



Research paper

Intraarticular slow-release triamcinolone acetate reduces allodynia in an experimental mouse knee osteoarthritis model



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ABSTRACT

Intraarticular steroid injection has been the mainstay of short-term treatment of knee osteoarthritis (OA) pain. However, the duration of therapeutic effect from a single injection is not as long as desired. In this study we use a viscous formulation of triamcinolone acetate (TCA) in hyaluronic acid to prolong the anti-allodynia effect of that steroid. OA was induced in mice by a partial medial meniscectomy. Over time the animals developed a mechanical allodynia in the injected leg. Mice were then given a single intraarticular injection of TCA in a short-acting DMSO formulation, or a standard commercial suspension, or the drug formulated in 5% hyaluronic acid for slow-release. Control injections in OA mice were PBS or 5% hyaluronic acid vehicle. Mechanical allodynia was then monitored over the therapeutic period. Organotypic spinal cord slices and DRG culture were performed to assess whether TCA attenuates expressions of pain mediators induced by interleukin 1 β . TCA 40 μ g in a fast-releasing DMSO formulation produced relief from mechanical allodynia for a few days compared to PBS control injections ($P = 0.007$). Similarly, the commercial suspension of TCA 40 μ g also produced relief from mechanical allodynia for a few days compared to PBS control injections ($P = 0.001$). However, TCA 100 μ g in 5% hyaluronic acid produced relief from mechanical allodynia for at least 28 days compared to PBS control or 5% hyaluronic acid vehicle injections ($P = 0.0005$). Furthermore, TCA significantly suppressed expression of pain mediators induced by interleukin 1 β in spinal cord and DRG organotypic culture. Intraarticular TCA in a sustained release formulation of viscous 5% hyaluronic acid will produce a long-term attenuation of mechanical allodynia in the OA knees of mice.

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Abbreviations: OA, osteoarthritis; TCA, triamcinolone acetate; TKR, knee replacement surgery; IA, intraarticular; DRG, dorsal root ganglion; NGF, nerve growth factor; CGRP, calcitonin gene-related peptide; TNF α , tumor necrosis factor- α ; CCL2, chemokine (C-C motif) ligand 2; HA, hyaluronic acid; DMM, destabilization of the medial meniscus; HBSS, Hanks Balanced Salt Solution; IL-1 β , interleukin 1 β ; qPCR, quantitative PCR.

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1. Introduction

Joint degeneration caused by osteoarthritis (OA) leads to chronic knee joint pain that affects > 100 million individuals all over the world. Pain accounts for most primary care visits for OA, and is one of the key reasons why people with OA choose to have knee replacement surgery (TKR) (Altman et al., 1995). In the United States alone, there are almost one million TKR each year to treat OA and OA pain, with the projected number of TKR for 2030 expected to reach 3.5 million (Kurtz et al., 2007). Prior to TKR, knee pain patients will have used noninvasive non-surgical alternatives such as systemic NSAIDs and analgesics for years. For more severe pain, intraarticular (IA) steroid injections have

been the mainstay of short-term treatment of knee OA pain (Dieppe et al., 1980; Valtonen, 1981; Raynauld et al., 2003; Arroll and Goodyear-Smith, 2004). However, the duration of therapeutic effect from a single injection is not as long as needed for a chronic pain condition, (Dieppe et al., 1980; Arroll and Goodyear-Smith, 2004) and so patients may drop the therapy due to injection frequency. To extend the period of pain relief, there are ongoing clinical trials on the efficacy of new IA triamcinolone acetate (TCA) formulations (Petrella et al., 2015; Bodick et al., 2015).

Physiological mechanisms of pain operate at the local joint level, the dorsal root ganglion (DRG) level, spinal level and higher brain processing centers. Several nociceptive molecules are recruited into the OA joint, including nerve growth factor (NGF), calcitonin gene-related peptide (CGRP), tumor necrosis factor- α (TNF α) and chemokine (C-C motif) ligand 2 (CCL2) and can cause activation of peripheral nociceptors (Kc et al., 2016; Dong et al., 2015; Dawes et al., 2013). During chronic OA, the nociceptive system can become sensitized, leading to a heightened sensitivity. Steroids are proven, both scientifically and clinically, to be effective at reducing pain in knee OA. It has been suggested that TCA exert a potent anti-inflammatory effect on joints by inhibiting inflammatory cytokines and thus reduces pain (Evans et al., 2014). However, the mechanism by which TCA alleviates knee OA pain is still not clearly defined.

