



## Original Article

## Effects of neuro-immuno-modulation on healing of wound combined with local radiation injury in rats

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## ABSTRACT

**Purpose:** To investigate effects of neuro-immuno-modulation on wound healing by observing changes of cytokines and hypothalamic-pituitary-adrenal (HPA) axis hormones in acute stress reaction in rats with wound and combined local radiation injury.

**Methods:** Sixty female Wistar rats (weighting  $200 \pm 20$  g) were randomly divided into normal control group, wound group and combined wound-local radiation (CWR) group (25 Gy local radiation post wound), 20 rats in each group. Contents of IL-1 $\beta$ , IL-6 and IFN- $\gamma$  and IL-4 in serum were measured and changes of adrenocorticotrophic hormone (ACTH) and glucocorticoid (GC) in serum were analyzed by using enzyme-linked immunosorbent assay and radioimmunologic assay, respectively at different time points post wound and radiation.

**Results:** (1) The level of IFN- $\gamma$ , one of the Th1 cell cytokines increased significantly at 14 d post CWR, which was markedly higher than that in control group and wound group. However, the level of IL-4, IL-1 $\beta$  and IL-6, one of the Th2 cell cytokines, did not show obvious change. (2) Ratio of Th1/Th2 (IFN- $\gamma$ /IL-4) in wound group and CWR group increased significantly at 7 d after wound and radiation, which suggested that Th1/Th2 balance drifted to Th1 immune response. The ratio of Th1/Th2 in wound group returned to the normal level up to 14 d after the wound and radiation, while the Th1/Th2 ratio in CWR group increased persistently and was much higher than that in control and wound groups. (3) Level of serum ACTH and GC in CWR group increased at 3 d post wound and radiation, and among them, level of GC showed statistically significant increase, which was much higher than that in control and wound groups.

**Conclusion:** Level of serum neurohormone GC in rats increased significantly immediately after wound and radiation; while the level of IFN- $\gamma$  showed significant increase only up to 14 d after wound and radiation, and the Th1/Th2 imbalance sustained till 28 d post wound and radiation. In order to reduce acute damage caused by CWR, organic immune system and nerve system showed up a marked regulate effects simultaneously and mutually. Nonetheless, the excessive stress induced by CWR causes disturbance of immunoregulation, which is one of the key reasons for delayed wound healing in CWR.

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## Introduction

Combined wound and radiation injury (CWR) occurs when wound is combined simultaneously or successively with radiation exposure at a dose that is sufficient to cause injury. This kind of combined injury is common in radiotherapy during surgical

treatment of malignant tumor, nuclear accident and nuclear-attacks.<sup>1</sup> The feature of CWR is decreased inflammation, decreased local white blood cell infiltration, serious bleeding and tissue necrosis, easily complicated infection, bad granulation tissue formation, and delayed healing and so on. Acute stress can induce sympathetic nervous system over excited, result in immune imbalance through the nerve-endocrine-immune network, and at last cause homeostasis imbalance.<sup>2</sup> It is confirmed that the levels of adrenocorticotrophic hormone (ACTH) and glucocorticoid (GC) significantly increase in mice with combined radiation-burn injury, and severe trauma can lead to accentuation of hypothalamo-

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pituitary-adrenal (HPA) axis.<sup>3</sup> Our previous study found that the proportions of regulatory T cells (Tregs) and Th17 cells increased, and Treg/Th17 imbalance occurred in rats with CWR, which indicate that CWR can lead to immunologic disorders.<sup>4</sup> However, the effect of HPA axis accentuation on immune function and wound healing post CWR is not clear. This paper observed the changes of neurohormonal, ACTH and GC, and the changes of Th1/Th2 balance, to explore the interaction of nerve-endocrine-immune post CWR and the effects of neuro-endocrine-immune (NEI) on wound healing.

## Materials and methods

### Animals

Sixty female Wistar rats (weighting  $200 \pm 20$  g) were purchased from the Laboratory Animal Center of Academy of Military Medical Sciences (AMMS) in Beijing. All rats were maintained in a specific-pathogen-free facility. The rats were randomly divided into control group, wound group and CWR group, 20 rats in each group. Enzyme-linked immunosorbent assay (ELISA) kits for IL-4 and IFN- $\gamma$  from Biolegend Company (the USA), and radioimmunoassay kits for ACTH and GC from IBL Company (Germany).

