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ORIGINAL RESEARCH

Impaired Function and *in vivo* Imaging of NF-κB Activation in a Mouse Model of Knee Joint Inflammation

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ABSTRACT

Objective: Osteoarthritis (OA) is a consequence of not only mechanical events such as joint instability, but also biological events that result in the upregulation of proinflammatory and catabolic mediators. The intra-articular injection of mono-iodoacetate (MIA) has been widely used to induce OA. NF-κB activity has been linked to increased expression of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, etc), metallo-proteinases (MMPs), chemokines and inducible enzymes, which all contribute to cartilage degradation and subsequent OA. The goal of this study was to use *in vivo* imaging (IVIS) of NF-κB activation to track longitudinal changes due to inflammation in a rodent model of OA.

Design: Twenty-four (24) NF-κB-luc reporter transgenic mice [BALB/C-Tg (NF-κB-RE-luc)-Xen, age 7-8 weeks] were given intra-articular knee injections with either MIA (n = 12) or normal saline (n = 12) to serve as a control. IVIS and *ex vivo* imaging of NF-κB and tactile allodynia measurements were performed, and correlations were recorded preoperatively and on days 1, 3, 7, 14, 21 and 28. Animals were euthanized on days 3 and 28 for *ex vivo* imaging, and tissues were stored for future immunohistochemical evaluation.

Results: NF-κB activity was significantly elevated in the MIA group on days 1 and 3 ($p < 0.05$) when compared to pre-operative levels and was significantly elevated compared to the normal saline group on day 3 ($p < 0.05$). There was a significant increase in tactile allodynia in the MIA group compared to pre-operative levels, as well as compared to the normal saline group at all time points ($p < 0.05$). *In vivo* NF-κB luminescence correlated with tactile allodynia ($p < 0.0001$) and with *ex vivo* imaging ($p < 0.0001$).

Conclusion: This study validates the use of IVIS imaging of NF-κB activity in a MIA rodent model of arthritis and provides evidence for the use of NF-κB luminescence imaging as an imaging biomarker of pain sensitivities. This can be utilized in the future to further elucidate NF-κB's role in inflammation and OA. In addition, it can help evaluate potential therapeutic agents that target NF-κB.

Keywords: Osteoarthritis, Inflammation, *In vivo* imaging.

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INTRODUCTION

Osteoarthritis (OA) results in the degradation of cartilage and sclerosis of the underlying bone. This is a consequence of not only mechanical events such as joint instability, but also biological events that result in the upregulation of proinflammatory and catabolic mediators.[1] OA affects over 20 million people in the United States alone and is considered the most common joint disease. It is anticipated that 20% of adults will be affected by pain or disability associated with OA by the year 2030.[2] For this reason, revealing mechanisms related to the onset or progression of OA and developing new clinical interventions will have enormous impact on the societal cost of OA.

Numerous animal models have been suggested and developed for the study of OA etiology, including the use of murine models.[3,4] Models for the study of OA in mice and rats generally fall into two categories; mechanically-induced instability, such as ACL transections and medial meniscal tear models, or chemically induced inflammation/pathology models that interfere with cartilage metabolism.[3,5] The intra-articular injection of mono-iodoacetate (MIA) has been widely used to induce OA for its repeatability and rapid onset of pathology. In the MIA model, histological evidence of disease and behavioral changes progress rapidly over a 4 to 6 week period.[5-13] This model is also associated with an inflammatory response that occurs in the first 1 to 3 days post-injection, which is followed by the subsequent dissipation of inflammation and the development of arthritis in the following weeks.[5,7] This model mimics some of the key features of acute injury mediated OA and can help to elucidate the role of the initial inflammatory process in the development of later disease.

The NF-κB transcription factor family induces the expression of more than 150 genes that play a role in immunity and inflammation, anti-apoptosis, cell proliferation and the negative feedback of NF-κB pathways.[14-17] The initiation of the NF-κB pathways is complex and there are multiple cellular stimuli that can cause its activation including inflammatory cytokines (TNF-κ, IL-1β), extracellular matrix degradation products and mechanical overload. Once activated, NF-κB induces biological changes in multiple cell types, including synoviocytes, chondrocytes and fibroblasts. NF-κB activity is involved in increased expression of proinflammatory cytokines (IL-1β, TNF-α, IL-6, etc.), metalloproteinases (MMPs), chemokines and inducible enzymes which all contribute to cartilage degradation and subsequent OA. Due to its involvement with the induction and propagation of inflammation, NF-κB has served as a target for possible therapeutic agents.[2,15,17]