In this study we use a viscous formulation of triamcinolone acetate (TCA) in hyaluronic acid (HA) to prolong the anti-allodynic effect of that steroid. While the use of HA alone as an IA-injected viscosupplement to reduce knee pain is a controversial issue (Jevsevar et al., 2015), we are using HA to entrap the TCA molecule and thus slow its release. Furthermore, to delineate analgesic mechanism of TCA, we also performed organotypic culture of spinal cord slices and DRG.

2. Methods

2.1. OA model

The study was approved by the IACUC of Rush University Medical Center. OA was induced by partial medial meniscectomy in C57/BL6 mice (20 g) (Knights et al., 2012). Briefly, mice were anesthetized with 1.5% isoflurane (Abbott Laboratories) in oxygen and the left knee was shaved and prepared for aseptic surgery. A medial para-patellar arthrotomy exposed the anterior medial meniscotibial ligament, which was elevated with a microprobe and severed using curved dissecting forceps. The medial meniscus was freed from its attachments to the margin of the tibial plateau and approximately one third of the medial meniscus was removed (approximately 1 mm of tissue). The patella was repositioned, and the skin incision closed with 5-0 polypropylene sutures. This surgically-induced OA model is slightly modified from OA model of destabilization of the medial meniscus, referred to as DMM. In prior studies this medial meniscus transection model has been shown to produce cartilage degeneration and mechanical allodynia by 4 weeks post-surgery (Knights et al., 2012).

2.2. Evaluation of mechanical allodynia

Force withdrawal thresholds, in response to mechanical stimuli, were assessed using von Frey filaments applied to the plantar hindpaw, using an iterative up-down method (Chaplan et al., 1994). Animals were tested prior to and at 3 day intervals after OA induction surgery to determine when mechanical allodynia had developed (force withdrawal threshold < 2 g) (Kc et al., 2016).

2.3. Drug treatment

Once mechanical allodynia developed (at least 6 weeks after surgery), mice were briefly anesthetized with 1.5% isoflurane and given a

single percutaneous intraarticular injection, through a 30-gauge needle, of drug or control solution. Withdrawal thresholds were assessed using von Frey filaments until the therapeutic effect had worn off in the drug group (threshold back below 2 g).

Experiment 1 (fast-release; 2 groups): Active drug was TCA powder (Ark Pharm Inc., Libertyville, IL), dissolved in DMSO at 8 mg/mL so a 5 μ L injection was 40 μ g total dose. Control injection was PBS.

Experiment 2 (slower-release; 2 groups): Active drug was a commercial TCA suspension (Kenalog-10; Bristol-Myers Squibb) at 10 mg/mL so a 4 μ L injection was 40 μ g total dose. Control injection was PBS.

Experiment 3 (sustained-release; 3 groups): Active drug was 50 mg TCA powder hand-mixed into 2.5 mL (2.5 g) of 5% HA solution producing a clear 20 mg/mL TCA mixture; so a 5 μ L injection was 100 μ g total dose. To produce the 5% HA vehicle, HA powder (sodium hyaluronate; 1000 kDa; Novozymes, Cincinnati, OH) was dissolved in PBS to produce a very viscous (200,000 cP), but easy to inject clear solution. The 5% HA solution was autoclaved for sterility. One control injection was PBS, and the other 5% HA alone.

The 40 μ g TCA dose for Kenalog-10 was based on the manufacturer's recommendation of 2.5 mg to 5 mg (assume 4 mg average) for the initial dose for an IA injection. Assuming a normal mouse knee joint synovial volume of 4–5 μ L (based on our preliminary dye-injection experiments) and a normal human knee joint synovial volume of 0.5 mL (Courtney and Doherty, 2005), 100 \times larger, the equivalent mouse dose was estimated as 4 mg/100 = 40 μ g (Experiment 2). We used the same 40 μ g dose for the TCA-DMSO injections (Experiment 1). For the TCA-HA injections (Experiment 3) we choose a larger dose, 100 μ g, since with the expected release to be spread out over weeks we wanted our daily dose to be above a threshold, so we would have an effect within the first few days after injection (as with TCA-DMSO or TCA-Kenalog).

