Original Article

Anti-hyperalgesic and Anti-Inflammatory Effects of Long Term Calcium Administration during Adjuvant-Induced Arthritis in Rats

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Abstract

Background: Inflammation and edema symptoms are physiological response to triggers that can be induced by different mediators such as cytokines. Rheumatoid arthritis is the most common form of arthritis which is characterized by chronic inflammation of the synovial membrane, severe and debilitating pain and progressive cartilage injury. It is clear that cytokines are involved in different stages of inflammation by inducing pro-inflammatory effects; TNF-α as a cytokine involved in the pathogenesis of rheumatoid arthritis. In this study we attempted to investigate the role of prescription of calcium to reduce inflammatory edema and serum TNF-α level during different stages of arthritic inflammation induced by Complete Freund Adjuvant (CFA) injection in male Wistar rats.

Materials and Methods: In this Applicable-Fundamental study, we used male Wistar rats and adjuvant arthritis was created by once subcutaneous injection of CFA in the right hind paw of animals on day zero in experimental groups. Various doses of calcium were prepared and injected within 21 days of the study. Hyperalgesia and paw volume changes were assessed by radiant heat and plethysmometer over several days, respectively. Serum levels of TNF-α were checked by ELISA standard kit for each rat during various phases and were measured according to the kit.

Results: The results indicated dose related effects of long term calcium administration on edema, hyperalgesia and serum TNF-α level reduction. Daily treatment with effective dose of calcium (5 mg/kg) in AA+ Ca group significantly decreased paw edema, hyperalgesia and serum TNF-α level in comparison to AA and AA+ Vehicle groups on days 7, 14, and 21 of study.

Conclusion: Findings of this study showed that long term administration of calcium in the proper dosage can act as an anti-inflammatory agent and pain modulator during adjuvant-induced arthritis and a part of those effects may conducted by decreasing of serum TNF-α level.

Keywords: Calcium, TNF-α, Edema, Complete Freund's adjuvant, Hyperalgesia, Inflammation

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by swelling, tenderness and destruction of synovial joints and leading to severe disability (1). Inflammation is the primary and essentially a salutary response that normally resolves with the restoration of normal tissue structure and function, however when inflammation persists (chronic inflammation) it can cause tissue damage and loss of function. It results in rapid elevation of the secretion of inflammatory mediators, chemokine and cytokines, such as interleukins 1 and 6 (IL-1 and IL-6) and tumor necrosis factors α (TNFα). Pain and disability are the principal clinical features of inflammation. This situation is associated with sensitization of specialized sensory neurons that comprise the nociceptive (pain) pathway (2). TNFα is a key cytokine involved in the pathogenesis of RA, resulting in a chronic inflammatory state in which the synovial membrane is the primary site of attack. Therapies directed against tumor necrosis factor (TNF) are effective for treatment of rheumatoid arthritis and reduce pain scores in this condition (3). Therapeutic approaches used in treatment of RA symptoms are traditional non-selective no steroidal anti-inflammatory drugs (NSAIDs), disease-modifying anti-rheumatic drugs (DMARDs), glucocorticoids and biologics, which have partial clinical benefit and are associated with significant toxicity (4). Due to the side-effects and high cost of these immune modulatory agents, patients with RA often seek alternative methods for symptomatic relief and are among the highest users of such methods. Nutritional supplements such as vitamins with fewer side effects have been proven for significantly reducing inflammation. Use of vitamin compounds and supplements as analgesics for pain treatment which associated with deficiency of these components are common. Recently they have been considered more than before because of their effectiveness and having no side effect (Unlike chemical drugs) and also be easy to access (5).

Calcium ion, as a usual supplement, plays an important role in release of inflammatory mediators or activating them and contributes to their effects. Also reported that, calcium activates phospholipase-A2 and ultimately increase the metabolism of arachidonic acid products (6), activate protein kinase-C, production of NO (7), and Releasing interleukins (8). It is known that releasing of these mediators, triggers exocytosis movement of inflammatory cells, neutrophils and macrophage chemicals secretes (9), increase level of LTB4, lymphocyte (10) and adhesion of the white blood cell to vascular endothelial cells and their migration to the site of inflammation (11). Therefore, the present study was carried out to evaluate the anti-hyperalgesic and anti-inflammatory effects of calcium long term treatment in adjuvant-induced arthritis with emphasis on pro-inflammatory cytokine, serum TNFα level.