### Combined wound and local radiation model

After anesthetization and focal sterilization, two round wounds in rats were made at thoracic segment in the back paralleled with spine, symmetrical on both sides. The diameter of each wound was 1.5 cm, deep to the full-thickness skin, and the interval between two wounds was 1.5 cm. The animals received local irradiation immediately post trauma. The rats in CWR group were given 25 Gy  $\gamma$  rays using a single <sup>60</sup>Co source (Beijing Institute of Radiation Medicine, China) at a dose rate of 278.98 cGy/min. Meanwhile, the rats in control and wound groups received sham exposure.

### ELISA assay

On the 3rd, 7th, 14th and 28th d after injury and radiation (simple injury), 5 rats from each group were sacrificed and serums were taken for ELISA. ELISA assay for IL-1 $\beta$ , IL-4, IL-6 and IFN- $\gamma$  were performed according to the manufacturer's instructions. Serum was diluted 1:2 in 2% bovine serum albumin of phosphate buffer saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich, PBST) and was added 100  $\mu$ l to the wells. After 90 min incubation at 37 °C with shaking, plates were washed (4  $\times$  PBST) and biotin-conjugated detection antibody was added. Following 1 h incubation at 37 °C, microwells were washed four times and streptavidin-HRP was added. Following 30 min incubation at 37 °C, microwells were washed four times and tetramethylbenzidine (TMB, Thermo Fisher Scientific, the USA) added for 10–15 min at 37 °C. Reactions were stopped by addition of 0.9 M H<sub>2</sub>SO<sub>4</sub> (50  $\mu$ l) and product absorbance was determined at 450 nm. Non-specific background (measured at 630 nm) was subtracted.

### Radioimmunologic assay

On the 3rd, 7th, 14th, and 28th d after injury, 5 rats from each group were sacrificed and serums were taken for radioimmunologic assay. According to the manufacturer's instructions, the samples were incubated and centrifugated, and then the radioactivity of precipitation was measured by a gamma counter. The concentration of ACTH and GC in serum was calculated according to the standard curve.

### Statistical analysis

All data are presented as means  $\pm$  SD. Student's unpaired *t*-test was used to compare the data among groups. *P* < 0.05 was considered statistically significant.

## Results

### Changes of IFN- $\gamma$ and IL-4 in serum post injury

There was no significant difference in IFN- $\gamma$  among three groups 3–7 d post injury. However, on the 14th d post injury, the level of serum IFN- $\gamma$  in CWR group increased significantly, and was markedly higher than that in the other two groups. The level of serum IL-4 in wound and CWR groups decreased in some extent post injury; but the change did not show statistical significance. Meanwhile, there were no statistically significant changes in the level of serum IL-4, IL-1 $\beta$  and IL-6 between the wound and CWR groups (Fig. 1).

