International Immunopharmacology 69 (2019) 337–346

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Anti-hyperalgesia effect of nanchelating based nano particle, RAc1, can be mediated via liver hepcidin expression modulation during persistent inflammation

Vida Nazemian[a], Somayeh Kalanaky[b], Homa Manaheji[a], Etrat Houshmandi[c], Mola Mohammadi[a], Jalal Zaringhalam[a,c,*], Somayeh Mirjafai[a]

[a] Department of Physiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Department of Research and Development, SodourAhrarShargh Company, Tehran, Iran
[c] Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Keywords:
Inflammation
Rheumatoid arthritis
Nanochelating technology
RAc1
Hepcidin

ABSTRACT

Objective: Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder accompanied with hyperalgesia, edema and pain. At least 30% of the patients failed to respond to the available treatments and medications, which yet have a lot of serious adverse effects on patients. So, using novel technologies to produce more efficient medications is needed. According to the role of iron manipulation in inflammatory process, we have synthetized RAc1 nano particle, which contains zinc and has iron chelating property. In the present study, we evaluated RAc1 nano particle effects on hyperalgesia and liver hepcidin and serum IL-1β and TNF-α expression levels during acute and chronic phases of adjuvant-induced inflammation in male rats and compared its effects with Deferoxamine.

Methods and materials: Complete Freund's adjuvant (CFA)-induced arthritis was caused by single subcutaneous injection of CFA into the rat's hind paw on day zero. RAc1 with 100, 200 and 400 ng/kg doses and deferoxamin with doses of 200 mg/kg after diluting in vehicles were administered daily (i.p.) during the 21 days of the study after CFA injection. Hyperalgesia, Edema, liver hepcidin and serum IL-1β and TNF-α expression levels were assessed on days 0, 7, 14 and 21 of the study.

Results: The results of this study indicated the role of RAc1 nano particle administration in reducing paw edema, thermal hyperalgesia, and liver hepcidin and serum IL-1β and TNF-α expression even in comparison with Deferoxamine during different phases of inflammation caused by CFA.

Conclusion: It seems that RAc1 nano particle exerts its immune modulatory effects by decreasing liver hepcidin expression and serum IL-1β and TNF-α levels.

1. Introduction

As a systemic autoimmune inflammatory disease, rheumatoid arthritis (RA) can cause tissue damage by chronic inflammation. RA is characterized by cartilage degradation, immune cell infiltration to the synovium, hyperalgesia, fibroblast-like synoviocyte expansion, edema, pain, and mobility impairment [1,2]. In the case of persistent inflammatory pain states, mounting evidence has identified that multiple immune cells and cytokines have pivotal roles in the pathogenesis of RA. Dysregulation of the immune system is a foremost contributing factor in the development of inflammation as reflected by altered levels of cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β), the presence of inflammatory cells, variations in immunoglobulins produced, and presence of autoantibodies in some types. Cytokines have been the topic of intense investigations not only with respect to inflammatory pain states, but also in several other disorders such as neurodegenerative and neurological diseases. However, the exact mechanisms are still unknown [3]. Besides, studies have declared that inflammatory states can increase serum Ferritin levels and may finally lead to hypoferraemia via an increase in Hepcidin with successive iron sequestration in macrophages and duodenal enterocytes, as well. Over and above that, inflammation can reduce erythropoietin and bone marrow activity by Hepcidin and inflammatory cytokines, including IL-1β and TNF-α [4].

Iron is an essential element required for organism's survival and serves as a cofactor for so many heme and non-heme iron proteins and

* Corresponding author at: Physiology Department, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
E-mail addresses: skalanaky@nanochelatingtechnology.com (S. Kalanaky), jzaringhalam@gmail.com (J. Zaringhalam), jzaringhalam@sbmu.ac.ir (S. Mirjafai).

https://doi.org/10.1016/j.intimp.2019.02.003
Received 26 November 2018; Received in revised form 14 January 2019; Accepted 3 February 2019
Available online 15 February 2019

also key redox enzymes [5]. Physiological iron balance is strongly controlled at the cellular and systemic level by iron regulatory proteins and hormones and it is demonstrated that iron overload is associated with tissue damage. Underlying mechanisms ordinarily intersect to achieve optimal iron utilization, in order to control immune responses and also prevent iron toxicity [6]. Data generated thus far revealed that systemic iron homeostasis is primarily regulated through the hepcidin/ferroportin axis. Hepcidin is a disulfide-rich peptide hormone which is produced by hepatocytes and restricts iron fluxes to the bloodstream. Hepcidin synthesis is notably induced by infection and inflammatory states. In this regard, hepcidin not only is an iron-regulatory hormone, but also links iron metabolism to host defense and inflammation [7]. As such, it deserves consideration as a therapeutic target. Nevertheless, research regarding the significance of different iron states in inflammatory diseases is conflicting, and guidelines for iron substitution are scattered [4].

A large body of evidence exists to substantiate that chronic inflammatory reactions can increase serum iron, change the iron metabolism and also change the redistribution of iron to the reticuloendothelial system, and in turn, iron within the synovial tissue can exacerbate the inflammatory reactions in RA. One of the most widely used iron chelators in medicine is deferoxamine (DFX), which has extremely high binding affinity for Ferritin. DFX has a high potential for inhibiting the damaging effects of inflammation in inflamed joints and central nervous system, as well [8,9]. More interesting, DFX can reduce inflammatory cytokines and reactive oxygen species production and also lipid peroxidation. In contrast, it can increase Collagen-I and tissue inhibitor metalloproteinase-1 mRNA levels [10].

Nanochelating technology, which has novelty for the time being, has the capacity to synthetize efficient structures in various science areas [11]. In our previous research studies, we have evaluated the effects of MSc1, a Nanochelating-based structure on animal models of multiple sclerosis. MSc1 could improve disabling features of experimental autoimmune encephalomyelitis, which was confirmed by decreased clinical scores versus increased body mass and 100% survival probability [12]. Also in other studies, Nanochelating-based structures showed therapeutic effects in different disease models in vitro and in vivo [13–15].

By applying mentioned technology, we have synthetize RAc1 nano particle, which has iron chelating property. In a dozen of studies and specially in some meta-analysis studies, zinc deficiency in autoimmune disorders is reported. Thus, based on these reports and also important role of zinc in regulating the immune system functions, we trapped zinc in RAc1 structure. In other words, it is a zinc chelate nano particle [16,17]. In this regard, due to the importance of offering effective treatments with fewer side effects, in this study we set out to evaluate hyperalgesia, edema, serum TNF-α and IL-1β and liver hepcidin expression levels variations due to nano particle RAc1 administration during acute and chronic phases of adjuvant-induced inflammation of in male rats.

2. Materials and methods

2.1. Adjuvant-induced inflammation induction

Arthritis inflammation was caused by single subcutaneous injection of 100 μL CFA (Complete Freund's Adjuvant), which was a heat-killed *Mycobacterium tuberculosis* suspended in sterile mineral oil, into the rats' hind paw (10 mg/mL; CFA; Sigma, St. Louis, MO, USA) [18].

2.2. Laboratory animals

In this research study, adult male Wistar rats weighing 180–220 g were selected. These rats were housed in polypropylene cages under standard hygienic environmental conditions (22 ± 2 °C, 12-hour light/dark cycle, and 60–70% humidity). All animals, except experimental time, had access to standard food and water. All procedures in this study were approved by the guidelines of ethical standards for investigations of experimental acute and chronic inflammation in animals and the ethical principles of the Declaration of Helsinki and National Institute of Health (NIH) (Zimmermann, 1983). In the sake of determining the liver hepcidin expression levels as well as serum TNF-α and IL-1β levels due to nano particle RAc1 administration a series of experiments were executed. Rats were randomly divided into 8 experimental groups, as follows: (a) CFA group, (b) CFA control group (Mineral Oil (MO)), (c) CFA + 100 ng/kg RAc1 (d) CFA + 200 ng/kg RAc1 (e) CFA + 400 ng/kg RAc1 (f) CFA + 200 mg/kg Deferoxamine (CFA + DFX) (g) CFA + Distilled Water (DW) (h) CFA + Normal Saline (NaCl) (19). According to the study, each group was divided into 4 subgroups based on different time points of the study (days 0, 7, 14, and 21) and there were 6 rats in each subgroup.

2.3. Experimental procedure

In the CFA group, 100 μL CFA was injected once into the rat's right hindpaw on day zero (under light anesthesia with methoxy isoflurane). In the MO group, 100 μL sterile mineral oil was injected once into the rat's right hind paw on day zero. The CFA + DW group was received distilled water once daily (i.p.). The CFA + NaCl group was received Normal saline once daily (i.p.). Different dosages of nano particle (RAc1) (100, 200 and 400 ng/kg) were prepared by dissolving in distilled water and injected intraperitoneally on a daily basis from the first day after CFA injection up to day 21. The CFA + DFX group was received 200 mg/kg Deferoxamine dissolved in normal saline on a daily basis from the first day after CFA injection up to day 21 (i.p.) [20,21]. In this research study, the liver hepcidin and serum TNF-α and IL-1β expression levels, hyperalgesia and edema were assessed on days 0 (before CFA injection), 7 (inflammatory phase), 14 and 21 (arthritic phase) [22] [Fig. 1].

