Original Article

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

Setareh Khashaee[1], Homa Manaheji[1], Nazanin Nikzad[2], Jalal Zaringhalam[1*]

Abstract

Background: Inflammation and edema Symptoms are physiological responses to triggers 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 is a cytokine involved in the pathogenesis of rheumatoid arthritis. In this study, we attempted to investigate the role of the prescription of calcium to reduce inflammatory edema and serum TNF-α levels during different stages of arthritic inflammation induced by Complete Freund Adjuvant (CFA) injection in males Wistar rats.

Materials and Methods: In this Applicable-Fundamental study, we used male Wistar rats and adjuvant arthritis was created by once the 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. The serum levels of TNF-α were studied by ELISA standard kit of rats 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 an effective dose of calcium (5 mg/kg) in the 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 the study.

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

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

Please cite this article as: Khashaee S, Manaheji H, Nikzad N, Zaringhalam J. Anti-hyperalgesic and Anti-Inflammatory Effects of Long Term Calcium Administration during Adjuvant-Induced Arthritis in Rats. J Cell Mol Anesth. 2016;1(1):12-8.

1. Neurophysiology Research Center, Physiology Department, Shahid Beheshti University of Medical Sciences, Tehran, Iran
2. International Branch of Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding Author:
Jalal Zaringhalam, MD; Neuro-physiology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. Email: jzaringhalam@yahoo.com
Received: November 20, 2015
Accepted: November 12, 2015

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 the 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 the attack. Therapies directed against tumor necrosis factor (TNF) are effective for the treatment of rheumatoid arthritis and reduce pain scores in this condition (3). Therapeutic approaches used in the 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 immunomodulatory 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 to significantly reducing inflammation. The use of vitamin compounds and supplements as analgesics for pain treatment which are associated with deficiency of these components are common. Recently they have been considered more than before because of their effectiveness and have no side effects (unlike chemical drugs) and also be easy to access (5).

Calcium ion, as a usual supplement, plays an important role in the release of inflammatory mediators or activating them and contributes to their effects. Also reported that calcium activates phospholipase-A2 and ultimately increases the metabolism of arachidonic acid products (6), activates 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 the 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 fasted overnight with free access to water before the experiment. Thereafter 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 a 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. The left hind paws of control rats were injected with sterile mineral oil only (100µl). The first day after CFA injection unilateral inflammation was established in

injected hind paw then the first week was considered as an inflammatory phase. The third week after the intervention was the 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 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 was 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 the inflammatory phase (acute arthritis), and the second and third weeks are known as chronic arthritis (14).

Behavioral test: For behavioral test especially 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. Then, the mean value for the affected paw (CFA-injected paw) was subtracted from that for the other paw and the result was 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 the 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 a 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). Kit's interaction with rat serum and TNF-α was 100% (15).

Statistical methods: Data are presented as mean±standard error of the mean (SEM). Statistical analysis of thermal hyperalgesia, paw edema, and serum TNF-α level was analyzed by one-way analysis of variance (SPSS software; version 18). Repeated measurement and post hoc analysis were performed with Tukey's multiple comparison tests where appropriate. For comparing variations between groups on the same days an unpaired *t*-test was 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 the volume of inflamed paw at days 14 and 21 were significantly increased compared to day 7 ($p<0.001$).

Calcium administration significantly reduced the increased volume of rats' paws within 21 days after CFA injection. The results showed; calcium long-term treatment at a 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 points out that, continuous daily administration of 5 mg /kg dose of calcium

