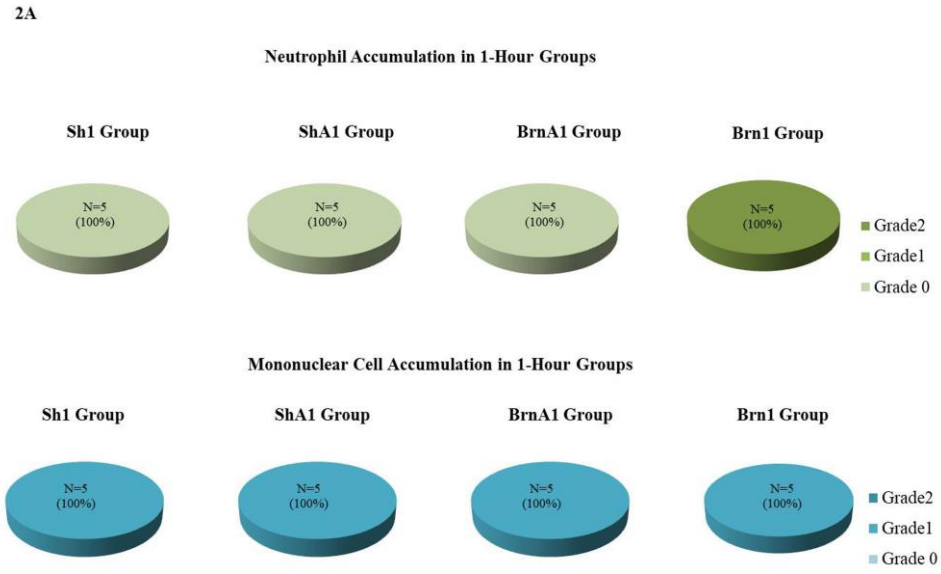

Figure 2B

2B

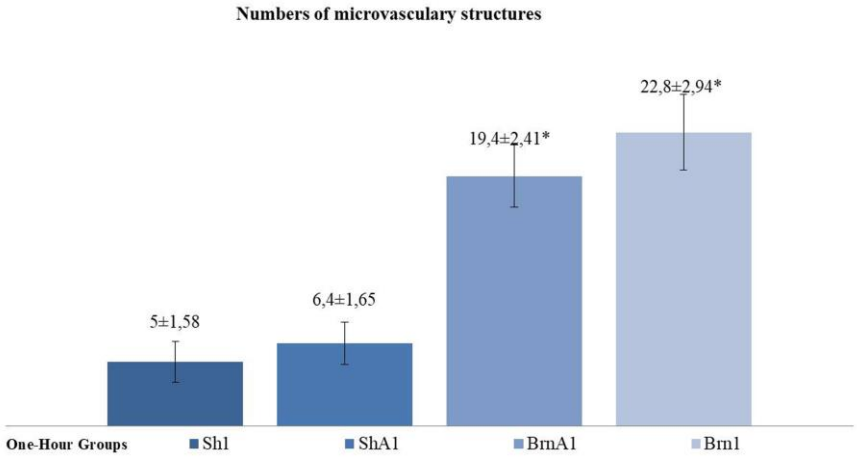

Figure 3

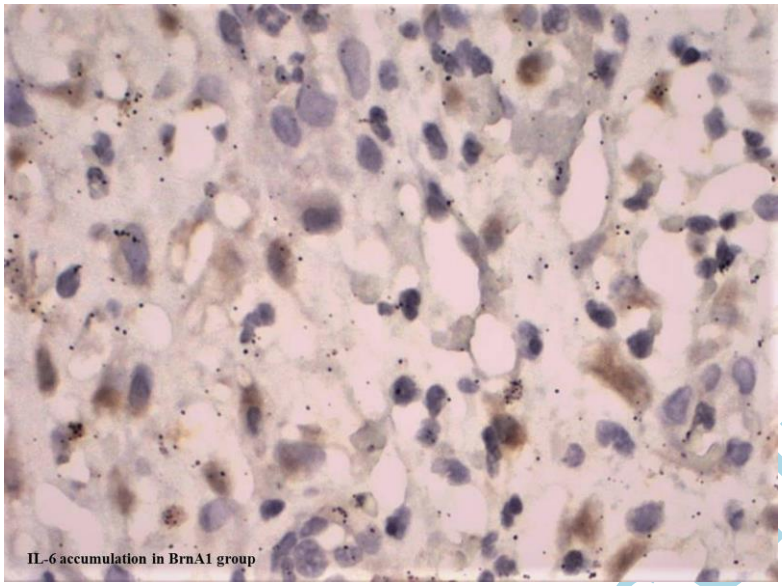

Figure 4A