2.4. RAc1 nano particle synthesis and imaging

RAc1 is a zinc chelate nano particle which has iron chelating property and is endotoxin free. RAc1 was synthetized by Sodour Ahrar Shargh Company (Tehran, Iran). This nano structure was synthetized based on Nanochelating technology which is patented under the name of Mohammad Hassan Nazaran in USPTO [11]. Scanning electron microscope (SEM) image from this nano particle was prepared in Institute for color science and technology (Tehran, Iran) by SEM microscope (VWGA//TESCAN-LUM model).

2.5. Paw edema assessment

Briefly, the rat's hindpaw was submerged to the tibiotarsal joint into a transparent chamber of plethysmometer containing an electrolyte solution. Following that, measuring the paw volume was conducted by displacement of an electrolyte solution in a plethysmometer chamber (model 7141; Ugo Basile, Comerio-Varese, Italy). The volume of displacement is equal to the paw volume and measurement was done twice for each paw and the average calculated [23].

2.6. Thermal hyperalgesia assessment

Paw withdrawal latency (PWL) in response to radiant heat by plantar test was performed in all experimental groups (Ugo Basilar, Verse, Italy). Rats were placed in a Plexiglas chambers 30 min before the test in order to habituate with the experimental environment. Infrared light was projected to the rats' hind paw and PWL was recorded. A cut off time of 20 s was considered to avoid hind paw injury. Three times for each paw at an interval of 5–10 min was done and the mean latency of the withdrawal responses for each paw was calculated. The mean amount of the injected paw was subtracted from other paw

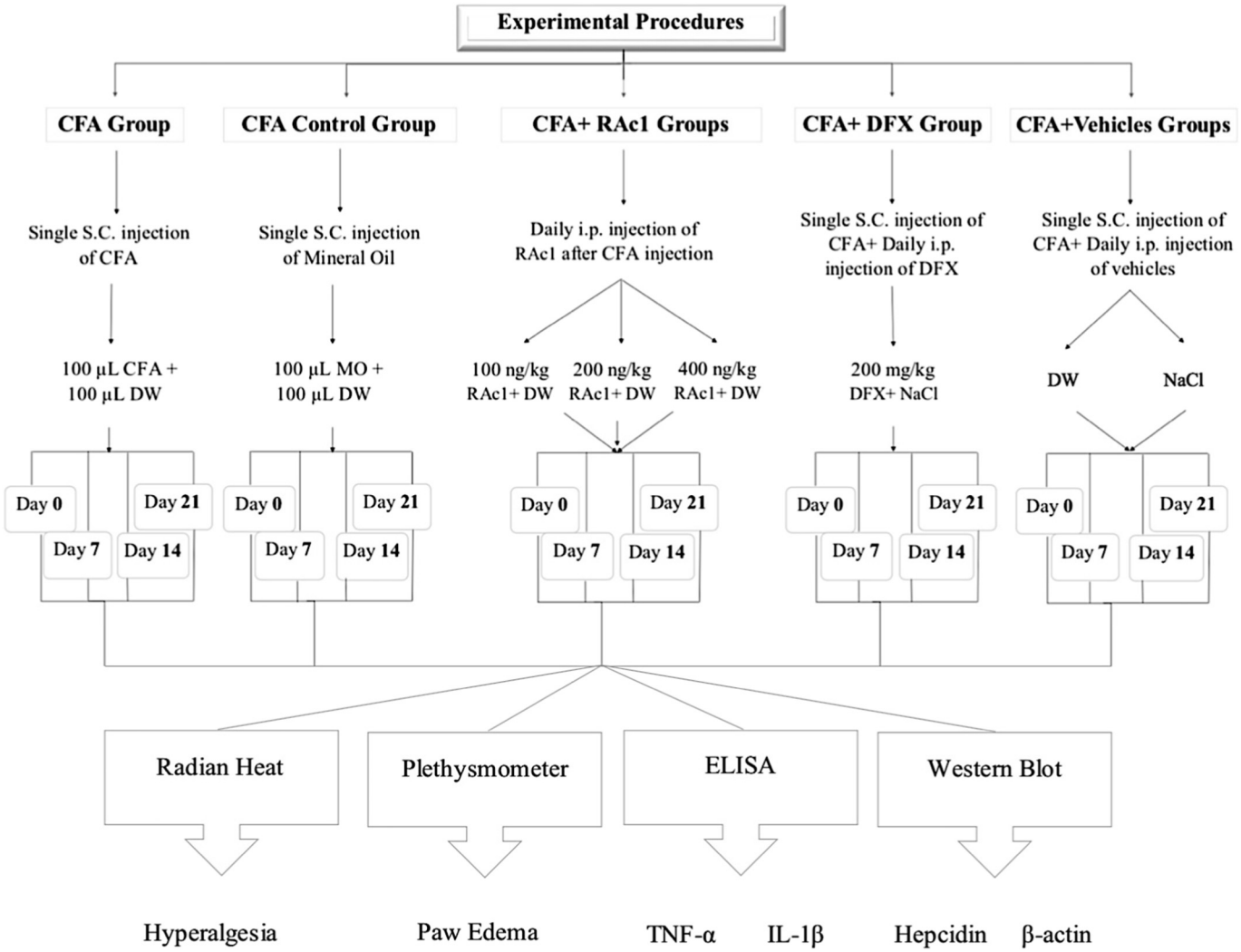

Fig. 1. Experimental procedure has been shown in Fig. 1 entirely. The liver hepcidin and serum IL-1β and TNF-α expression levels variations, hyperalgesia and edema were assessed on days 0 (before CFA injection), 7 (inflammatory phase), 14 and 21 (arthritic phase).

and the value obtained represented the hyperalgesia in the injured paw [24].

2.7. Blood sampling and serum TNF-α and IL-1β levels measurements

Rats were anesthetized moderately with methoxy isoflurane and retro-orbitally blood samples were prepared and collected in heparinized tubes and centrifuged (in 4 °C and 13,000 rpm). Serum TNF-α and IL-1β levels were evaluated by enzyme-linked immunosorbent assay (ELISA) kit (Bender MED System, UK) according to the manufacturer's instruction. The procedure summarized as follow: Blood serums were added to 96-well micro plates coated with rat IL-1β and TNF-α-specific polyclonal antibody. After the incubation at room temperature for 2 h, followed by washing, enzyme-linked polyclonal antibodes specific for TNF-α and IL-1β were then added. After incubation for 2 h at room temperature and washing again, the color reagents were added. The color intensity was measured by Microplate reader at 450 nm. Standard curves were drawn and the IL-1β and TNF-α levels (pg/mL) of the samples were calculated [24,25].

2.8. Liver tissue extraction

In order to identify the expression of liver hepcidin in each group, rats were anesthetized with methoxy isoflurane, heads removed and liver was separated fast on ice and then frozen in liquid nitrogen at a temperature of −80 °C for western blotting [26].

2.9. Evaluation of liver hepcidin expression

Liver samples in lysis buffer which contained EDTA, Tris-HCl (pH = 7.4), NP40 1%, PMSF, NaCl, aprotinin, and leupeptin were homogenized and then centrifuged. Protein extracts or supernatants were harvested for analysis of protein concentration through Bradford method. After diluting proteins with sample buffer, each cocktail was loaded in each lane on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then run. Subsequently, separated proteins were transferred to Immobilon-PVDF membranes (Millipore, Bedford, MA, USA) via using the miniprotean II (Bio-Rad). Incubation with blocking buffer considerably helped to block nonspecific binding sites on the PVDF membrane. Afterwards, the membrane was incubated with primary antibodies (Anti-hepcidin antibody, ab187778 and β-actin, ab8227) and secondary antibodies (Anti-rabbit antibody) which were diluted in blocking buffer (Abcam, CA). Immunoreactivity of the proteins on the PVDF membrane was detected by chemiluminescence detection system (ECL, Amersham). Band intensities were densitometrically assessed via using of J-Image software. For evaluating the liver hepcidin expression variations, the ratio of hepcidin/β-actin was calculated [26,27].