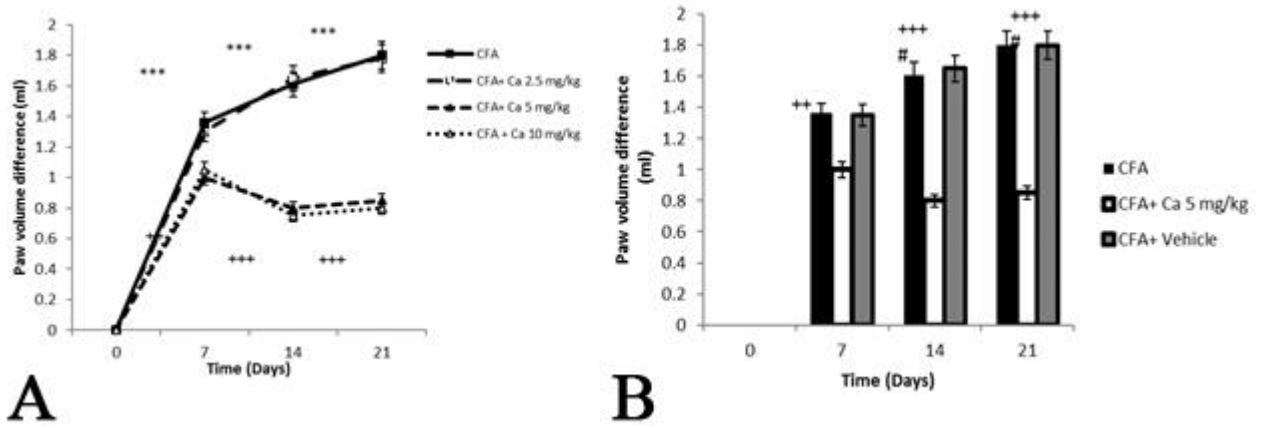

Fig. 1. Effects of administration of different doses of calcium on reducing paw volume during 21 days inflammation induced by CFA. (A): 5 mg/ kg doses of calcium significantly reduced paw edema in AA+Ca compared to AA groups. (B): Paw volume showed significant decrease on 14 and 21 compared to day 7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

significantly reduced paw volume on days 14 and 21 in comparison to day 7 in the AA+Ca group ($p<0.05$). There was no statistically significant difference between 5 and 10 mg/kg doses of calcium in edema reduction during our study in the AA+Ca group, therefore 5 mg /kg was considered as the effective dose. Also, results showed that 2.5 mg/kg did not affect 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 on different days of the study (Figure 1A and B).

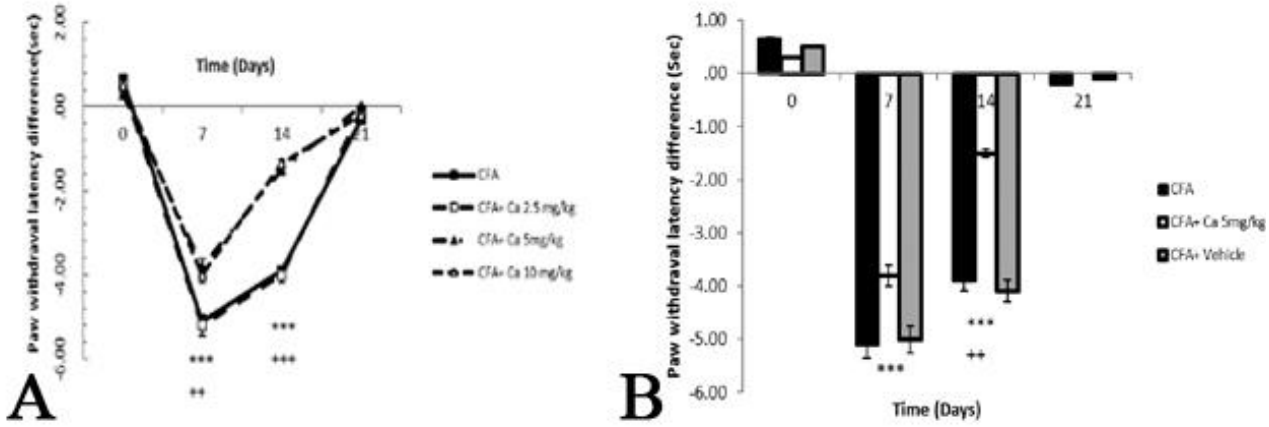

Fig. 2. Effects of treatment by different doses of calcium long term administration on thermal hyperalgesia variations in AA rats during 21 days of study. (A): 5 mg/kg Calcium long term treatment significantly reduced hyperalgesia in AA+Ca compared to AA groups. (B): Hyperalgesia indicated significant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