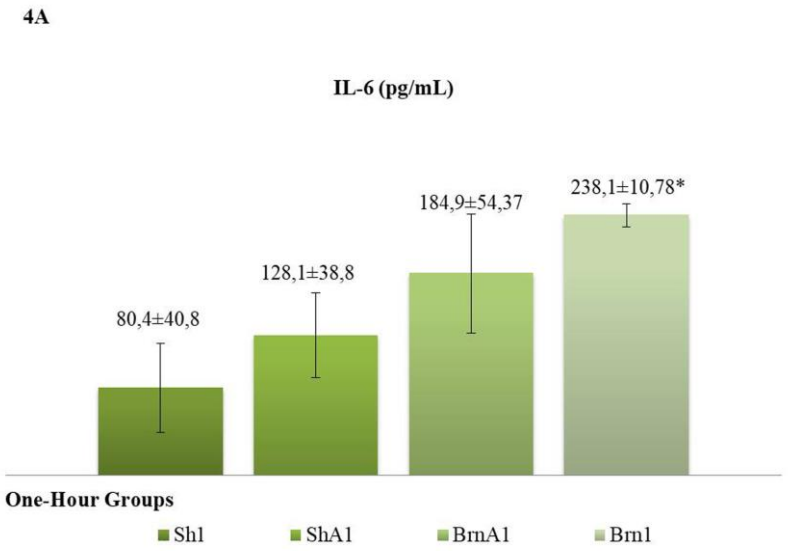

Figure 4B

4B

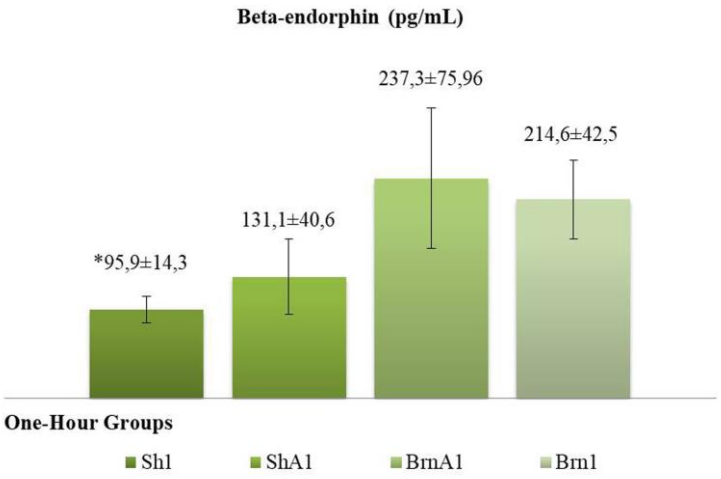

Figure 5A

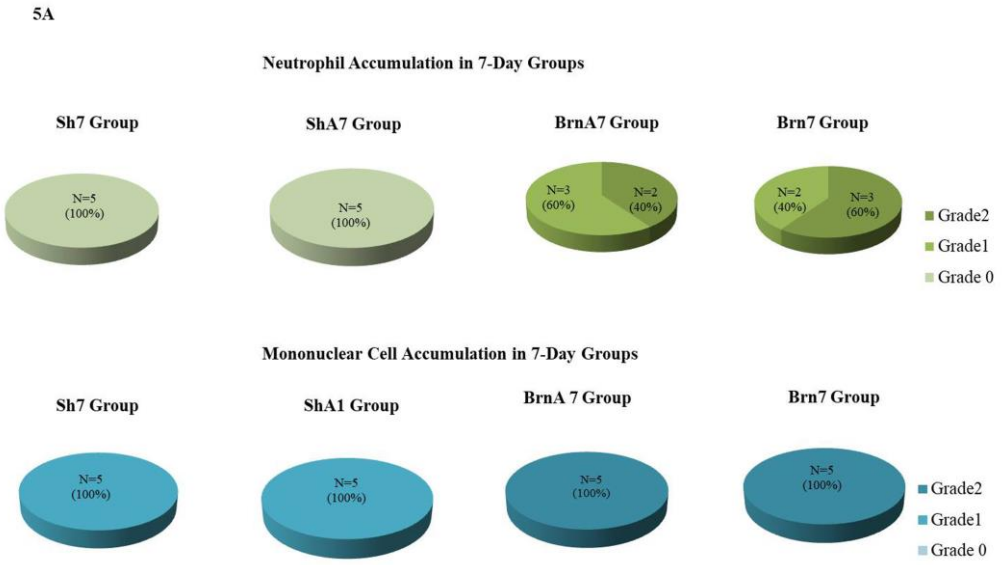

Figure 5B