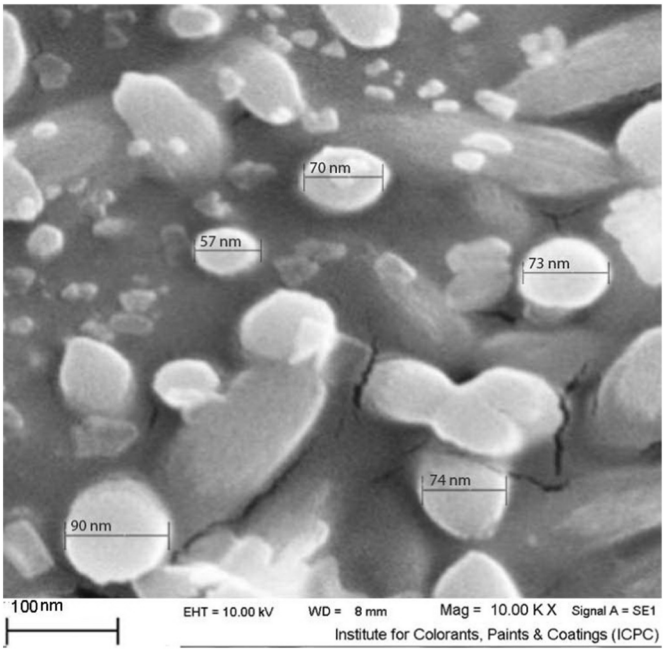

Fig. 2. The SEM image showed that RAc1 nano particle size is 70 nm.

2.10. Statistical analysis

Data were expressed as Mean ± SEM. For comparison of variants within the groups, repeated measurement ANOVA test (One-way ANOVA) and post hoc-*Tukey* were used. To compare more accurately the changes of variants on the same days between the two groups, unpaired student *t*-test was used.

3. Results

3.1. RAc1 nano particle size

The SEM image showed that RAc1 nano particle size is 70 nm [Fig. 2].

3.2. Paw edema variations during different phases of inflammation

CFA injection into the rat's right hind paw induced paw edema, which continued up to day 21 ($p < 0.001$). No significant discrepancies were observed in the paw edema in different days of the study compared with baseline in the MO group. The results showed that intraperitoneal administration of RAc1 with a dosage of 100 ng/kg did not have a significant effect in changes of paw edema in the CFA + 100 ng/kg RAc1 group compared with the CFA group during different days of the study. Intraperitoneal administration of RAc1 with a dosage of 200 and 400 ng/kg in the CFA + 200 and 400 ng/kg RAc1 groups significantly decreased paw edema in different days of the study compared with CFA group ($p < 0.01$ for day 7 and $p < 0.001$ for days 14 and 21). There were no significant differences in paw edema between the two groups receiving RAc1 with doses of 200 and 400 ng/kg. Therefore, the dose of 200 ng/kg for RAc1 was selected as an effective dose for this study [Fig. 3A].

On the other hand, administration of DFX significantly reduced paw edema in the CFA + DFX group on different days of the study in comparison with the CFA group ($p < 0.05$ for day 7 and $p < 0.001$ for days 14 and 21). Comparison of the two groups, CFA + 200 ng/kg RAc1 and CFA + DFX showed that the effective dose of RAc1 (200 ng/kg) significantly reduced paw edema on different days compared to the same days in the CFA + DFX group ($p < 0.05$ for day 7 and $p < 0.01$ for days 14 and 21). Paw edema in the CFA + DW and CFA + NaCl groups showed no significant differences compared with the CFA group (Therefore, the results of these groups are not shown graphically) [Fig. 3B].

3.3. Thermal hyperalgesia variations during different phases of inflammation

The results showed that hyperalgesia significantly increased on day 7 after CFA injection compared with baseline ($p \leq 0.001$). But, hyperalgesia significantly decreased on days 14 and 21 of the study compared with day 7 in the CFA group ($p \leq 0.001$). Significant discrepancies during different days of the study compared with baseline were not observed in the MO group. The results showed that intraperitoneal administration of RAc1 with different dosages decreased PWL dose dependently in different days of the study in comparison with CFA group ($p < 0.001$ for days 7 and 14 and $p < 0.01$ for day 21). There were no significant differences in PWL between the two groups receiving RAc1 with doses of 200 and 400 ng/kg. Therefore, the dose of 200 ng/kg for RAc1 was selected as an effective dose for this study [Fig. 4A].

On the other hand, administration of DFX significantly reduced PWL in the CFA + DFX group on different days of the study in comparison with the CFA group ($p < 0.01$ for day 7 and $p < 0.05$ for days 14 and 21). Comparison of the two groups, CFA + 200 ng/kg RAc1 and CFA + DFX showed that the effective dose of RAc1 (200 ng/kg) significantly reduced PWL on different days compared to the same days in the CFA + DFX group ($p < 0.01$). Thermal hyperalgesia in the CFA + DW and CFA + NaCl groups showed no significant differences compared with the CFA group (Therefore, the results of these groups are not shown graphically) [Fig. 4B].

3.4. Serum TNF-α and IL-1β expression variations during different phases of inflammation

Inflammation caused by CFA injection, considerably increased serum levels of TNF-α on different time points of the study in the CFA group compared with day zero ($P \leq 0.001$). No significant discrepancies in serum levels of TNF-α were observed in the MO group compared with baselines. The results showed that intraperitoneal administration of RAc1 with a dosage of 100 ng/kg did not have a significant effect in changes of serum levels of TNF-α in the CFA + 100 ng/kg RAc1 group compared with the CFA group during different days of the study. Intraperitoneal administration of RAc1 with a dosage of 200 and 400 ng/kg in the CFA + 200 and 400 ng/kg RAc1 groups significantly decreased serum levels of TNF-α in different days of the study compared with CFA group ($p < 0.001$). There were no significant differences in serum levels of TNF-α between the two groups receiving RAc1 with doses of 200 and 400 ng/kg. On the other hand, administration of DFX significantly reduced serum levels of TNF-α in the CFA + DFX group on different days of the study in comparison with the CFA group ($p < 0.01$ for day 7 and $p < 0.001$ for days 14 and 21). Serum levels of TNF-α in the CFA + DW and CFA + NaCl groups showed no significant differences compared with the CFA group (Therefore, the results of these groups are not shown graphically) [Fig. 5].

Inflammation caused by CFA injection, considerably increased serum levels of IL-1β on different time points of the study in the CFA group compared with day zero ($P \leq 0.001$). No significant discrepancies in serum levels of IL-1β were observed in the MO group compared with baselines. The results showed that intraperitoneal administration of RAc1 with a dosage of 100 ng/kg did not have a significant effect in changes of serum levels of IL-1β in the CFA + 100 ng/kg RAc1 group compared with the CFA group during different days of the study. Intraperitoneal administration of RAc1 with a dosage of 200 and 400 ng/kg in the CFA + 200 and 400 ng/kg RAc1 groups