### Changes of ratio of IFN- $\gamma$ /IL-4 (Th1/Th2) post injury

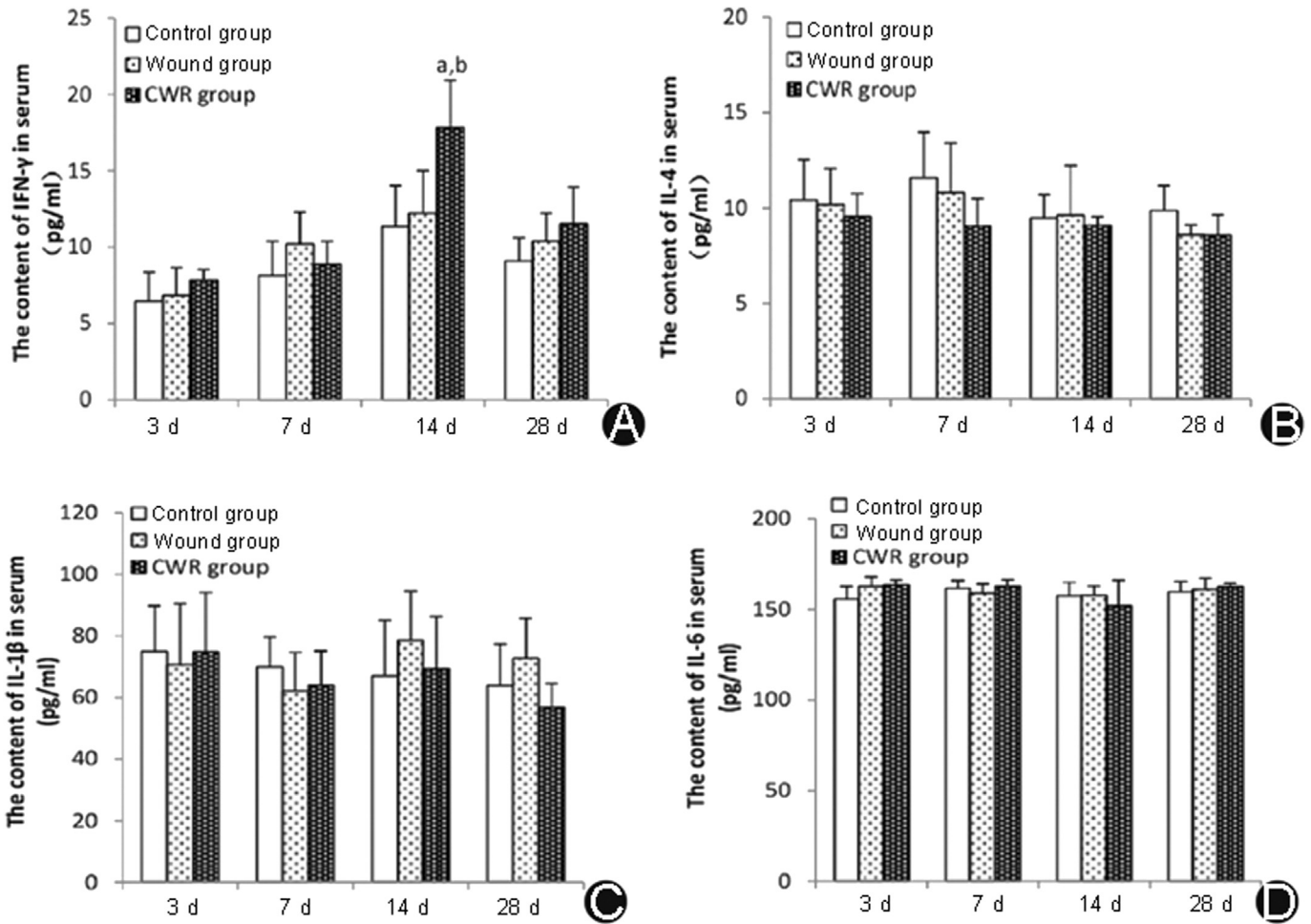
IFN- $\gamma$  and IL-4 represented the characterized cytokines of Th1 cells and Th2 cells respectively, so the ratio of IFN- $\gamma$ /IL-4 could objectively stand for the balance of Th1/Th2.<sup>5</sup> As shown in Fig. 2, the ratio of IFN- $\gamma$ /IL-4 in wound and CWR groups was significantly higher than that in control group at 7 d post injury. At 14 d post injury, the level IFN- $\gamma$  in the wound group recovered to the controlled level. However, because of durative high level of IFN- $\gamma$ , the ratio of IFN- $\gamma$ /IL-4 in CWR group was significantly higher than that in the control and the wound groups, that is, the Th1/Th2 balance showed a shift to the Th1 immune response. Until 28 d post injury, the ratio of IFN- $\gamma$ /IL-4 in CWR group was still significantly higher than that in control group.

### Changes of ACTH and GC in serum post injury

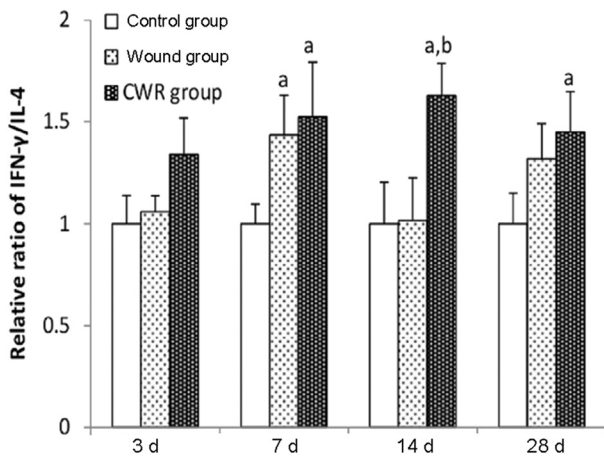
Analysis of serous ACTH and GC contents showed that the level of ACTH and GC in CWR group increased at 3 d post injury, and the level of GC significantly increased. At 7 d post injury, the level of serous ACTH and GC in CWR group was lower than that in wound group; however there was no significant difference between two groups (Fig. 3).