In addition to therapeutic agents, different models have been developed to study NF-κB activation, including mRNA

expression, DNA binding, reporter genes, transgenic reporter mice and molecular imaging.[14] Carlsen et al described the use of an NF-κB-luciferase reporter system in mice capable of studying the transient kinetics of NF-κB activation in any tissue in the animal.[14] Transgenic mice were engineered to carry cDNA for luciferase downstream of an NF-κB response element, and then they were treated systemically with stressors, such as TNF-α, IL-1α and lipopolysaccharide (LPS). Results of *in vivo* luminescence imaging following stimulation demonstrated a systemic activation of NF-κB in response to these challenges and demonstrated the potential for non-invasive longitudinal NF-κB molecular imaging. The nonterminal and quantitative nature of luminescence *in vivo* imaging (IVIS) provides the ability to quantitatively assess molecular level events.

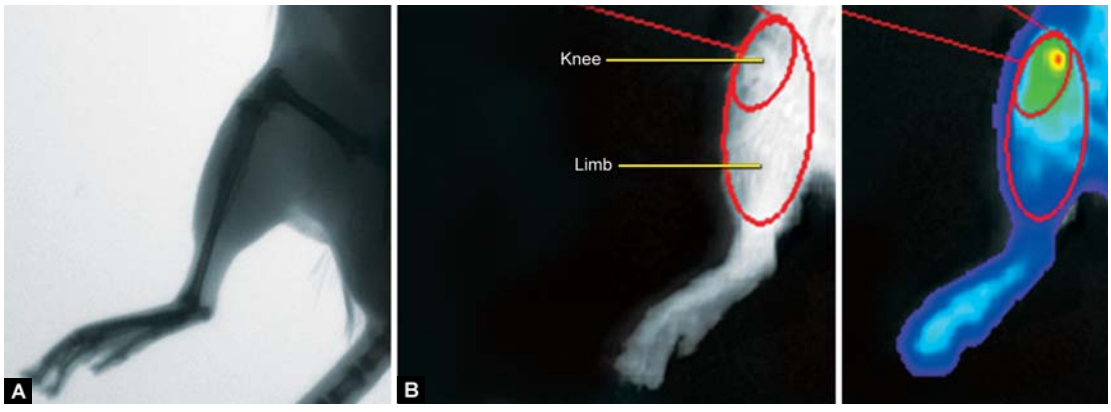
The goal of this study was to use IVIS imaging of NF-κB activation to investigate the relationship between NF-κB activity and pain related behavior in a rodent model of OA. This study describes a model of knee joint inflammation induced by an intra-articular injection of MIA and the correlation between NF-κB activation and the development of pain sensitivities. Results illustrated an initial increase in both systemic and localized knee activity of NF-κB with a peak onset 1 day after the initial chemical insult. Significant pain-related behaviors persisted throughout the full length of the study and are significantly correlated with NF-κB activity. Validation of this model allows for future investigations of early therapeutic interventions for OA and its associated symptoms, such as pain. Work is ongoing to design treatment strategies targeted at NF-κB inhibition, whose effectiveness can be screened using the IVIS imaging modalities presented in this study.

MATERIALS AND METHODS

Intra-articular injections and experimental timeline: Twenty-four (24) NF-κB-RE-luc reporter transgenic mice [BALB/C-Tg (NF-κB-RE-luc)-Xen, age 7-8 weeks] were acquired from Taconic, Inc. The animals were acclimated to housing facilities at the Duke University Vivarium for approximately 1 week. Baseline *in vivo* imaging and tactile allodynia (methods described below) were performed on all animals. On day 0, animals were anesthetized using 2% isoflurane inhalation (n = 24). These mice then underwent intra-articular knee injections of either 5 µl of normal saline to serve as a control (n = 12, CONT) or 5 µl of MIA at a concentration of 10 mg/ml (n = 12, MIA). Animals were then divided into two groups. One set of animals (n = 6/group, CONT and MIA) underwent *in vivo* imaging and tactile allodynia testing on days 1 and 3. Immediately following *in vivo* imaging on day 3, animals were sacrificed and tissues harvested for *ex vivo* imaging. A second group of animals (n = 6/group, CONT and MIA) underwent *in vivo* imaging and tactile allodynia testing on days 1, 3, 7, 14, 21 and 28. Immediately after *in vivo* imaging on day 28, organs and relevant tissues were harvested from a subset of animals (n = 3 per group) for *ex vivo* imaging immediately after sacrifice. Synovial fluid was collected from the joints (ipsilateral and contralateral) of the remaining animals in each group. All mice were euthanized via CO_2 with cervical dislocation as the secondary method of euthanasia. All procedures were performed with approval of the Duke University IACUC.