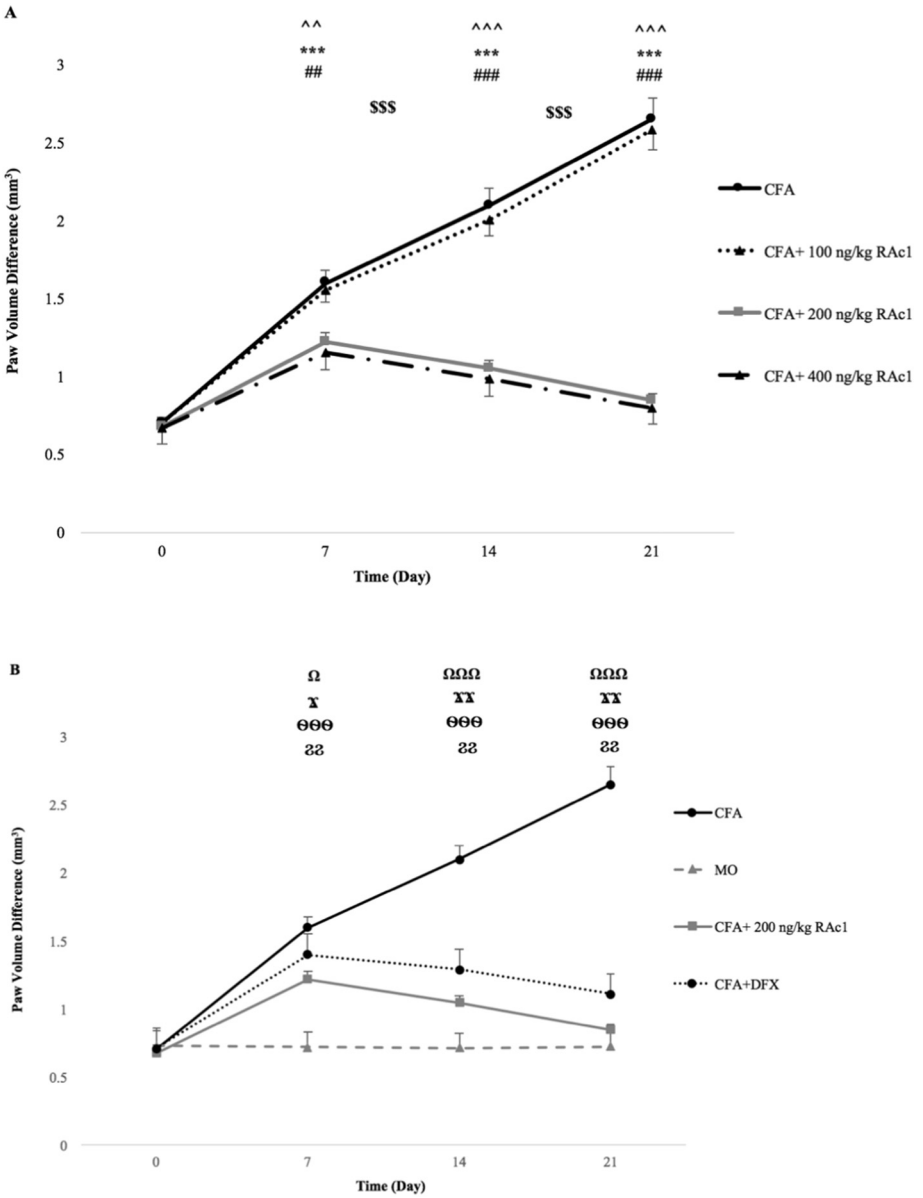

Fig. 3. A. Paw edema significantly increased in CFA group compared with baseline in different time points of the study. Long-term administration of RAc1 nano particle was noticeably downturned paw edema. Results as Mean ± SEM (n = 6/group) stated. ***$P \leq 0.001$ for comparison of paw edema variations between baseline and different days of the study in CFA group. $$$P ≤ 0.001 for indicating the changes in paw edema at days 14 compared with day 7 and day 21 compared with day 14 in CFA group. ##$P \leq 0.01$ and ###P ≤ 0.001 for comparison of paw edema variations between baseline and different days of the study in the CFA + 200 ng/kg RAc1 group. ^^P ≤ 0.01 and ^^^P ≤ 0.001 for comparison of paw edema variations between baseline and different days of the study in the CFA + 400 ng/kg RAc1 group. B. CFA injection considerably increased paw edema, while effective dose of intraperitoneal administration of RAc1 caused a significant reduction in paw edema compared with CFA group. Results as Mean ± SEM (n = 6/group) stated. ƸƸP ≤ 0.01 and ƸƸƸP ≤ 0.001 for comparison of paw edema variations between CFA and CFA + 200 ng/kg RAc1groups in the same days. ΩP ≤ 0.05 and ΩΩΩP ≤ 0.001 for comparison of paw edema variations between CFA + DFX and CFA groups in the same days. ӾP ≤ 0.05 and ӾӾP ≤ 0.01 for comparison of differences in paw edema variations between CFA + DFX and CFA + 200 ng/kg RAc1groups in the same days. ϴϴP ≤ 0.001 for comparison of paw edema variations between CFA and CFA Control groups in the same days.

significantly decreased serum levels of IL-1β in different days of the study compared with CFA group ($p < 0.001$). On the other hand, administration of DFX significantly reduced serum levels of IL-1β in the CFA + DFX group on different days of the study in comparison with the CFA group (p < 0.001) [Fig. 6].

3.5. Variations in liver hepcidin expression during different phases of inflammation

Protein expression of hepcidin indicated similar bands intensity with molecular masses of nearly 55 kDa in the liver tissues of all experimental groups. β-Actin was considered as a loading control protein (43 kDa) [Fig. 7A]. Data which obtained from the Western blotting technique demonstrated that expression of hepcidin in the liver of rats in CFA group significantly increased in different days of the study compared with baseline ($p < 0.001$). Sterile mineral oil injection to the rat's hind paw in the MO group, did not cause considerable discrepancies in the liver hepcidin expression compared with baseline in different days of the study. The results showed that intraperitoneal administration of RAc1 with different dosages decreased hepcidin expression dose dependently in different days of the study in comparison with CFA group (p < 0.001). There were no significant differences in hepcidin expression between the two groups receiving RAc1 with doses of 200 and 400 ng/kg. Therefore, the dose of 200 ng/kg for RAc1 was selected as an effective dose for this study [Fig. 7B]. DFX administration in the CFA + DFX group, did not cause considerable discrepancies in the liver hepcidin expression compared with baseline in different days of the study [Fig. 7C].

4. Discussion

The present study documented that administration of effective dose of RAc1 nano particle could reduce edema, hyperalgesia, serum IL-1β and TNF-α levels and liver hepcidin expression during acute and chronic phases of CFA-induced inflammation. Additionally, DFX administration could reduce hyperalgesia, edema and serum IL-1β and TNF-α levels, however, could not reduce liver hepcidin expression in both acute and chronic phases of inflammation.

Data from diverse animal models support the idea that peripheral inflammation induced by CFA, as an animal usual model of RA, can cause diverse changes in the central nervous system, which is accompanied with mechanical allodynia, thermal hyperalgesia and edema.

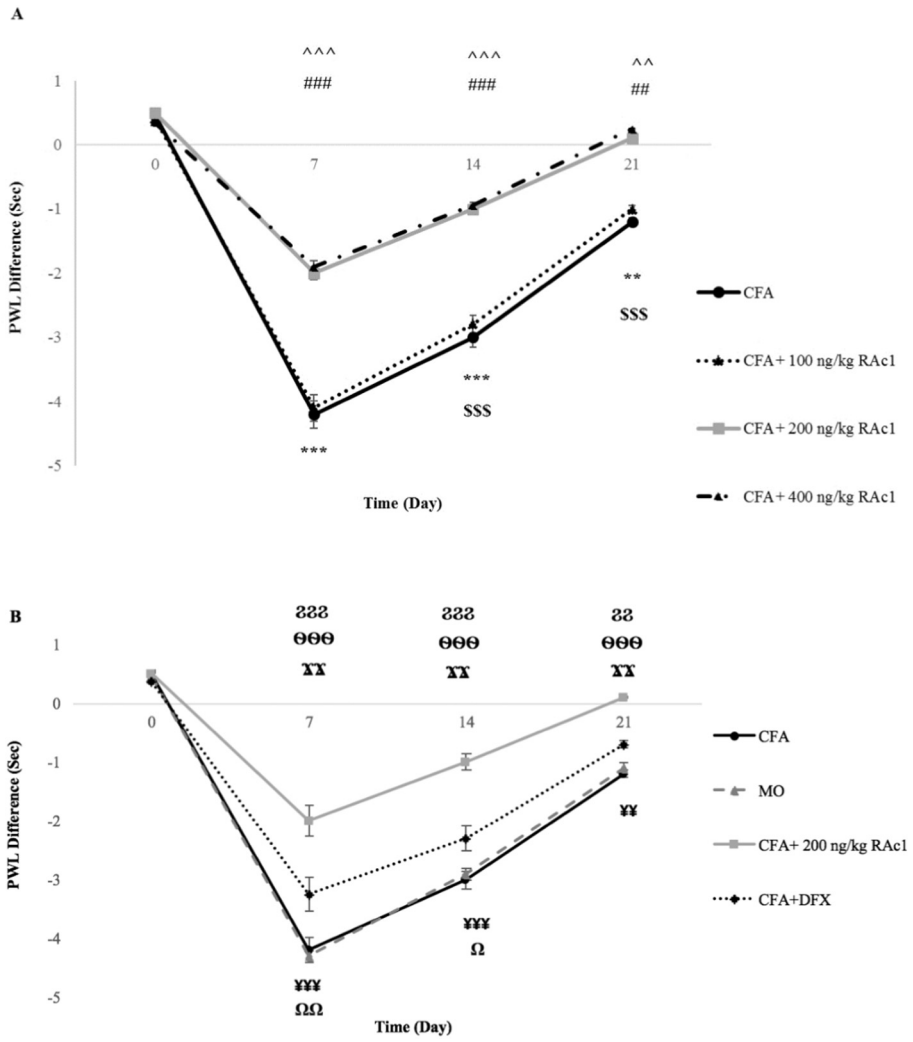

Fig. 4. A. Hyperalgesia significantly increased in different time points of the study in the CFA group compared with baseline and long-term administration of RAc1 remarkably decreased thermal hyperalgesia. Results as Mean ± SEM (n = 6/group) stated. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ for comparison of the PWL variations between baseline and different days of the study in CFA group. $\$\$\$P \leq 0.001$ for indicating the changes in PWL at days 14 compared with day 7 and day 21 compared with day 14 in CFA group. $\#\#P \leq 0.01$ and $\#\#\#P \leq 0.001$ for comparison of PWL variations between baseline and different days of the study in the CFA + 200 ng/kg RAc1 group. $\hat{}\hat{}P \leq 0.01$ and $\hat{}\hat{}\hat{}P \leq 0.001$ for comparison of PWL variations between baseline and different days of the study in the CFA + 400 ng/kg RAc1 group. B. CFA injection considerably increased PWL, while effective dose of intraperitoneal administration of RAc1 caused a significant reduction in PWL compared with CFA group. Results as Mean ± SEM (n = 6/group) stated. ßßP ≤ 0.01 and ßßßP ≤ 0.001 for comparison of PWL variations between CFA and CFA + 200 ng/kg RAc1groups in the same days. ¥¥P ≤ 0.01 and ¥¥¥P ≤ 0.001 for comparison of PWL variations between baseline and different days of the study in the CFA + DFX group. ΩP ≤ 0.05 and ΩΩP ≤ 0.01 for comparison of PWL variations between CFA + DFX and CFA groups in the same days. ƔƔP ≤ 0.01 for comparison of differences in PWL variations between CFA + DFX and CFA + 200 ng/kg RAc1groups in the same days. ΘΘΘP ≤ 0.001 for comparison of PWL variations between CFA and CFA Control groups in the same days.