5B

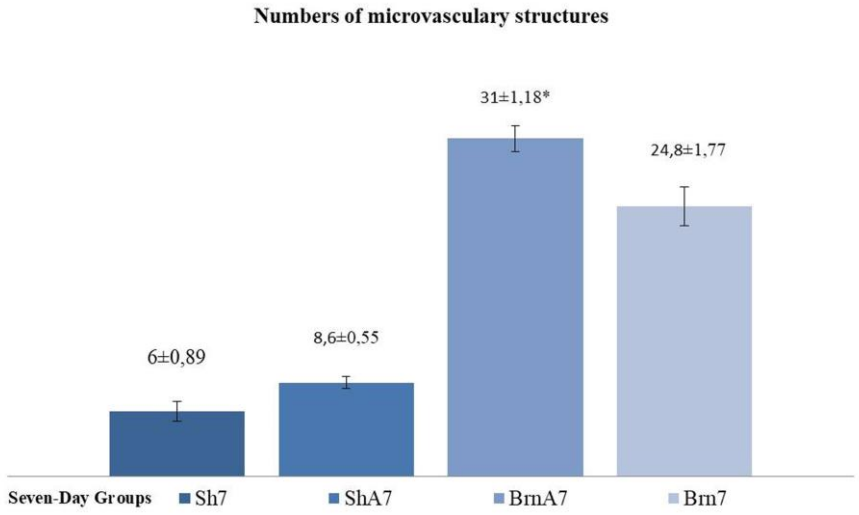

Figure 6

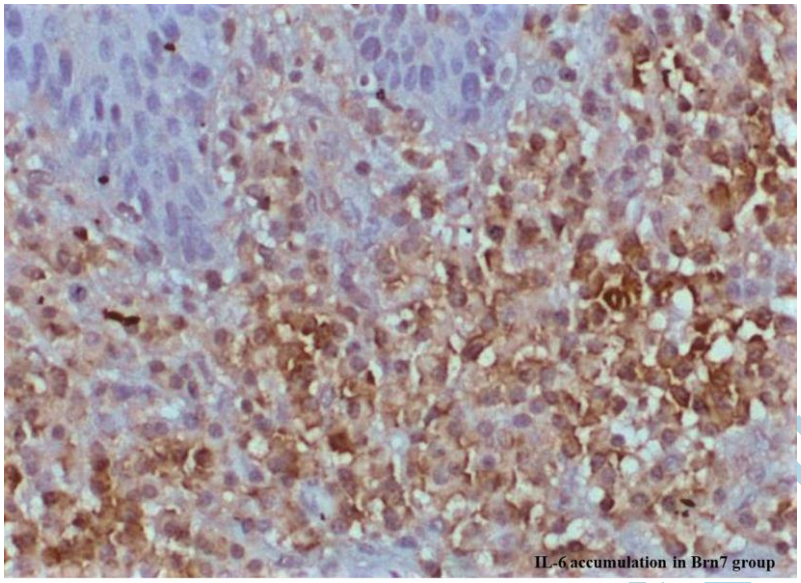

Figure 7A

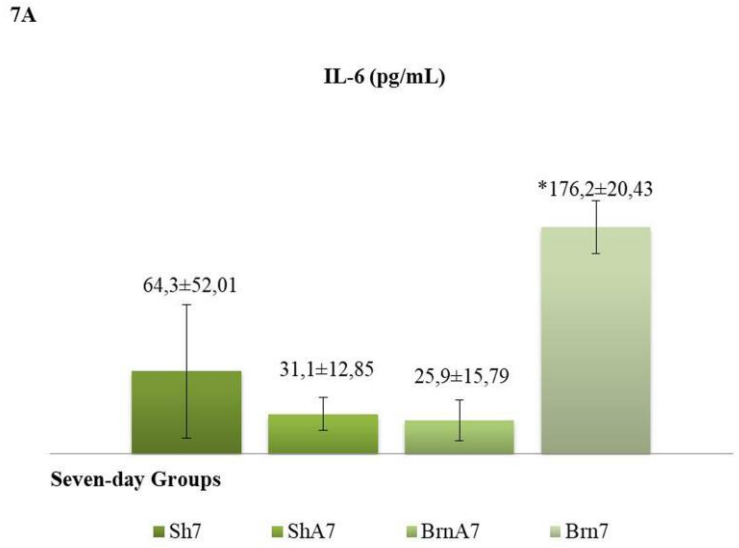

Figure 7B

7B