In vivo imaging (IVIS): Mice were anesthetized with 2% isoflurane inhalation and administered luciferin (120 mg/kg) intraperitoneally. Ten minutes post-luciferin injection, the mice underwent NF-κB luminescence imaging on the IVIS 100 imaging system (Caliper, Optical and Molecular Imaging Analysis Core, Duke University). Luminescence was integrated over a 30 second exposure. Regions of interest (ROIs) were selected as corresponding to the knee joint and whole limb. ROIs of the knee and limb were determined using X-rays and anatomical markers to ensure an essentially unbiased selection in each limb (Fig. 1A). These regions were then manually selected based on the anatomical markers determined from the X-rays, first on plain IVIS images, and then overlaid on the luminescent IVIS images (Fig. 1B). Knee and limb ROIs were obtained for the ipsilateral and contralateral limbs. ROI luminescence data was normalized to the animal-matched preoperative baseline values and plotted against time. Analysis of variance (ANOVA) was used to detect differences in normalized ROI luminescence for the ipsilateral limbs between CONT and MIA groups at each time point.

Ex vivo imaging: Mice were anesthetized with 2% isoflurane inhalation. The mice then received an intraperitoneal injection of luciferin (120 mg/kg) 10 minutes prior to *in vivo* imaging as described previously. *In vivo* imaging was performed on the mice, which were then euthanized, and the knee joints and adjacent muscle immediately harvested. The patellae were isolated from the knee joint as separate *ex vivo* samples. The isolated knee joints, patellae and adjacent muscle were placed in 6 well plates overlaid with 300 mg/ml luciferin in PBS for 5 minutes. At the end of 5 minutes, NF-κB luminescence imaging was performed on the explanted tissue on the IVIS 100 imaging system with an exposure time of 30 seconds. Results for luminescence levels of *ex vivo* imaging regions were used



Figs 1A and B: Regions of interest (ROIs): Plain films were first taken to evaluate the true location of knee joint while the mouse was in a prone position (A). Based on this, the two ROIs, including the knee joint and the limb, were chosen for each mouse on plain imaging, and this area was then overlaid onto the image showing the luminescence (B)

only to confirm spatial localization and specificity of the *in vivo* signal. Ipsilateral and contralateral limb *ex vivo* luminescence imaging of each knee joint, patella, and muscle were correlated with knee and limb ROIs luminescence across all time points using linear correlation analysis.

Tactile allodynia: Mechanical paw withdrawal thresholds were determined using the Chaplan up-down protocol.[18] Briefly, mice were acclimated to wire-bottom caging for 30 minutes. Von Frey filaments were then applied to the plantar surface of rats' hind paws, both ipsilateral and contralateral limbs. If withdrawal was observed, the next smallest filament was applied; if withdrawal was not observed, the next largest filament was applied. Using this up-down method, the mechanical force where the probability of paw withdrawal reflects the animals stimulus tolerances (paw withdrawal threshold) is determined.[18] Withdrawal thresholds were then normalized to each animal's pre-operative paw withdrawal threshold and plotted overtime following knee joint injection. A two-factor ANOVA was used to detect differences between CONT and MIA groups

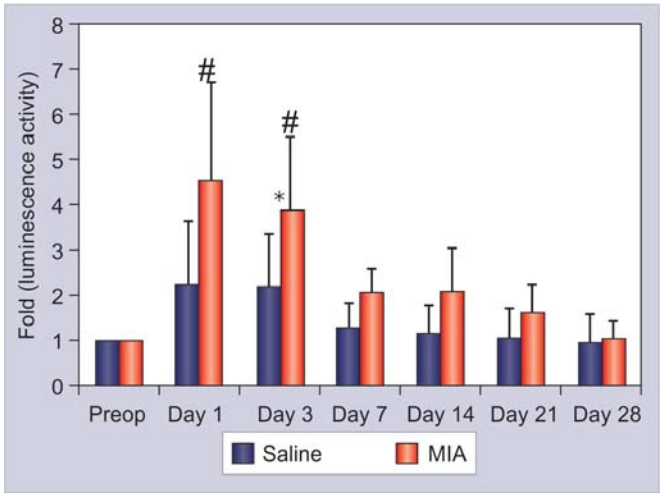


Fig. 2A: NF-κB luminescence/activity *in vivo*: Luminescence activity from the knee ROI. The normal saline injected group (CONT) did not show any significant increase in activity compared to preoperative levels at any time point. There was a significant difference in the luminescence activity in the MIA group on days 1 and 3 from preoperative levels (#, $p < 0.05$). There was also a significant difference between the MIA and CONT groups on day 3 (*, $p < 0.05$)