The results of this study in line with our previous research studies have shown that edema and hyperalgesia induced by CFA were started 2 h and continued for at least 21 days after CFA injection [22].

Evidence is accumulating to indicate that dysregulation of glial functions can result in inflammation and sensitization. Such observations also raise the possibility that activated microglia have crucial role in releasing inflammatory mediators, thereby decreasing the pain threshold. The activated microglia work with astrocytes in releasing prototypic inflammatory cytokines such as interleukin IL-1β, IL-6, interferon-gamma and TNF-α, which is accompanied and often

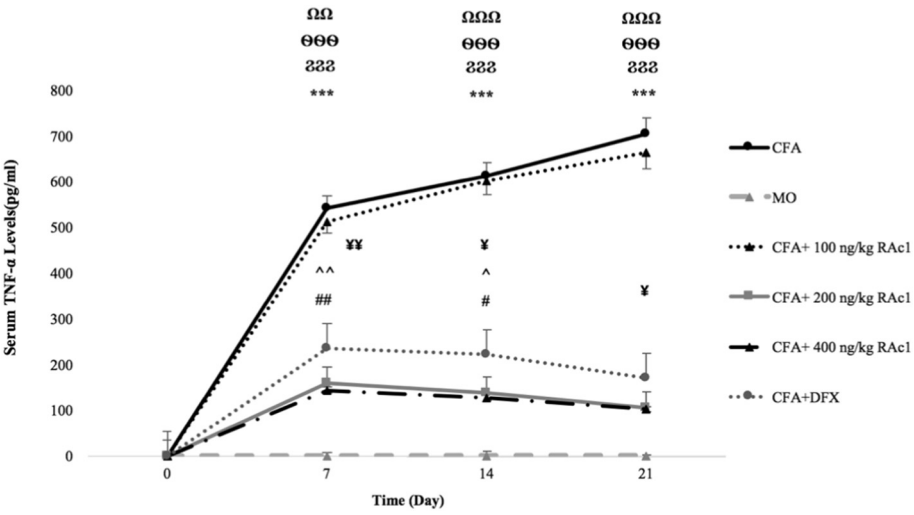

Fig. 5. Serum levels of TNF-α significantly increased in CFA group compared with baseline in different time points of the study. Long-term administration of RAc1 nano particle was noticeably downturned TNF-α expression levels. Results as Mean ± SEM (n = 6/group) stated. $^{***}P \leq 0.001$ for comparison of serum TNF-α expression variations between baseline and different days of the study in CFA group. $\#P \leq 0.05$ and $\#\#P \leq 0.01$ for comparison of serum TNF-α variations between baseline and different days of the study in the CFA + 200 ng/kg RAc1 group. $\hat{}P \leq 0.05$ and $\hat{}\hat{}P \leq 0.01$ for comparison of serum TNF-α variations between baseline and different days of the study in the CFA + 400 ng/kg RAc1 group. ßßßP ≤ 0.001 for comparison of serum TNF-α variations between CFA and CFA + 200 ng/kg RAc1groups in the same days. ΩΩP ≤ 0.01 and ΩΩΩP ≤ 0.001 for comparison of serum TNF-α variations between CFA + DFX and CFA groups in the same days. ΘΘΘP ≤ 0.001 for comparison of serum TNF-α variations between CFA and CFA Control groups in the same days.

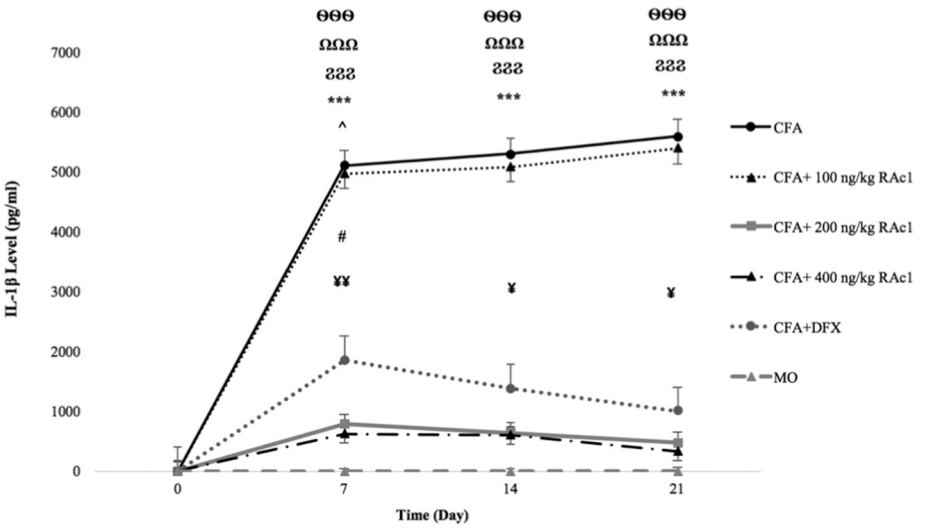

Fig. 6. Serum levels of IL-1β significantly increased in CFA group compared with baseline in different time points of the study. Long-term administration of RAc1 nano particle was noticeably downturned IL-1β expression levels. Results as Mean ± SEM ($n = 6$/group) stated. ***$P \leq 0.001$ for comparison of serum IL-1β expression variations between baseline and different days of the study in CFA group. #$P \leq 0.05$ for comparison of serum IL-1β variations between baseline and different days of the study in the CFA + 200 ng/kg RAc1 group. ^P ≤ 0.05 for comparison of serum IL-1β variations between baseline and different days of the study in the CFA + 400 ng/kg RAc1 group. ßßßP ≤ 0.001 for comparison of serum IL-1β variations between CFA and CFA + 200 ng/kg RAc1groups in the same days. ΩΩΩP ≤ 0.001 for comparison of serum IL-1β variations between CFA + DFX and CFA groups in the same days. ΘΘΘP ≤ 0.001 for comparison of serum IL-1β variations between CFA and CFA Control groups in the same days. ¥P ≤ 0.05 and ¥¥$P \leq 0.01$ for comparison of serum IL-1β expression levels between baseline and different days of the study in the CFA + DFX group.

followed by a cascade of down-stream inflammatory events (i.e. activation of toll-like receptors, nuclear factor-kappa B (NF-kB), complement system, chemokines, and acute phase proteins). Cytokines and other inflammatory mediators have contribution in both excitotoxic and apoptotic neuronal death, highlighting the possibility that production and release of inflammatory cytokines during inflammation by glia and adaptive immune system's cells may contribute to neuronal damage. The deleterious effects of inflammatory cytokines on neuronal survival may involve in the production of various neurotoxic compounds via autocrine and/or paracrine mechanisms. Data generated thus far illustrated that TNF-α and IL-1β through endothelial damage and also increasing the vascular permeability can cause edema in RA. TNFα usually precedes IL-1β in the inflammatory cytokine cascade, and via direct action on neurons and stimulation of pain receptors as well as joint inflammation and destruction has hyperalgesic actions. So, it seems that neutralizing of TNF-α and IL-1β may decrease hyperalgesia and edema during different stages of inflammation. Therefore, it seems that elevated levels of serum TNF-α and IL-1β within 21 days of the study may play crucial role in continuation of edema and hyperalgesia during RA [19,25].

Mounting evidence has shown that iron is an active component of wide ranges of enzymes, which are essential for function of dynamic cell compartments, such as immune cells and erythropoiesis, as well. As iron homeostasis and cellular immunity are closely linked to each other, it is suggestive that, immune cells such as monocytes and macrophages have evoked different immunological pathways by using iron. Taylor Thorburn, et al., have suggested that in one hand in infection and inflammation, utilization of iron by innate immune cells can induce reactive oxygen species generation. On the other hand, the host's immune system endeavors to down regulate circulating iron levels in order to decrease invading pathogen growth. However, in cases of systemic inflammation, iron catalyzes immoderate production of reactive oxygen species, which causes cellular damage of different organs and tissues [28]. Hence, it is thoroughly transparent that iron deposition in immune cells can sensitize host towards invading pathogens via blocking essential immune functions and cytokines activities. Such in vitro findings are supported by clinical evidence [29].