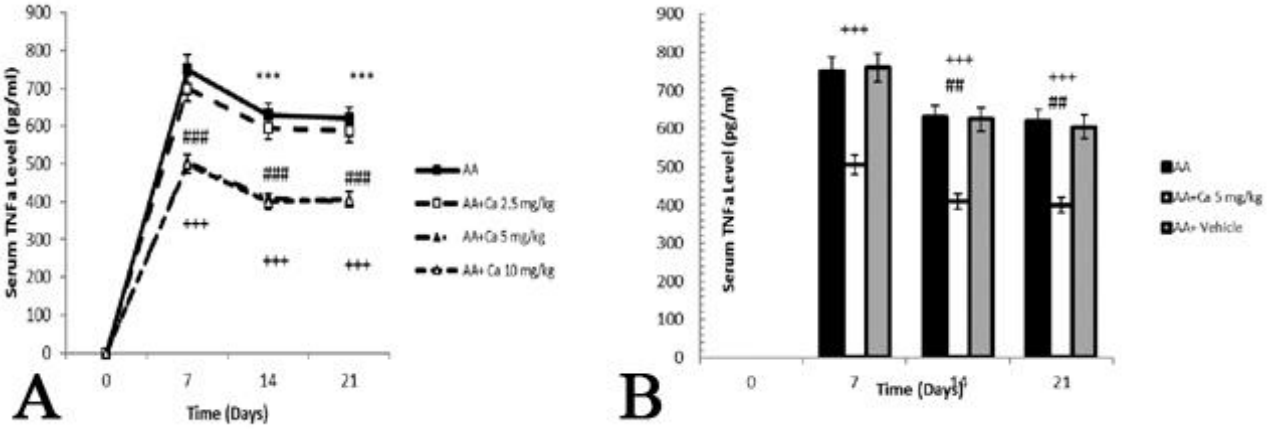

Fig. 3. Effects of administration of different doses of calcium on serum TNF-α level during different stages of study. (A): Administration of 5 mg/ kg Calcium significantly reduced serum TNF-α levels in AA+Ca rats. (B): Serum TNF-α level indicated significant reduction on 14 and 21 compared to day 7 in AA+Ca group when received effective dose of calcium.

Results showed that when paw inflammation continued hyperalgesia significantly decreased at days 14 and 21 compared to day 7 of the study in the 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 the AA+Ca group. It was showed a 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 did not affect hyperalgesia variations during 21 days treatment in AA+Ca rats. It should be noted that injection of calcium solvent in the AA + vehicle group indicated no significant effect on hyperalgesia variations when compared with the 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 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 were significantly higher than 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 the 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 the AA+Ca group significantly reduced TNF-α levels 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 the AA+Ca group. Furthermore, administration of calcium solvent in the AA + vehicle group had no significant effect on serum TNF-α level fluctuations when compared to the AA group during different days of study (Figure 3A and B).

Discussion

This study along with other studies showed that the

inflammation model induced by CFA is a two-phase model. In the first phase, hyperalgesia and paw edema were increased by the presence and significant increase in the serum level of the pro-inflammatory cytokine, TNF-α. Although the molecular mechanisms of TNF-α are not exactly clear, the use of antagonists 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 the release of mediators such as cytokines, particularly TNF-α and IL-1ß (12, 16, 17). Our results also indicated that hyperalgesia significantly decreased in the second phase of CFA inflammation (next two weeks) which was along with a 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 the 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 the 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 mice (24). Although some other studies stated that the 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 be concluded that long-term administration of the effective dose of calcium can eliminate hyperalgesia and edema as inflammatory symptoms which it seems that, can be due to serum TNF-α level reduction during calcium treatment. The molecular and cellular aspects of these variations need more investigation.

Acknowledgment

This study was done as the MD degree thesis in Neurophysiology Research Center of Shahid Beheshti University of Medical Sciences.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

References

1. Astin JA, Beckner W, Soeken K, Hochberg MC, Berman B. Psychological interventions for rheumatoid arthritis: a meta-analysis of randomized controlled trials. Arthritis Rheum. 2002;47: 291–302.
2. Akhtari Z, Zaringhalam J, Eidi A, Manaheji H, Tekieh E. Bidirectional effects of serum TNF alpha level and spinal p38mapk