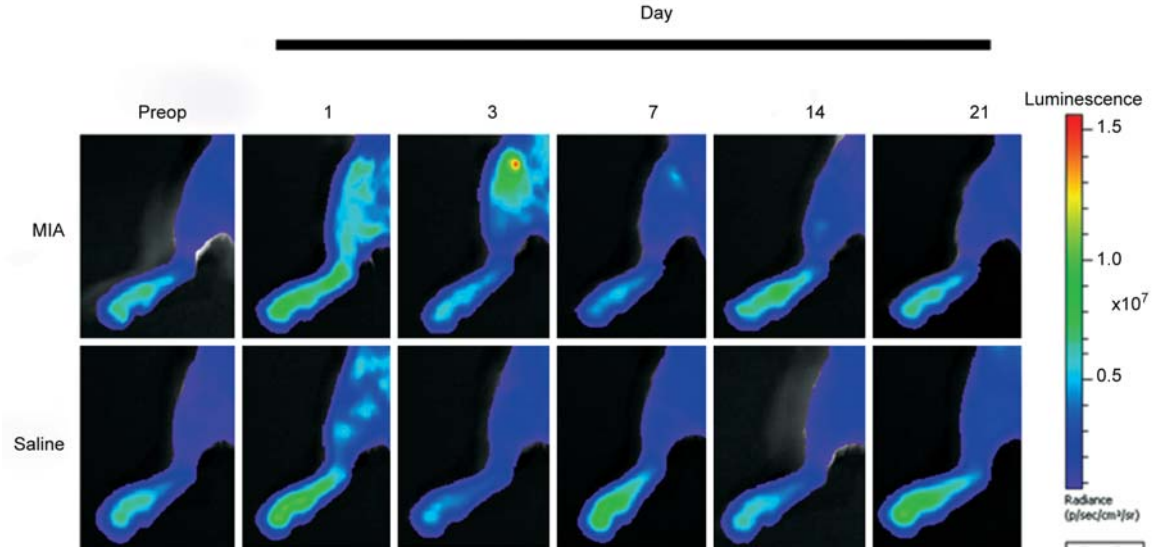


Fig. 2B: NF-κB luminescence/activity *in vivo*: Representative imaging of luminescence from the limb in the NS (CONT) and MIA groups preinjection and on days 1, 3, 7, 14, 21. Day 28 is not shown. There was no subjective or objective difference from day 21

at each time point for measurements in the ipsilateral limb. A linear regression model was use to correlate *in vivo* luminescence levels and tactile allodynia.

RESULTS

In vivo imaging: MIA injection into the knee joint was associated with a significant increase in luminescence in the affected mice at day 3 after injection as compared to CONT animals receiving normal saline injection (Figs 2A and B, $p < 0.05$). There was a significant increase in MIA knee ROI luminescence of approximately 4.5-fold at day 1 and almost 4-fold at day 3 over preoperative values, providing evidence of a significant increase in localized NF-κB activity (Figs 2A and B, $p < 0.05$). There was also an increase in the knee ROI in the normal saline (CONT) group over preoperative values at days 1 and 3, but this increase was not statistically significant.