Notwithstanding the fact that little is known about the direct or indirect cellular and molecular mechanisms involved in iron- and inflammation-induced regulation of hepcidin, several lines of evidence strongly stated that hepcidin, as a negative regulator of dietary iron absorption, is responsible for the regulation of iron recycling and iron balance and also can inhibit release of recycled iron from macrophages [30,31]. It is vividly depicted in recent studies that, in contrast with in vivo conditions, iron cannot induce hepcidin gene expression in isolated hepatocytes in vitro. In systemic iron homeostasis, hepcidin presumably through interaction with cell surface signaling receptors and inhibition of iron export via modifying iron transport molecules may limit iron release from enterocytes and macrophages [31]. Several studies have demonstrated roles for inflammatory stimuli and infections for induction of hepcidin gene expression. Nemeth, et al., have indicated that hepcidin production induction by inflammation is a type II acute-phase response [30]. As outlined above, Gaël Nicolas, et al., following their animal experimental measurements have suggested that hypoxia, anemia, and inflammation can regulate hepcidin gene expression in mice. Latest findings suggest that hepatocytes can be directly stimulated to produce hepcidin via inflammatory cytokines such as IL-6, IL-1β, and TNF-α. IL-1β can play a momentous role in the anemia of inflammation through up-regulating hepcidin. Most notably this may hold true for TNF-α [5]. Moreover, Dan-Qing Lou, et al., through in vivo induction of hepcidin by inflammation mentioned that hepcidin is a long-anticipated central regulatory hormone and has crucial role in the progressive iron accumulation and anemia in chronic diseases [31]. In this regard, iron chelation and depletion appears to have selective effects in reducing joint inflammation and be a useful approach in modifying the inflammatory process in adjuvant-induced inflammatory diseases [8]. Therefore, in order to investigate further the role of iron in inflammatory conditions, we have examined the effect of iron chelation using DFX in adjuvant induced-inflammation in male rats.

DFX, a ferric iron chelator, has been used for treating iron overloaded patients in the last forty years [9]. Luis Henrique Rapucci Moraes, et al., have represented that inasmuch as iron plays a notable role in inflammatory processes, iron chelating agents can ameliorate oxidative stress and muscle damage in dystrophic muscle via mechanisms involving oxidative and inflammatory pathways, however, they cannot be fully effective. They also mentioned that DFX can reduce strength loss, muscle fiber degeneration, and lipid peroxidation in DFX treated mice compared with none treated ones [10]. Evidence has begun to accumulate that DFX can inhibit activation of microglia and inflammatory cascades linked to inflammatory response and then decreases inflammatory cytokines such as TNF-α and IL-1β. Moreover, elevated levels of iron may affect the functional properties of activated microglia at the secretory and gene expression levels and that DFX

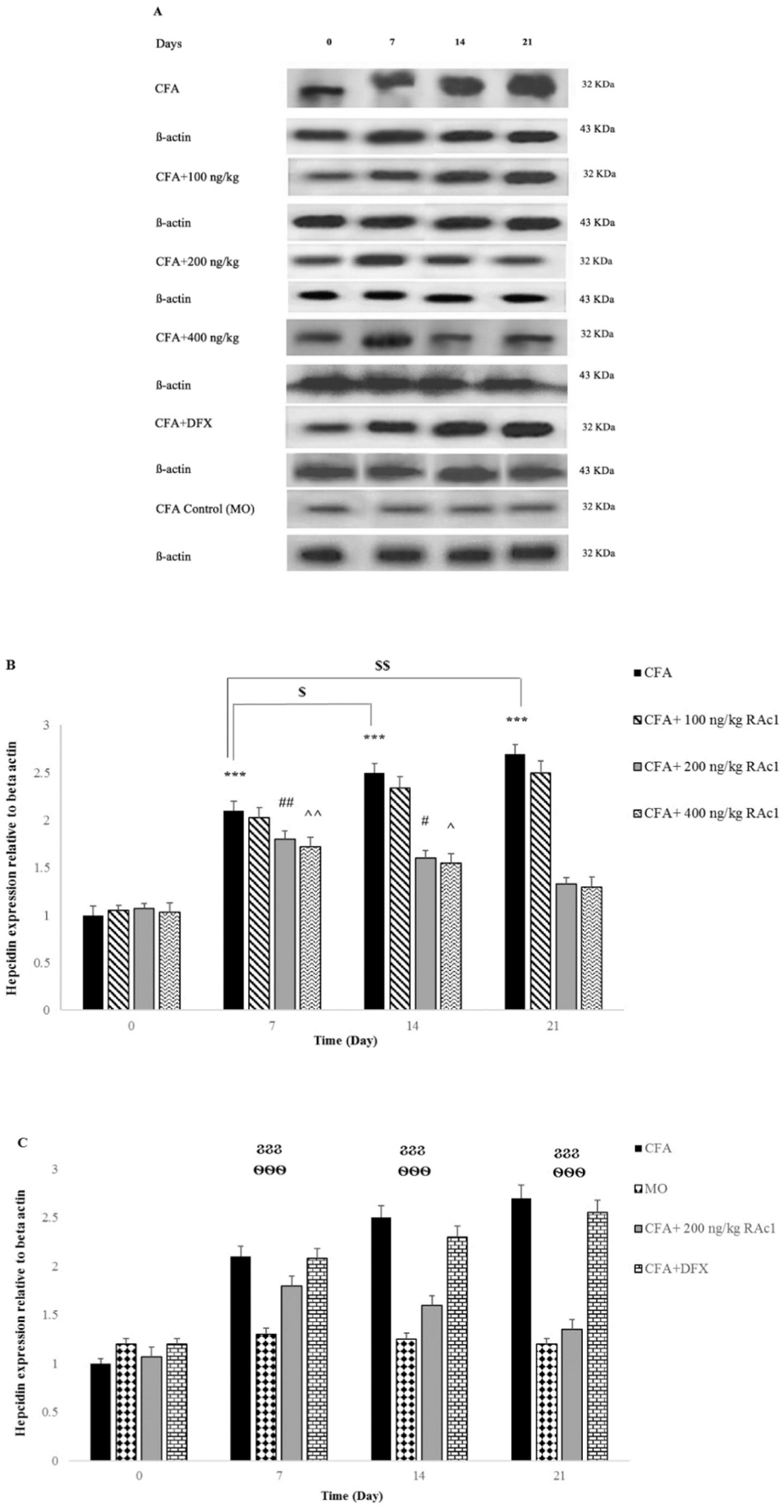

Fig. 7. A, B, C: A Immunoblots of hepcidin expression during different stages of persistent inflammation (day 0, 7, 14 and 21) in CFA and CFA + RAc1 groups. All densitometry data were demonstrated as hepcidin/β-actin ratio. Data are represented as Mean ± SEM (n = 6/group). **B** Hepcidin expression during different stages of persistent inflammation in all experimental groups. The hepcidin/β-actin ratio significantly increased in days 7, 14 and 21 after CFA injection compared with day 0 in CFA group. Hepcidin expression significantly increased in different time points of the study in the CFA group compared with baseline and long-term administration of RAc1remarkably decreased hepcidin expression. Results as Mean ± SEM (n = 6/group) stated. ***$P \leq 0.001$ for comparison of the hepcidin expression variations between baseline and different days of the study in CFA group. \$$P \leq 0.05$ and \$ \$$P \leq 0.01$ for indicating the changes in hepcidin expression at days 14 and 21 compared with day 7 in CFA group. #P ≤ 0.05 and ##P ≤ 0.01 for comparison of hepcidin/β-actin ratio variations between baseline and different days of the study in the CFA + 200 ng/kg RAc1 group. ^P ≤ 0.05 and ^^P ≤ 0.01 for comparison of hepcidin/β-actin ratio variations between baseline and different days of the study in the CFA + 400 ng/kg RAc1 group. **C** CFA injection considerably increased hepcidin/β-actin ratio, while effective dose of intraperitoneal administration of RAc1 caused a significant reduction in hepcidin/β-actin ratio compared with CFA group. Results as Mean ± SEM (n = 6/group) stated. ƨƨƨP ≤ 0.001 for comparison of hepcidin expression variations between CFA and CFA + 200 ng/kg RAc1groups in the same days. ΘΘΘP ≤ 0.001 for comparison of PWL variations between CFA and CFA Control groups in the same days.