## Discussion

Stress is a non-specific response of the body to the external or internal stimuli. When the body receives stimuli and shows stress reaction, the stress signal can cause the secretion of corticotropin releasing hormone (CRH) in hypothalamus, and then lead to the secretion of ACTH in pituitary gland, afterwards, ACTH can induce secretion of GC in adrenal cortex, and thereby results in excitation of HPA axis. The neurotransmitters, neuropeptides and hormones secreted from HPA axis play an important role in the regulation of immune system by binding to the receptor. And the immune system achieves the feedback regulation of neuroendocrine system through various biological active molecules generated in immune response,<sup>6,7</sup> and thus forms the NEI network, which also plays an important role in the maintenance of homeostasis. Stress has a dual role; appropriate stress can improve the body's adaptability, while excessive stress can lead to dysfunction of the body. In the study of single radiation on neuroimmuno-modulation, Marquette et al.<sup>8</sup> found that IL-1 $\beta$  levels increased in the hypothalamus, thalamus and hippocampus, and TNF- $\alpha$  and IL-6 levels increased significantly in the hypothalamus post irradiation, indicating that radiation has an important influence on the neuroimmunomodulation. In CWR,



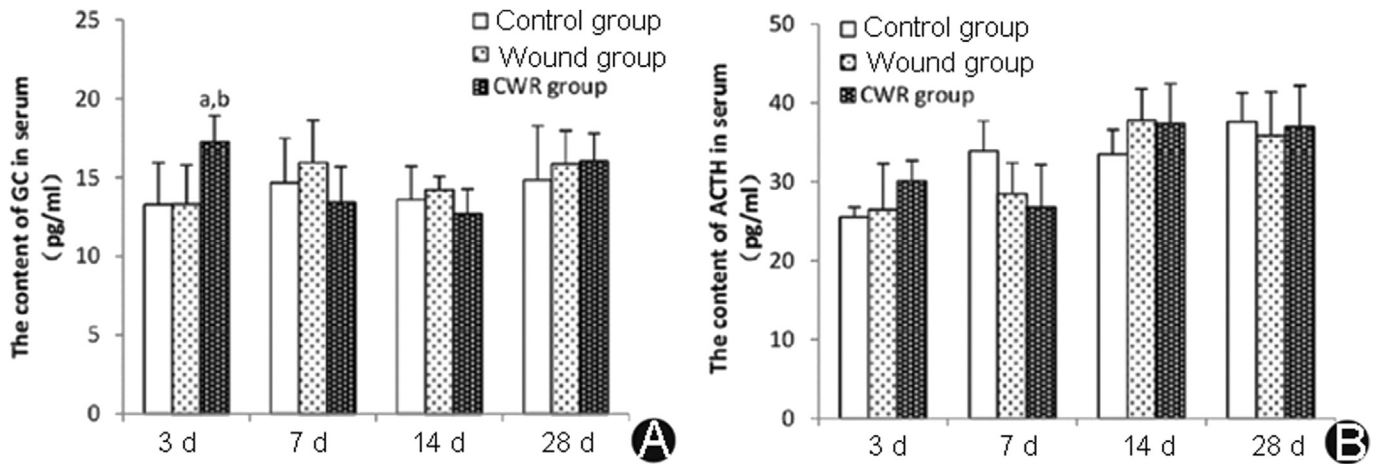
**Fig. 1.** ELISA assay of serum IFN- $\gamma$  and IL-4 3–28 d post CWR. The content of IFN- $\gamma$  in CWR group increased significantly at on the 14th d post CWR, and the level of IFN- $\gamma$  in wound group did not change markedly (A). Meanwhile, there was no statistically significant changes in the level of serum IL-4, IL-1 $\beta$  and IL-6 between the wound and CWR groups (B–D). a: compared with control group,  $p < 0.05$ ; b: compared with wound group,  $p < 0.05$ ,  $n = 5$ .



**Fig. 2.** Relative ratio of IFN- $\gamma$ /IL-4 post CWR. The ratio of IFN- $\gamma$ /IL-4 in wound and CWR groups increased significantly post CWR, and the ratio in CWR group did not recover until 28 d post CWR. a. compared with control group,  $p < 0.05$ ; b. compared with wound group,  $p < 0.05$ ,  $n = 5$ .

the body will produce a corresponding stress reaction after receiving double injury of trauma and irradiation. Therefore, we observed the changes of Th1, Th2, GC and ACTH in serum in order to explore the mutual regulatory relationship between the neuroendocrine and immune system post CWR.