Methods

Animals

The study protocol was approved by the local ethics committee for the use of animals in research, and we followed the guidelines of ethical standards for investigation of experimental pain in animals (2). Adult male Wistar rats (180–220 g) were used for all animal experiments. The photoperiods were adjusted to 12 hours (h) light and 12 h darkness daily, and the environmental temperature was constantly maintained at 25±2°C and humidity 55%. The rats were kept in cages housing 4-6 animals and were given ad libitum access to food and water. The study was carried out after a period of 21 days of acclimatization.

Four groups of male Wistar rats (n=6) were used in this study. Animals were fasted overnight with free access to water before the experiment. There after group I (control group) received 0.1 ml Oil emulsion (as CFA solvent), group II (AA group) received 0.1ml CFA and groups III (AA+ vehicle group) received double distill water (calcium solvent) via intraperitoneal (IP) injection with the same volume injected in therapy group for 21 days after CFA injection, groups IV (AA+ Ca group). This group also had three sub-groups, which after CFA injection they have received calcium in doses of 2/5, 5 and 10 mg/kg (IP). The volume of all IP treatments was 1ml/rat on each day.

Induction of adjuvant arthritis (AA)

AA was induced at day 0 as expressed in our previous studies. Briefly, a single subcutaneous injection (100µl) of heat-killed mycobacterium
tuberculosis suspended in sterile mineral oil (10 mg/ml), CFA (Sigma, St Louis, MO, USA), into the left hind paw was performed. Left hind paws of control rats were injected with sterile mineral oil only (100 µl). First day after CFA injection unilateral inflammation was established in injected hind paw then first week was considered as inflammatory phase. The third week after intervention was arthritic phase (12).

Paw volume assessment

Paw volume variations were assessed by plethysmometer (Ugo Basile; model7141; Comerio VA, Italy) during different stages of study (days 0, 7, 14 and 21) as it was mentioned previously in our lab (12, 13). In brief, rats’ hind paw was submerged to the tibiotarsal joint into an electrolyte-filled Perspex cell of the plethysmometer after taken out of their cages. The volume of liquid displacement, which is equal to the paw volume, was indicated on a digital display. Volume measurement was done twice for each paw, and the average calculated. The amount of paw edema was calculated by measuring the difference in volume between baseline and various times.

It should be noted that according to previous studies, the first week of illness is considered inflammatory phase (acute arthritis), and the second and third week are known as chronic arthritis (14).

Behavioral test

For behavioral test specially thermal hyperalgesia (Paw Withdrawal Latency (PWL)) from noxious heat) was assessed by using the plantar test (Ugo Basile, Verse, Italy) in different experimental and control rats on days 0, 3, 7, 14, and 21 as previously described (12). Briefly, rats were habituated to the apparatus that consisted of three individual Perspex boxes on a glass table. A mobile radiant heat source was located under the table and focused onto the desired paw. PWLs were recorded three times for both hind paws of each animal, the mean of which represented baseline for left (CFA-injected paw) and right hind paws. A mobile radiator heat source was located under the table and focused onto the desired paw. PWLs were recorded three times for both hind paws of each animal, the mean of which represented baseline for left (CFA-injected paw) and right hind paws. Then, the mean value for the affected paw (CFA-injected paw) was subtracted from that for the other paw and the result considered as the hyperalgesia sign in the injured paw. To prevent tissue damage of the plantar zone, a 20-s cut-off was observed.

Measuring blood levels of TNF-α

The serum TNF-α levels of rats were assayed by a rat standard ELISA kits (Koma Biotech, Seoul, Korea) on day 0 (before CFA injection) and at different phases of study (days 7, 14, and 21) according to the manufacturer’s protocol. Rats were anesthetized with isoflurane and their blood samples were taken from the vessel retro-orbital corners of their eyes with heparin capillary tube. These samples were centrifuged and the serum was stored at -80°C. TNF-α was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (12, 13). It's interaction with rat serum and TNF-α was 100% (15).