exerts its protective properties against elevated iron levels and neural injury. In this regard, Wei-Jian Zhang, et al., have reported that DFX can inhibit TNF-α and IL-1β expression and LPS-induced superoxide production by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in vivo in experimental mice [32]. F.J. Andrews, et al., have indicated that adjuvant disease is an appropriate model of iron deposit in inflamed synovium, which has close approximation to RA and is accompanied with erosive bone damage. They also showed that DFX induced mild iron deficiency and reduced the severity and incidence of joint inflammation associated with adjuvant disease in experimental rats, however, could not alter the local primary inflammatory response or the systemic sequelae in joint mediated inflammation. Moreover, the iron dextran exacerbated joint inflammation selectively in RA. In line with our results, they stated that effective dose of DFX could reduce both acute and chronic inflammatory symptoms such as hyperalgesia and edema [8]. Whilst, DFX could not decrease expression of liver hepcidin in both acute and chronic phases of inflammation. It is tempting to speculate that DFX exerts its effects by affecting inflammatory cytokines expressions and intracellular pathways activities, not hepcidin expression. George J. Kontoghiorghes, et al., have mentioned that DFX has many toxic adverse effects, such as neutropenia, thrombocytopenia, and pancytopenia, and also swelling and soreness at the site of the injection. So, the wide ranges of research to define new therapeutic approaches with fewer side effects including new iron chelators are in progress [33].

There are numerous chelating agents with different ability to bind with excess iron based on their metal ion affinity, cell permeability, and molecular weight. Thorburn, et al., have stated that administration of iron chelating agents can reduce leukocytes activation and recruitment within the intestinal microcirculation in sepsis induced by LPS via reducing the formation of reactive oxygen species. Moreover, Iron chelation therapy may be beneficial for treating infections with low susceptibility/resistance to antibiotics [28].

Nanotechnology and nanomedicine are complementary disciplines with the aim of amending human's life. Nanotechnology is an emerging branch of science for designing tools and devices of size 1–100 nm, with unique functions at the cellular, atomic and molecular levels. The concept of using nanotechnology in medical research and clinical practice is known as nanomedicine [34]. In the previous research studies, therapeutic effects of nano structures, which were synthetized based on Nanochelating technology, were evaluated and proved. Kalanaky et al. stated that BCc1 nano structure can induce neoplastic cell apoptosis and reduce tumor growth in breast cancer-bearing balb/c mice [13]. Also, Fakharzadeh et al. have shown that MSc1 nano structure could decrease disability signs in experimental autoimmune encephalomyelitis mice and increase survival [12]. RAc1 nano particle is synthetized based on mentioned technology and has iron chelating properties. In the present study, RAc1 could reduce paw edema, thermal hyperalgesia and TNF-α and IL-1β expression levels compared to CFA and DFX groups. It is of interest to note that RAc1 could efficiently decrease hepcidin expression in contrast with DFX. In support of the latter, RAc1 nano particle has iron chelating properties and exerts part of its effects via iron metabolism. It is crystal clear that zinc is an essential trace element for living organisms and their biological processes [35]. Subsequent studies have elucidated a broad array of effective functions beyond zinc chelation which implicate its momentous role in therapies via regulating the immune system in zinc deficiency diseases and inflammation as well [17]. Sanna et al. in their Meta-Analysis study have shown that zinc level is decreased in serum and plasma of patients with autoimmune disorders compared with controls [16]. They suggested that “in populations with higher genetic risk of autoimmunity, it would be therefore interesting to have clinical trial investigations in the field of zinc supplementation for preventing and/or treating autoimmune diseases”. Also, Xin et al. have reported that decreased serum level of zinc is generally present in RA patients [36]. There is clear evidence in the literature supporting a role for zinc supplementation in controlling inflammatory processes and autoimmune disorders. In the study by Hadj Abdallah, using zinc acexamate could reduce pro-inflammatory cytokines such as TNF-α and IL-1β in rat model of renal warm ischemia [37]. Also, Stoye et al. have stated that zinc aspartate could suppress T cell activation in vitro and relapse experimental autoimmune encephalomyelitis in SJL/J mice [38]. So, it is therefore conceivable that RAc1 nano particle may exert part of its therapeutic effects through zinc supplement in its structure. As such, it deserves consideration as a therapeutic target.

In agreement with this notion, our data therefore revealed that anti-inflammatory effects of RAc1 nano particle can be partially related to the reduction of liver hepcidin and serum TNF-α and IL-1β expression levels, which were aligned with hyperalgesia and edema reduction during CFA-induced inflammation.

5. Conclusion

The results of the present experiment suggest that immunomodulatory effects of long term treatment by RAc1 may be mediated via decrement of liver hepcidin and TNF-α and IL-1β expression levels. Interestingly, the potent anti-edematogenic, anti-hyperalgesic and principally anti-inflammatory effects of RAc1 nano particle, raise the possibility to be a good candidate for the control of inflammatory symptoms. In light of the principal role of RAc1 nano particle, the identification of involved anti-inflammatory pathways during long term treatment with RAc1 should bring forth novel therapeutics for the treatment of inflammatory diseases. Also, further clinical studies are needed to evaluate the anti-hyperalgesic and inflammatory effects of RAc1 compared with synthetic drugs.

Acknowledgements

This work was scientifically supported by Neuroscience Research Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran. The RAc1 nanoparticle was patented by Mohammad Hassan Nazaran, United States Patent US 8288587 B2, 2012.

Conflict of interests

The authors declare that they have no conflict of interest.

References

[1] W. Jia, W. Wu, D. Yang, C. Xiao, M. Huang, F. Long, Z. Su, M. Qin, X. Liu, Y.Z. Zhu, GATA4 regulates angiogenesis and persistence of inflammation in rheumatoid arthritis, Cell Death Dis. 9 (2018) 1–15, https://doi.org/10.1038/s41419-018-0570-5.
[2] V. Nazemian, M. Shadnoush, H. Manaheji, J. Zaringhalam, Probiotics and inflammatory pain: a literature review study, Middle East J. Rehabil. Health Stud. 3 (2016) 1–11, https://doi.org/10.17795/mejrh-36087.
[3] M. Lv, J. Miao, P. Zhao, X. Luo, Q. Han, Z. Wu, K. Zhang, P. Zhu, CD 147-mediated chemotaxis of $CD4^+CD161^+$ T cells may contribute to local inflammation in rheumatoid arthritis, Clin. Rheumatol. 37 (2018) 59–66, https://doi.org/10.1007/s10067-017-3800-9.
[4] C. Chrobak, J.A. Sidler, A. O'Meara, S. Schaedelin, B.L. Hug, Iron homeostasis in inflammation: a single centre prospective observational study in medicalinpatients, Swiss Med. Wkly. 147 (2017) 1–6, doi:https://doi.org/10.4414/smw.2017.14431.
[5] G. Nicolas, C. Chauvet, L. Viatte, J.L. Danan, X. Bigard, I. Devaux, C. Beaumont, A. Kahn, S. Vaulont, The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation, J. Clin. Invest. 110 (2002) 1037–1044, https://doi.org/10.1172/JCI15686.
[6] G. Papanikolaou, K. Pantopoulos, Systemic iron homeostasis and erythropoiesis, IUBMB Life 69 (2017) 399–413, https://doi.org/10.1002/iub.1629.
[7] T. Ganz, E. Nemeth, Iron imports. IV. hepcidin and regulation of body iron metabolism, Am. J. Physiol. Gastrointest. Liver Physiol. 290 (2006) 199–203, https://doi.org/10.1152/ajpgi.00412.2005.
[8] F.J. Andrews, C.J. Morris, G. Kondratowics, D.R. Blake, Effect of iron chelation on inflammatory joint disease, Ann. Rheum. Dis. 46 (1987) 327–333.
[9] N.A. Rao, J.L. Romero, M.A.S. Fernandez, A. Sevanian, G.E. Marak Jr., Effect of iron chelation on severity of ocular inflammation in an animal model, Arch. Ophthalmol. 104 (1986) 1369–1371, https://doi.org/10.1001/archopht.1986.01050210123038.