Ex vivo imaging: In order to confirm that NF-κB generated luminescence assigned to the knee ROI originated within the knee tissues, explants were harvested from the knee and other tissues in a subset of animals on days 3 and 28. Imaging appearance subjectively confirmed that knee joints generated higher levels of luminescence *ex vivo* for ipsilateral, rather than contralateral, joints and for MIA, as compared to CONT, joints. In addition, higher luminescence values were observed in these harvested tissues. Finally, there was a significant correlation between the *in vivo* knee ROI luminescence and *ex vivo* knee luminescence for all ispsilateral and contralateral limbs that were injected with either normal saline or MIA ($R^2 = 0.711$, $p < 0.0001$ Fig. 3). Comparing the knee ROI *vs ex vivo* knee correlation ($R^2 = 0.711$, $p < 0.0001$) versus the knee ROI *vs ex vivo* patella ($R^2 = 0.516$, $p < 0.0001$), the limb ROI *vs ex vivo* knee ($R^2 = 0.496$, $p < 0.0001$), and the limb ROI *vs ex vivo* patella

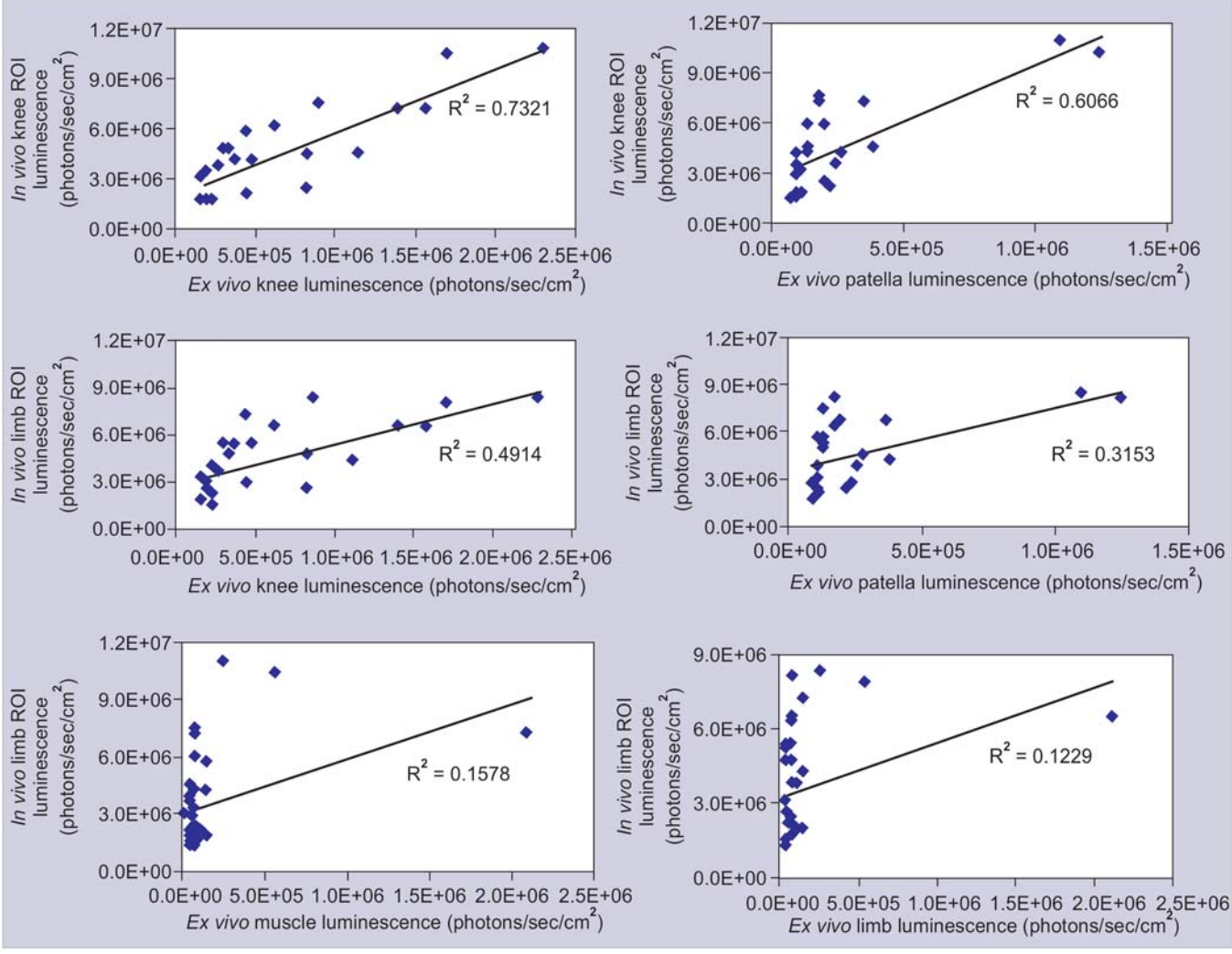


Fig. 3: *In vivo* and *ex vivo* correlations: The top two graphs represent the significant correlations between the *in vivo* knee ROI luminescence and the *ex vivo* knee and patellar luminescence with R^2 values of 0.711 and 0.516, respectively ($p < 0.001$). These correlations are stronger than the *in vivo* ROI limb luminescence and the *ex vivo* knee and patellar luminescence, as seen in the middle two graphs with R^2 values of 0.496 and 0.266 ($p < 0.0001$). There was not a strong correlation noted between the *in vivo* knee ROI or limb ROI luminescence and the *ex vivo* adjacent muscle with R^2 values of 0.157 and 0.123, respectively ($p = 0.0164$ and $p = 0.0360$)