2.4. Organ culture experiments

Organotypic slice cultures of spinal cord and dorsal root ganglia (DRG) were prepared from adult mice (20 g) as previously described (Aoki et al., 2007; Marsh et al., 2000). After decapitation, the spinal cord and bilateral lumbar DRGs from L1 to L6 were aseptically removed and placed into ice-cold Hanks Balanced Salt Solution (HBSS) (Life Technologies, Carlsbad, CA). Lumbar spinal cords were transversely sliced into 1 mm thickness for spinal cord organ culture. The ganglia and spinal cord slices were transferred in serum free DMEM/F12 (Life Technologies, Carlsbad, CA) containing interleukin 1 β (IL-1 β) (10 ng/mL) (PeproTech, Rocky Hill, NJ), TCA in DMSO (1 mg/mL) or combination of IL-1 β and TCA and incubated for 24 h at 37 $^{\circ}$ C in a humidified 95% air/5% CO $_2$ incubator. At the end of the culture period the ganglia and spinal cord slices were harvested, quickly frozen on dry ice, and stored at -80° C until they were assayed.

2.5. Reverse transcription and quantitative polymerase chain reaction

Total RNA was isolated using the Trizol reagent (Life Technologies, Carlsbad, CA) following the instructions provided by the manufacturer. Reverse transcription (RT) was carried out with 1 μ g total RNA using the qScriptTM cDNA SuperMix (Quanta Biosciences Inc., Gaithersburg, MD) for first strand cDNA synthesis. For quantitative PCR (qPCR), cDNA was amplified using the MyiQ Real-Time PCR Detection System (Bio-Rad Hercules, CA). Relative mRNA expression was determined using the $\Delta\Delta C_T$ method, as detailed by the manufacturer (Bio-Rad Hercules, CA). GAPDH was used as an internal control. The values represent the mean of three separate experiments. The primer sequences will be provided upon request.

2.6. Statistics

Withdrawal thresholds (g) over time were compared between groups with repeated measures general linear model (SPSS software).

Group comparisons at individual time points were by *t*-test or ANOVA, with step-down Bonferroni correction for multiple comparisons. For qPCR results, statistical significance was determined by Student's *t*-test. *P* values lower than 0.05 were considered to be statistically significant.

3. Results

3.1. TCA-DMSO experiment

The von Frey force withdrawal thresholds are presented in Fig. 1. Before DMM surgery, thresholds were 5–6 g. After surgery, thresholds gradually decreased to <2 g (allodynia). Mice receiving an intraarticular injection of TCA in DMSO vehicle had higher withdrawal thresholds than PBS-injected mice over the day 1–7 post-injection period ($F = 10.3, P = 0.007$). On days, 1 and 3, these differences were also significant.

3.2. Commercial TCA suspension experiment

The von Frey force withdrawal thresholds are presented in Fig. 2. Mice receiving an intraarticular injection of commercial TCA (Kenalog-10) had higher withdrawal thresholds than PBS-injected mice over the day 1–11 post-injection period ($F = 22.6, P = 0.001$). On days, 3 and 5, these differences were also significant.

3.3. TCA-HA experiment

The von Frey force withdrawal thresholds are presented in Fig. 3. Mice receiving an intraarticular injection of TCA in 5% HA had higher withdrawal thresholds than PBS-injected mice or mice injected with 5% HA vehicle over the days 3–35 post-injection period ($F = 17.8, P = 0.0005$). On days 3, 7, 14, 21, and 28 these differences were also significant. The 5% HA alone did not increase force withdrawal thresholds compared to PBS.

3.4. Organ culture experiments

TCA exerts a potent anti-inflammatory effect on joints by inhibiting inflammatory cytokines and reduces OA associated pain (Evans et al., 2014), however, little is known about the mechanism of action of TCA in sensory neurons and reduction in OA associated pain. To delineate analgesic mechanisms of TCA, we performed organotypic slice cultures of spinal cord and DRG and assessed whether TCA can inhibit mediators

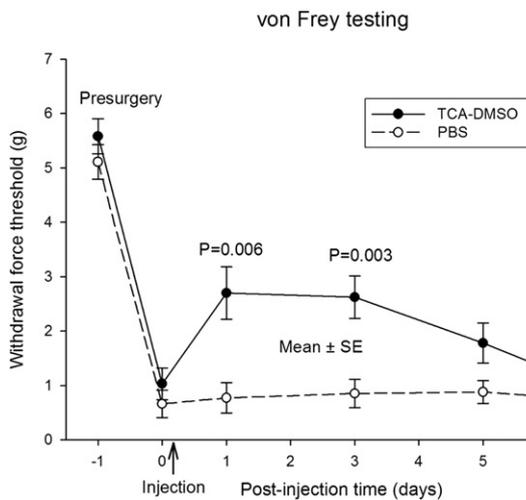


Fig. 1. von Frey force withdrawal thresholds in mice with knee OA. Time 0 represents the chronic OA allodynic condition. Animals received a single IA injection of 40 μ g TCA in DMSO or PBS alone. *N* = 8/group. *P* values are of the TCA-DMSO group vs. the PBS group.