[10] L.H. Moraes, R.R. de Burgos, A.B. Macedo, T. de Almeida Hermes, F.M. de Faria, E. Minatel, Reduction of oxidative damage and inflammatory response in the diaphragm muscle of mdx mice using iron chelator deferoxamine, Biol. Trace Elem. Res. 167 (2015) 115–120, https://doi.org/10.1007/s12011-015-0290-y.
[11] M.H. Nazaran, Chelate compounds, United States patent US 8288587 B2. (2012).
[12] S. Fakharzadeh, M.A. Sahraian, M. Hafizi, S. Kalanaky, Z. Masoumi, M. Mahdavi, N. Kamalian, A. Minagar, M.H. Nazaran, The therapeutic effects of MSc1 nanocomplex, synthesized by nanochelating technology, on experimental autoimmune encephalomyelitic C57/BL6 mice, Int. J. Nanomedicine 9 (2014) 3841–3853 (doi:10.2147%2FIJN.S64630).
[13] S. Kalanaky, M. Hafizi, S. Fakharzadeh, M. Vasei, L. Langroudi, E. Janzamin, S.M. Hashemi, M. Khayamzadeh, M. Soleimani, M.E. Akbari, M.H. NAzaran, BCc1, the novel antineoplastic nanocomplex, showed potent anticancer effects in vitro and in vivo, Drug Des. Devel. Ther. 10 (2016) 59–70 (doi:10.2147%2FDDDT.S89694).
[14] A. Maghsoudi, S. Fakharzadeh, M. Hafizi, M. Abbasi, F. Kohram, S. Sardab, A. Tahzibi, S. Kalanaky, M.H. Nazaran, Neuroprotective effects of three different sizes nanochelating based nano complexes in MPP(+) induced neurotoxicity, Apoptosis 20 (2015) 298–309, https://doi.org/10.1007/s10495-014-1069-x.
[15] S. Kalanaky, M. Hafizi, S. Safari, K. Mousavizadeh, M. Kabiri, A. Farsinejad, S. Fakharzadeh, M.H. Nazaran, TLc-A, the leading nanochelating-based nanochelator, reduces iron overload in vitro and in vivo, Int. J. Hematol. 103 (2016) 274–282, https://doi.org/10.1007/s12185-015-1932-8.
[16] A. Sanna, D. Firinu, P. Zavattari, P. Valera, Zinc status and autoimmunity: a systematic review and meta-analysis, Nutrients 10 (2018) 1–17, https://doi.org/10.3390/nu10010068.
[17] A.S. Prasad, Zinc: role in immunity, oxidative stress and chronic inflammation, Curr. Opin. Clin. Nutr. Metab. Care. 12 (2009) 646–652, https://doi.org/10.1097/MCO.0b013e3283312956.
[18] M. Shadnoush, V. Nazemian, H. Manaheji, J. Zaringhalam, Effect of probiotic administration on acute inflammatory pain, Middle East J. Rehabil. Health 4 (2017) 1–10 http://dx.doi.org/10.17795/mejrh-42022.
[19] V. Nazemian, H. Manaheji, A.M. Sharifi, J. Zaringhalam, Long term treatment by mesenchymal stem cells conditioned medium modulates cellular, molecular and behavioral aspects of adjuvant-induced arthritis, Cell. Mol. Biol. (Noisy-le-grand) 64 (2018) 19–26 https://doi.org/10.14715/cmb/2018.64.2.5.
[20] D.R. Blake, N.D. Hall, P.A. Bacon, P.A. Dieppe, B. Halliwell, J.M. Gutteridge, Effect of a specific iron chelating agent on animal models of inflammation, Ann. Rheum. Dis. 42 (1983) 89–93.
[21] I. Sakaida, K. Kayano, S. Wasaki, A. Nagatomi, Y. Matsumura, K. Okita, Protection against acetaminophen-induced liver injury in vivo by an iron chelator, deferoxamine, Scand. J. Gastroenterol. 30 (1995) 61–67, https://doi.org/10.3109/00365529509093237.
[22] J. Zaringhalam, A.M. khanizadeh, H. Manaheji, V. Nazemian, A. Sonboli, M. Ayyari, Treatment by hydroalcoholic and chloroformic extracts of *salvia candidissima* reduced hyperalgesia, edema and serum TNF–α level in arthritis model, Entomol. Appl. Sci. Lett. 3 (2016) 13–22.
[23] V. Nazemian, B. Nasseri, H. Manaheji, J. Zaringhalam, Effects of mesenchymal stem cells conditioned medium on behavioral aspects of inflammatory arthritic pain induced by CFA adjuvant, J. Cell. Mol. Anesth. 1 (2016) 47–55, https://doi.org/10.22037/jcma.v1i2.11429.
[24] J. Zaringhalam, A. Akbari, A. Zali, H. Manaheji, V. Nazemian, M. Shadnoush, S. Ezzatpanah, Long-term treatment by vitamin B_1 and reduction of serum proinflammatory cytokines, hyperalgesia, and paw edema in adjuvant-induced arthritis, Basic Clin. Neurosci. 7 (2016) 331–340 https://dx.doi.org/10.15412%2FJ.BCN.03070406.
[25] M. Shadnoush, V. Nazemian, H. Manaheji, J. Zaringhalam, The effect of orally administered probiotics on the behavioral, cellular, and molecular aspects of adjuvant-induced arthritis, Basic Clin. Neurosci. 9 (2018) 325–336 http://dx.doi.org/10.32598/bcn.9.5.325.
[26] D.M. Frazer, S.J. Wilkins, E.M. Becker, C.D. Vulpe, A.T. McKie, D. Trinder, G.J. Anderson, Hepcidin expression inversely correlates with the expression of duodenal iron transporters and iron absorption in rats, Gastroenterology 123 (2002) 835–844.
[27] B. Nasseri, V. Nazemian, H. Manaheji, J. Zaringhalam, Microglia are involved in pain related behaviors during the acute and chronic phases of arthritis inflammation, J. Cell. Mol. Anesth. 1 (2016) 137–145, https://doi.org/10.22037/jcma.v1i4.13557.
[28] T. Thorburn, M. Aali, L. Kostek, C. LeTourneau-Paci, P. Colp, J. Zhou, B. Holbein, D. Hoskin, C. Lehmann, Anti-inflammatory effects of a novel iron chelator, DIBI, in experimental sepsis, Clin. Hemorheol. Microcirc. 67 (2017) 241–250, https://doi.org/10.3233/CH-179205.
[29] C. Gasche, M.C. Lomer, I. Cavill, G. Weiss, Iron, anaemia, and inflammatory bowel diseases, Gut 53 (2004) 1190–1197, https://doi.org/10.1136/gut.2003.035758.
[30] E. Nemeth, S. Rivera, V. Gabayan, C. Keller, S. Taudorf, B.K. Pedersen, T. Ganz, IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin, J. Clin. Invest. 113 (2004) 1271–1276, https://doi.org/10.1172/JCI.20945.
[31] D.Q. Lou, J.C. Lesbordes, G. Nicolas, L. Viatte, M. Bennoun, N. Van Rooijen, A. Kahn, L. Renia, S. Vaulont, Iron- and inflammation-induced hepcidin gene expression in mice is not mediated by Kupffer cells in vivo, Hepatology 41 (2005) 1056–1064, https://doi.org/10.1002/hep.20663.
[32] W.J. Zhang, H. Wei, B. Frei, The iron chelator, desferrioxamine, reduces inflammation and atherosclerotic lesion developmentin experimental mice, Exp. Biol. Med. (Maywood) 235 (2010) 633–641, https://doi.org/10.1258/ebm.2009.009229.
[33] G.J. Kontoghiorghes, K. Pattichi, M. Hadjigavriel, A. Kolnagou, Transfusional iron overload and chelation therapy with deferoxamine and deferiprone (L1), Transfus. Sci. 23 (2000) 211–223.
[34] K.H. Basavaraj, Nanotechnology in medicine and relevance to dermatology: present concepts, Indian J. Dermatol. 57 (2012) 169–174 (doi:10.4103%2F0019-5154.96186).
[35] D. Beyersmann, H. Haase, Functions of zinc in signaling, proliferation and differentiation of mammalian cells, Biometals 14 (2001) 331–341, https://doi.org/10.1023/A:1012905406548.
[36] L. Xin, X. Yang, G. Cai, D. Fan, Q. Xia, L. Liu, Y. Hu, N. Ding, S. Xu, L. Wang, X. Li, Y. Zou, F. Pan, Serum levels of copper and zinc in patients with rheumatoid arthritis: a meta-analysis, Biol. Trace Elem. Res. 168 (2015) 1–10, https://doi.org/10.1007/s12011-015-0325-4.
[37] N.H. Abdallah, A. Baulies, A. Bouhlel, M. Bejaoui, M.A. Zaouali, S. Ben Mimouna, I. Messaoudi, J.C. Fernandez-Checa, C. García Ruiz, H. Ben Abdennebi, The effect of zinc acexamate on oxidative stress, inflammation and mitochondria induced apoptosis in rat model of renal warm ischemia, Biomed. Pharmacother. 105 (2018) 573–581.
[38] D. Stoye, C. Schubert, A. Goihl, K. Guttek, A. Reinhold, S. Brocke, K. Grüngreiff, D. Reinhold, Zinc aspartate suppresses T cell activation in vitro and relapsing experimental autoimmune encephalomyelitis in SJL/J mice, Biometals 25 (2012) 529–539, https://doi.org/10.1007/s10534-012-9532-z.