phosphorylation on hyperalgesia variation during CFA-induced arthritis. EXCLI J 2012; 11:373-385.
3. Tekieh E , Zaringhalam J, Akhtari Z. Relationship between cytokines and spinal mu opioid receptor expression during adjuvant-induced arthritis in rats. ARRB. 2014;4(11):1854-66.
4. Goldbatt F, Isenberg DA. New therapies of rheumatoid arthritis. Clin Exp Immunol. 2005;140:195–204.
5. Mader R, Deutsch H, Siebert GK, Gerbershagen HU, Gruhn E, Behl M, et al. Vitamin status of inpatients with chronic cephalgia and dysfunction pain syndrome and effects of a vitamin supplementation. Int J Vitam Nutr Res. 1988;58(4):436 .
6. Rodler S, Roth M, Nauck M, Tamm M, Block LH. $Ca^{(2+)}$-channel blockers modulate the expression of inter lukin-6 and inter lukin 8 genes in human vascular smooth muscle cells. J Mol Cell Cardiol. 1995;27(10):2295-302
7. Warren JB. Vascular control of inflammatory edema. Clin Sci (Lond). 1993;84(6):581-4.
8. Ogle CW, Cho CH, Tong MC, Koo MW. The influence of verapamil on the gastric effects of stress in rats. Eur J Pharmacol. 1985;112(3):399-404 .
9. Broecke RT, Leusink-Muis T, Hiberdink R, et al. Specific modulation of calmdulin activity induces a dramatic production of superoxide by alveolar macrophages. Lab Invest 2004; 84(1): 29-40 .
10. Green WC, Parker CM, Parker CW. Calcium lymphocyte activation. Cell Immunol. 1976;25(1):74-89.
11. Dugal D, Scott T. Acute joint inflammation mechanisms and mediators. Gen Pharmacol. 1994;25(7):1285-96 .
12. Zaringhalam J, Tekieh E, Manaheji H, Akhtari Z. Cellular events during arthritis-induced hyperalgesia are mediated by Interleukin-6 and p38 MAPK and their effects on the expression of spinal mu-opioid receptors. Rheumato Int. 2013;33(9);2291-9.
13. Mirzaei V, Manaheji H, Maghsoudi N, Zaringhalam J. Comparison of changes in mRNA expression of spinal glutamate transporters following induction of two neuropathic pain models. Spinal Cord. 2010;48(11):791-7.
14. Smith M, Numata Y, Ghosh P. Effects of calcium pentosan polysulfate on joint inflammation and pouch fluid levels of leukocytes, nitric oxide, and Interleukin-6 in a rat model of arthritis. Curr Ther Res. 1999;60(11):561-76.
15. Chover-Gonzalez AJ, Harbuz MS, Tejedor-Real P, Gibert-Rahola J, Larsen PJ, Jessop DS. Effects of stress on susceptibility and severity of inflammation in adjuvant-induced arthritisa. Ann NY Acad Sci. 1999;876(1):276-86 .
16. Hall JE. Pocket Companion to Guyton and Hall Textbook of Medical Physiology, 11nd ed. Elsevier: Saunders; 2006. p.67-72.
17. Park JY, Pillinger MH. Interleukin-6 in the pathogenesis of rheumatoid arthritis. Bulletin of the NYU hospital for joint Diseases. 2007;65(1):4-10.
18. Prado W. A. Involvement of calcium in pain and antinociception. Braz J Med Biol Res. 2001;34:449-61.
19. Eskandari F, Webster JI, Sternberg EM. Neural immune pathways and their connection to inflammatory diseases. Arthritis Res Ther. 2003;5(6):251-65.
20. Raghavendra V, Tanga FY, DeLeo JA. Complete freunds adjuvant induced peripheral inflammation evokes glial activation and proinflammatory cytokine expression in the CNS. Eur J Neurosci. 2004;20(2):467-73.
21. Taniguchi N, Kanai S, Kawamoto M, Endo H, Higashino H. Study on application of static magnetic field for adjuvant arthritis rats. Evid Based Complement Alternat Med. 2004;1(2):187-91 .
22. Anonymous. Guidelines for the Testing of Chemicals. Paris: Organ Econo Coop Develo (OECD). 2008;407.
23. Vinger R, Truax JF, Selph JL, Johnston PR, Venable AL, McKenzie KK. Pathway to carrageenan-induced inflammation in the hind limb of the rat. Fed Proc. 1987;46:118–26.
24. Diaz A, Dickenson AH. Blockade of spinal N- and P-type, but not L-type, calcium channels inhibits the excitability of rat dorsal horn neurons produced by subcutaneous formalin inflammation. Pain. 1997;69:93-100.
25. Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. Front Biosci. 1997;2(1):12-26.