(R^2 = 0.266, p < 0.0001) correlations indicate the higher specificity of the knee ROI to the knee joint NF-κB activity. Furthermore, there was no meaningful correlation between *in vivo* knee or limb imaging and the *ex vivo* imaging of the adjacent muscle indicating that the increased activity observed *in vivo* was due to the knee joint and not surrounding tissue (Fig. 3).

Tactile allodynia and correlation to in vivo imaging: All mice exhibited heightened sensitivity to mechanical stimuli as evidenced by lower paw withdrawal thresholds at day 3 compared to preoperative values, for both MIA and CONT groups. When compared to CONT values, MIA-injected mice had decreased paw withdrawal thresholds at all time points (p < 0.05, Fig. 4). Paw withdrawal thresholds for the CONT groups reversed to nearly preoperative values by day 7. In contrast, paw withdrawal thresholds for the MIA maintained a significant difference from preoperative values for the duration of the study (p < 0.05, Fig. 4). There was evidence of a significant correlation between knee ROI and limb ROI luminescence and tactile allodynia with R^2 values of 0.509 and 0.444 respectively (p < 0.0001, Fig. 5).

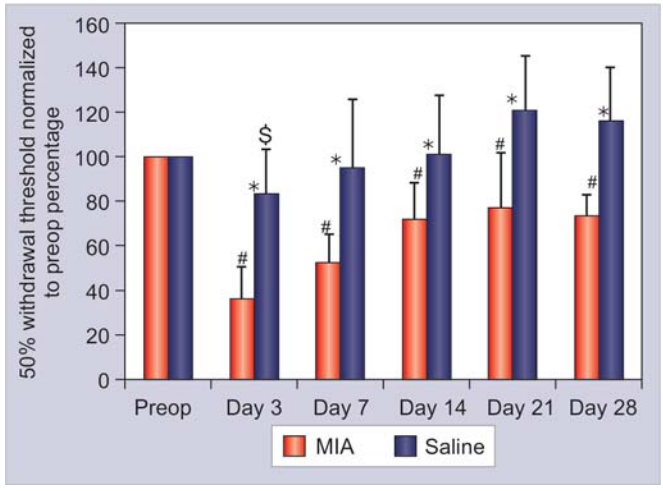


Fig. 4: Tactile allodynia: The MIA group and the normal saline group (CONT) both showed heightened sensitivity on day 3 with lower paw withdrawal thresholds, which were significantly different from their preoperative values (#,$, p < 0.05). The MIA group continued to maintain a significantly lower paw withdrawal threshold for the rest of the time points (#, p < 0.05). This difference was not maintained in the CONT group. There was also a significant difference at all time points between the MIA and CONT groups (*, p < 0.05)

DISCUSSION

In vivo imaging (IVIS) of NF-κB activity is non-invasive, spatially located to the knee joint and correlates with pain in the MIA model of osteoarthritis.

IVIS has been used in the past to evaluate systemic responses that involve NF-κB activation, but it has not been previously used in evaluating a mono-articular model of osteoarthritis. We observed that a localized injury to the knee joint causes a more diffuse activation of NF-κB. Although *in vivo* knee ROI best correlated with the *ex vivo* knee and patella samples, making this a valid method of measuring localized NF-κB activation in a mono-articular model of OA, there was still a significant correlation between the whole limb and what appeared to be a diffuse activation during early time points. The significance of this is not yet clear.

While cartilage histopathology was not assessed, previous studies utilizing this model have shown that OA will develop over 4 weeks and is characterized by synovial inflammation, cellular death, progressive loss of proteoglycans with cartilage erosion and, ultimately, formation of osteophytes.[5,7,8] The synovial inflammation in these previous studies was shown to peak from days 1 to 3 and dissipate by week 1, which correlates with our findings of initial activation of NF-κB on days 1 and 3 before decreasing to preoperative levels by day 28.