Statistical methods

Data are presented as mean±standard error of mean (SEM). Statistical analysis of thermal hyperalgesia, paw edema and serum TNF-α level were analyses by one-way analysis of variance (SPSS software; version 18). Repeated measurement and post HOC analysis was performed with Tukey’s multiple comparison tests where appropriate. For comparing variations between groups on same days an unpaired t-test were done. Statistical significance was accepted at p<0.05.

Results

Paw volume changes during different stages of study

CFA injection in the right hind paw of rats at day zero induced inflammation and edema in the same paw. This increase continued until day 21 after the injection. Paw volume on days 7, 14, and 21 after CFA injection significantly increased compared to baseline on day zero (p<0.001). Also our results indicated that, volume of inflamed-paw at days 14 and 21 was significantly increased compared to day 7 (p<0.001).

Calcium administration significantly reduced the increased volume of rats’ paw within 21 days after CFA injection. The results showed; calcium long term treatment at dose of 5 mg/ kg significantly reduced the volume of paw at days 7, 14, and 21 after CFA injection (p<0.01 for day 7 and p<0.001 for days 14 and 21). Our study also point out that, continuous daily administration of 5 mg /kg dose of calcium significantly reduced paw volume on days 14 and 21.
There was no statistically significant difference between 5 and 10 mg/kg doses of calcium in edema reduction during our study in AA+Ca group, therefore 5 mg/kg was considered as the effective dose. Also results showed that, 2.5 mg/kg had no effect on paw volume variation during 21 days injection in our experimental groups. It should be noted that injection of calcium solvent in AA+Vehicle group demonstrated no difference in paw volume variations when compared with AA group in different days of the study (Figure 1A and B).

**Fig. 1.** Effects of administration of different doses of calcium on reducing paw volume during 21 days inflammation induced by CFA. (A): 5 mg/kg doses of calcium significantly reduced paw edema in AA+Ca compared to AA groups. (B): Paw volume showed significant decrease on 14 and 21 compared to day 7 in AA+Ca with 5 mg/kg doses.

**Fig. 2.** Effects of treatment by different doses of calcium long term administration on thermal hyperalgesia variations in AA rats during 21 days of study. (A): 5 mg/kg Calcium long term treatment significantly reduced hyperalgesia in AA+Ca compared to AA groups. (B): Hyperalgesia indicated significant decrease on 14 and 21 compared to Day 7 in AA+Ca group when received effective dose of calcium (5 mg/kg).

**Thermal hyperalgesia variations during different stages of study**

Arthritic rats by injection of CFA showed thermal hyperalgesia changes in the injected paw during different stages of inflammation. Hyperalgesia was significantly increased on day 7 after CFA injection compared to day zero and the control group (p<0.001 for both).

Results showed that when paw inflammation continued hyperalgesia significantly decreased at days 14 and 21 compared to day 7 of the study in AA group (p<0.001 for both). In addition, hyperalgesia...
showed a significant decrease on day 21 compared to day 14 in AA rats (p<0.001).

Daily calcium administration reduces hyperalgesia in AA+Ca group. It was showed significant reduction in hyperalgesia after taking 5 mg/kg calcium supplementation in AA+Ca compared with AA groups on days 7, 14 of the study (p<0.001 for both days). There was no statistically significant difference between 5 and 10 mg/kg calcium on reducing hyperalgesia in AA+Ca rats, therefore 5 mg/kg was considered as the effective dose. Also results showed that, 2.5 mg/kg had no effect on hyperalgesia variations during 21 days treatment in AA+Ca rats. It should be noted that injection of calcium solvent in AA + vehicle group indicated no significant effect on hyperalgesia variations when compared with AA group during different stages of study (Figure 2A and B).