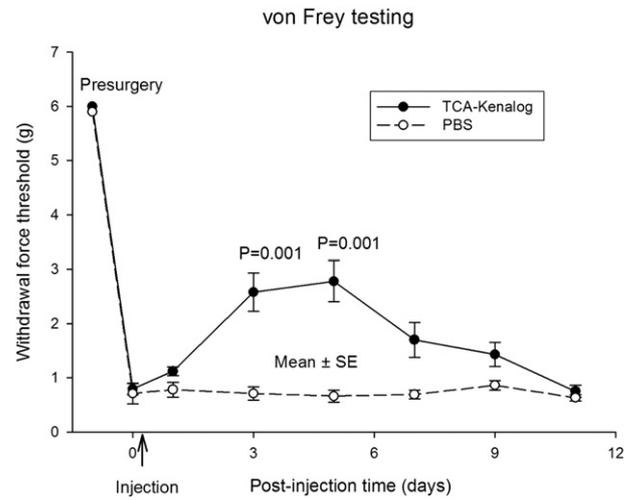


Fig. 2. von Frey force withdrawal thresholds in mice with knee OA. Time 0 represents the chronic OA allodynic condition. Animals received a single IA injection of a 40 μ g TCA suspension (Kenalog-10) or PBS alone. *N* = 8/group. *P* values are of the TCA-Kenalog group vs. the PBS group.

of chronic pain induced by pro-inflammatory cytokine IL-1 β . In chronic pain stage, pro-inflammatory cytokine such as IL-1 β leads to release and/or activation of nociceptive molecules like NGF, CGRP, and inflammatory cytokines thereby altering sensitivity of sensory neurons (Fehrenbacher et al., 2005; Oprea and Kress, 2000; Ren and Torres, 2009). We, therefore incubated spinal cord slices and DRGs in the presence of IL-1 β (10 ng/mL), TCA (1 mg/mL) or combination of IL-1 β and TCA for 24 h, and the mRNA levels of pain mediators – NGF, CGRP, TNF α and CCL2 – were measured by qPCR. Concentration of IL-1 β was chosen based on the previous studies (Fehrenbacher et al., 2005; Kawasaki et al., 2008), while concentration of TCA was selected to closely match clinically relevant concentration. Our data show that TCA significantly suppressed IL-1 β -induced mRNA expression levels of NGF, CGRP, TNF α and CCL2 in DRG (Fig. 4) and NGF, TNF α and CCL2 in spinal cord slices (Fig. 5) organotypic culture.

4. Discussion

HA is naturally found in knee joints: in the synovium, hyaline cartilage, and in the synovial fluid. Although HA solutions above 2% have

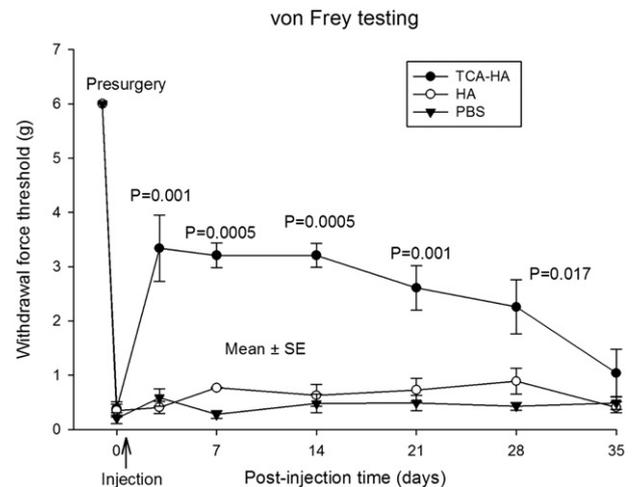


Fig. 3. von Frey force withdrawal thresholds in mice with knee OA. Time 0 represents the chronic OA allodynic condition. Animals received a single IA injection of 100 μ g TCA in 5% HA, 5% HA alone, or PBS alone. *N* = 8/group. *P* values are of the TCA-HA group vs. the other 2 groups.