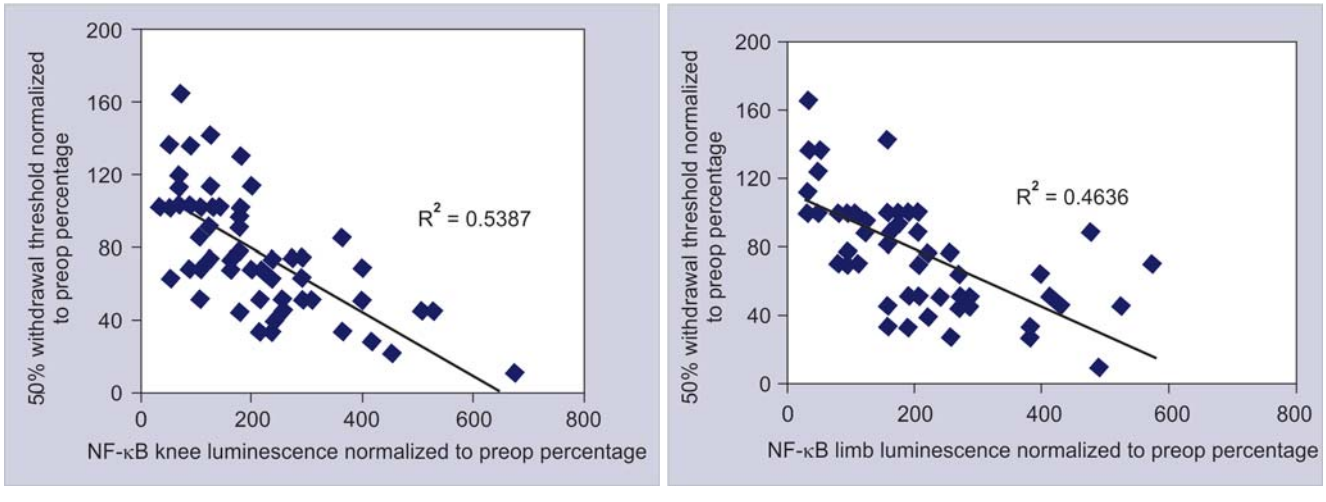


Fig. 5: Correlation between tactile allodynia and NF-κB luminescence: There was significant correlation between the MIA and NS *in vivo* knee ROI and limb ROI luminescence and tactile allodynia with R^2 values of 0.539 and 0.464, respectively (p < 0.0001)

Our behavioral data, tactile allodynia, was also consistent with previously published data on the MIA model.[8,12,13,18-21] There is an initial peak of heightened sensitivity which seems to be related to the inflammatory response. This sensitivity then decreases but does remain significantly elevated over baseline levels. The observation that pain persists while the activation of NF-κB rapidly resolves may either reflect a lack of involvement of inflammation in the perception of pain or, more likely, an initial 'programming' of pain experienced during the acute injury. There have been previous studies showing central sensitization in the MIA model that would support this theory.[19,22,23] It seems less likely that continued pain is related to the development of anatomic changes, although this cannot be completely ruled out at this time.

NF-κB is involved in the short-term responses to injury and remains a viable candidate for therapeutic intervention. Additional work would be needed to determine which components of the immune system are involved in regulating this activation. Certainly, the development of intra-articular treatments and, thereby, local inhibition of NF-κB could be advantageous given the involvement of NF-κB in the immune system throughout the body.

The use of IVIS in evaluating the activation of NF-κB has advantages over previously used methods such as mRNA expression, DNA binding and reporter genes. The largest advantage is the ability to evaluate the effectiveness of therapeutic interventions of NF-κB nonterminally, *in vivo*, while simultaneously collecting measures of pain and dysfunction. This reduces the necessary number of animals and provides a more rapid screening of therapeutics. Furthermore, the significant relationship between the NF-κB luminescence imaging and tactile allodynia indicates that NF-κB may be used as an imaging biomarker for pain in this model.

There are many therapeutic agents being studied at this time that inhibit NF-κB activation.[17] The utilization of this model could provide the ability to quickly evaluate the efficacy of such agents. This model can also help serve as a tool in evaluating the role of NF-κB in the pain response, including the possible role in 'programming' pain. Ongoing research involves the evaluation of serum cytokines, synovial cytokines and anatomic histopathology with IVIS imaging, which will allow even further validation of this model.

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