Th cells can be divided into two cell subsets (Th1 and Th2) according to the different secreted cytokines. Th1 cells secrete a lot of cytokines such as IFN- $\gamma$  and IL-12 and Th2 cells mainly secrete IL-4, IL-5 and IL-6, which play important regulatory roles respectively in cellular immunity and humoral immunity.<sup>5,9</sup> IL-1, IFN- $\gamma$  and IL-4, secreted by Th1 and Th2 cells, are closely related with HPA axis.<sup>10</sup> This study shows that the level of IFN- $\gamma$  significantly increases at 14 d post CWR, which indicates that the inflammatory reaction is still serious at this stage. The result is similar with the study of Tajima et al.<sup>11</sup> On the other hand, it has been reported that IFN- $\gamma$  is also a potent inhibitor of collagen gene transcription and can block TGF- $\beta$ -induced collagen synthesis.<sup>12,13</sup> Therefore, it is suggested that the increase of IFN- $\gamma$  may inhibit collagen synthesis, and then, leading to delayed wound healing in our experiment. This is consistent with the results of our previous pathological experiments.<sup>14</sup> Furthermore, IFN- $\gamma$  and IL-4 are the representative cytokines of Th1 and Th2 respectively,<sup>9</sup> and thus



**Fig. 3.** Radioimmunologic assays of GC and ACTH in serum 3–28 d post CWR. The level of GC in CWR group increased significantly at 3 d post CWR (A). And the level of ACTH did not change markedly post injury (B). a: compared with control group,  $p < 0.05$ ; b: compared with wound group,  $p < 0.05$ ,  $n = 5$ .

the ratio of IFN- $\gamma$ /IL-4 could reflect immune balance of Th1/Th2 to a certain extent. After analyzing the ratio of IFN- $\gamma$ /IL-4, we find that the Th1/Th2 balance significantly shifts to Th1 7–28 d post CWR, which reveals that the immunologic balance in rats with CWR is in disorder, and does not completely recover to the normal level until 28 d post CWR (Fig. 2). However, the contents of serum IL-1 $\beta$  and IL-6 after CWR do not show significant changes in our experiment, which is different from the results reported by Marquette et al.<sup>8</sup> There are two explanations for this problem: firstly, cytokines are detected in peripheral blood in our experiment, while the same cytokines are detected in hypothalamus, thalamus and hippocampus by Marquette et al.<sup>8</sup> Generally speaking, the contents of cytokines in peripheral blood may be fewer than those in tissues. Secondly, our previous experiment<sup>15</sup> shows that, after acute stress induced by millimeter-wave, peripheral IL-1 $\beta$  levels immediately peak at 6 h and IL-6 level peaks at 1 d. However, the earliest time point in this experiment is 3rd day post CWR. Therefore, further experiments will be needed to obtain objective results.

At the same time, the results of radioimmunologic assay show that single wound does not induce significant change of the ACTH and GC levels in serum; however, there is a transient increase in serum GC at 3 d post CWR, and no significant changes are found in ACTH, which reveals that the stress response is obviously enhanced post CWR. As part of the stress response, trauma results in activation of the HPA axis with the release of cortisol.<sup>16</sup> Through further analysis of correlation between changes of ACTH and GC and Th1/Th2 type cytokines, it is found that the level of GC significantly increases after responding to CWR; nevertheless, the level of IFN- $\gamma$  shows increment until 14 d after responding to the injury. Therefore, it is supposed that this may be the results from the feedback inhibition of neuroendocrine system in order to relieve the immune hyperactivity caused by excessive stress. However, the level of IFN- $\gamma$  in CWR group significantly increases 14 d after injury, and the balance of Th1/Th2 is still significantly shifted to Th1 even till 28 d post injury, which may be related to the immunodissonance induced by excessive stress of CWR. On the other hand, excessive stress can result in excessive secretion of GC, which can affect the expression of GR and thereby reduce the anti-inflammatory activity, and even lead to glucocorticoid resistant.<sup>17</sup>

Lu et al.<sup>3,18</sup> studied the effect of cervical sympathetic ganglia block (SB) on mice of combined radiation and burn injury. The results show that SB can promote homeostatic restoration of HPA axis

in mice with combined injury, suppress the excessive inflammatory reaction, and promote the healing of combined radiation and burn wound. All of these confirm that the nerve-immune regulation is abnormal in CWR. Considering our previous experimental data that wound healing delay is closely related to the increment of regulatory T cells (Tregs) and Th17 cells, especially the Treg/Th17 balance shifting to Th17,<sup>4,18</sup> it can be speculated that, CWR-induced stress can cause excitement of HPA axis, which attempts to regulate the immune dissonance; however, excessive stress of CWR still leads to the disorder of immunological regulation, for instance, persistent inflammatory in organism, then affects the synthesis of extracellular matrix and recruitment of neutrophils in wound area, and eventually leads to wound healing delay.

In conclusion, CWR can induce early increase of GC, succedent increase of IFN- $\gamma$ , and imbalance of Th1/Th2. Therefore, it is suggested that, CWR-induced dysfunction of nerve-immune regulation, especially the immune dissonance, might be one of main causes of delayed CWR healing.

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