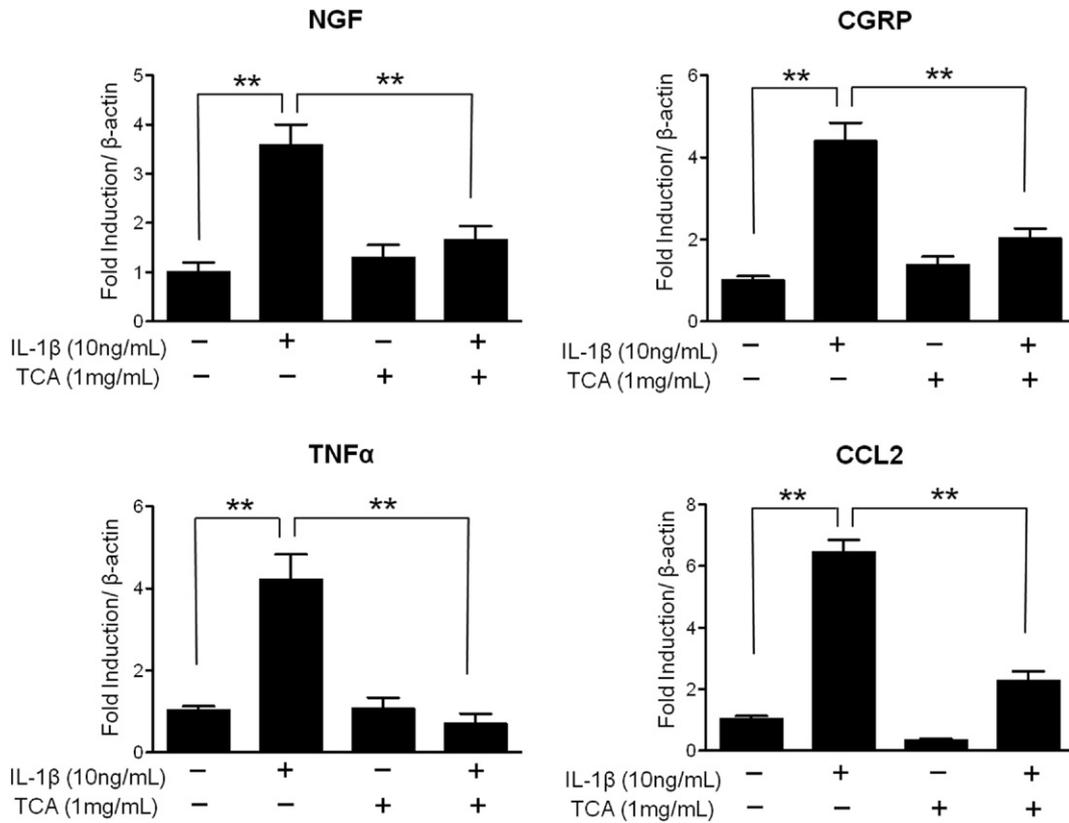


Fig. 4. qPCR analyses of NGF, CGRP, TNF α and CCL2 genes in organotypic DRG culture treated with IL-1 β (10 ng/mL), TCA (1 mg/mL) or combination of IL-1 β and TCA. The data are represented as mean \pm SD. The level of statistical significance is indicated by asterisks ($P < 0.01$,**).