**Serum TNF-α levels changes during different stages of study**

CFA injection induced a significant increase in serum levels of TNF-α and this continued up to the day 21 after the injection. Plantar CFA injection significantly increased serum levels of TNF-α level on days 7, 14 and 21 after injection compared with day zero (p<0.001). TNF-α levels on days 14 and 21 of the study was significantly higher than the day zero but showed a significant decrease over day 7 (for both p<0.001).

Daily calcium administration reduces serum levels of TNF-α. The results showed a significant reduction in serum levels of TNF-α after taking 5 mg/kg calcium supplementation in AA+Ca compared with AA group on days 7, 14 and 21 of the study (p<0.001 for all three days).

Moreover, continues treatment by 5 mg/kg calcium in AA+Ca group significantly reduced TNF-α level on days 14 and 21 when compared with day 7 (p<0.01). There was no statistically significant difference between 5 and 10 mg/kg doses of calcium in TNF-α level reduction then, 5mg/kg was considered as the effective dose. Also our results demonstrated that 2.5 mg/kg dose of calcium had no significant effect on serum TNF-α level change during 21 days of administration in AA+Ca group. Furthermore, administration of calcium solvent in AA + vehicle group had no significant effect on serum TNF-α level fluctuations when compared to AA group during different days of study (Figure 3A and B).

**Discussion**

This study along with other studies showed that inflammation model induced by CFA is two-phase model. In the first phase, hyperalgesia and paw edema were increased by the presence and significant increase in the serum level of pro-inflammatory cytokine, TNF-α. Although the molecular mechanisms of TNF-α is not exactly clear, but the use
of antagonists, it is now a widely used method in clinical treatment and may have a role in alleviating the symptoms of the inflammation (12, 19). It is clear that the first phase inflammatory response during CFA-induced local response is along with release of mediators such as cytokines, particularly TNF-α and IL-1β (12, 16, 17). Our results also indicated that hyperalgesia significantly decreased on second phase of CFA inflammation (next two weeks) which was along with significant reduction of TNF-α level. Previous studies indicated that activating of pain modulatory systems such as anti-inflammatory cytokines secretion increase and opioid system activation may cause pain modulation during inflammation (2, 3).

Moreover; long term administration of calcium in CFA-inflamed rats significantly caused dose-dependent reduction (5 mg/kg) in hyperalgesia and paw edema throughout the observation period. Our findings also revealed that serum TNF-α level which plays important role in inflammatory symptoms induction and progression (2, 13, 24) decreased due to effective dose administration of calcium and it was along with the paw volume and hyperalgesia variations. One previous study showed that calcium pentosan polysulfate (CaPPS), a polysulfated polysaccharide, modulated TNF-α and IL-6 activity in fluids of the rat air-pouch model without affecting total pouch volume (14), but according to our previous studies reduction of serum levels of pro-inflammatory cytokines is one of the important targets for treatment of inflammatory symptoms (2, 13). Studies suggested that CaPPS could represent a new class of disease modifying anti-arthritic agents, but neither the anti-arthritic activity of the compound nor its effects on inflammatory mediators had been adequately investigated.

Furthermore; transient changes in cytoplasmic calcium ion concentration represent a key step for neurotransmitter release and the modulation of cell membrane excitability. Evidence has accumulated for the involvement of calcium ions also in nociception and anti-nociception, including the analgesic effects produced by opioids (18). Our different studies also indicated during chronic inflammation spinal mu opioid receptor expression significantly increased (12). Intra-cerebra ventricular calcium administration caused naloxone sensitive anti-nociception effects in mouse (24). Although some other studies stated that combination of opioids with drugs able to inhibit calcium ion functions in neurons has been pointed out as a useful alternative for safer clinical pain management (25) but others insist that calcium treatment via inhibition of voltage-gated Na+ channels can reduce the pain (24).

**Conclusion**

Then according to our study it can concluded that, long term administration of effective dose of calcium can eliminate hyperalgesia and edema as inflammatory symptoms which it seems that, it can be due to serum TNF-α level reduction during calcium treatment. The molecular and cellular aspects of these variations need to more investigation.

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**Conflicts of Interest**

The authors declare that there are no conflicts of interest.

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