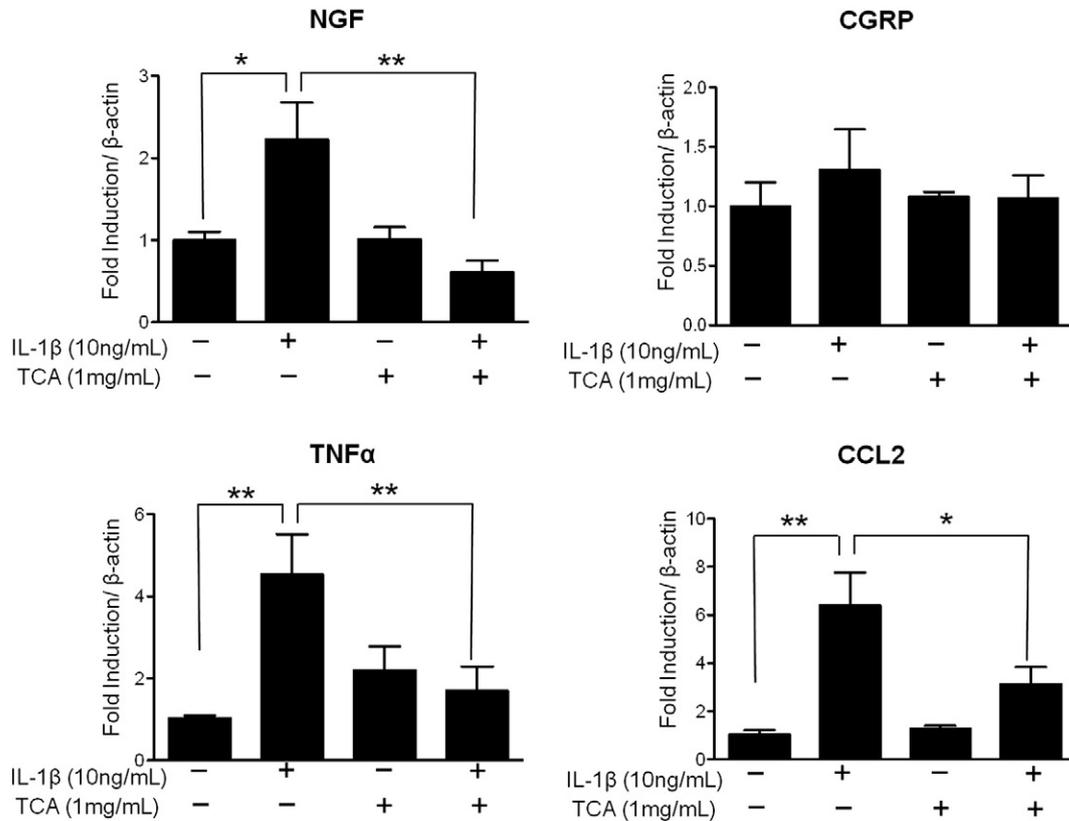


Fig. 5. qPCR analyses of NGF, CGRP, TNF α and CCL2 genes in organotypic spinal cord slices culture treated with IL-1 β (10 ng/mL), TCA (1 mg/mL) or combination of IL-1 β and TCA. The data are represented as mean \pm SD. The level of statistical significance is indicated by asterisks ($P < 0.05$,*; $P < 0.01$,**).

high viscosity, they can be easily injected through fine-gauge needles (we used 30-gauge in the mouse study). Intraarticular injections of TCA are widely used to reduce OA knee pain in patients, yet the duration of action of the analgesic effect is often only weeks (Dieppe et al., 1980; Arroll and Goodyear-Smith, 2004). Therefore combining TCA with a bio-compatible matrix that could greatly prolong the duration of analgesic action is much needed.

The present IA injection experiments demonstrate that indeed 100 µg TCA in a 5% HA matrix can produce a sustained anti-allodynia effect in mice with chronic surgically-induced OA. Interestingly, 5% HA alone had no therapeutic effect, which matches the meta-analysis of patient studies in which in large, double-blinded sham-controlled trials there were no clinically important differences of HA treatment over placebo (Jevsevar et al., 2015). One limitation of our study is that we did not perform a TCA dose-response for each of the formulations. The months involved in generating OA mice and the post steroid injection monitoring time (possibly over a month) somewhat restricted our animal numbers for the study.

Although use of TCA provides excellent results for OA-related chronic pain, however, the mechanism by which TCA exerts potent analgesic effect has not been fully elucidated. It is known that TCA exerts a potent anti-inflammatory effect on joints by inhibiting inflammatory cytokines and reduces chronic OA pain (Evans et al., 2014). However, there are no studies to date that have examined the effect of TCA on sensory neurons. Our results from organotypic culture of spinal cord slices and DRG showed that TCA significantly attenuated mRNA expression levels of nociceptive molecules induced by IL-1β in both spinal cord and DRG. During OA associated pain, mediators of pain such as NGF, CGRP, TNFα, CCL2 and IL-1β have shown to be recruited into the OA joint and cause activation of peripheral nociceptors (Kc et al., 2016; Dong et al., 2015; Dawes et al., 2013; Daheshia and Yao, 2008). The activation of these nociceptors is subsequently transmitted via the DRG, spinal cord and up through higher brain centers where signals are processed and perceived as pain. Thus, our current findings provide direct evidence of the mechanism by which TCA exerts analgesic effect.

Finally, these data in mice together with ex vivo provide rationale for evaluation of HA-based TCA formulations in man to mitigate and control pain for extended durations as compared to HA viscosupplementation and/or standard TCA injections alone. Such formulations may provide a welcome alternative for long-term control of chronic OA pain and may delay the need for knee replacement surgery.

Competing interest

The authors declare that there are no competing interests involved.

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