Title: Levels of neuropeptide Y in synovial fluid relate to pain in patients with knee osteoarthritis

Authors:

Lei Wang (wldzzs@163.com)
Haobo Pan (hb.pan@siat.ac.cn)
Songlin Peng (songlin824@gmail.com)
Li Zhang (lilyxjtu@163.com)
Minmin Lv (minminlv@gmail.com)
Zhen Miao (miaoz1987@163.com)
William Weijia Lu (jw.lv@siat.ac.cn)

Version: 6  Date: 12 June 2014

Author's response to reviews: see over
Dear editor and reviewers,

We have substantially revised our manuscript after reading the comments provided by the two reviewers again.

We would like to thank the reviewers and the editor for the positive and constructive comments and suggestions.

Lei Wang

Answers to reviewers:

Reviewer 1: Nicole Cattano

**Major Compulsory Revisions:**

[1]. Abstract:
1. Methods section in abstract is still very unclear. The subcategories should actually be in the results portion. Your methods appear to be (and should logically flow): Participant recruitment, Pain assessment, Radiographic assessment, Arthrocentesis.
2. Results: First sentence does not need Watanabe's pain score lead in. Remove this and start with “NPY in synovial fluid was significantly higher in…”
3. Many spacing and capitalization issues throughout Results.
4. Results should be broken down into sub-analyse of pain score and stage.
5. Need spaces in between means and standard deviations
6. Results: unclear by what ‘upward trend’ is.
7. Conclusions: state that a correlation was found yet no correlational tests were run? Primary finding is that NPY concentrations were higher in KOA than in healthy controls.
8. Key Words: only 1 word is NOT in the title. Select words that are not represented in title (correct spelling error of synovie?)

**Answer:**

**Background:** The precise aetiology of knee osteoarthritis (KOA) pain remains highly controversial, there is no known effective treatment and the side effects of commonly used analgesic agents often limit the effectiveness of current therapies. Due to the known or suggested effects of neuropeptide Y (NPY) on pain, we have sought to investigate the relationship between the concentration of NPY in synovial fluid of knee and the pain of KOA.

**Methods:** One hundred KOA patients and twenty healthy participants (volunteers) as control group were recruited. The pain and the radiographic grade of KOA were
assessed separately by Hideo Watanabe’s pain score and Tomihisa Koshino’s scoring system. Synovial fluid of knee from all participants was collected by arthrocentesis. Radioimmunoassay was used to examine the concentration of NPY in synovial fluid of knee.

**Results:** NPY in synovial fluid was significantly higher in KOA patients (124.7 ± 33.4pg/ml) compared with healthy participants (64.8 ± 26.3pg/ml). According to Hideo Watanabe’s pain score, 100 KOA patients were divided into 5 groups: no pain group 12, mild pain group 25, moderate pain group 37, strong pain group 19 and severe pain group 7. Within KOA group, significantly higher NPY concentrations were found in each subgroup as pain intensified (no pain group 81.4 ± 11.7pg/ml, mild pain group 99.1 ± 23.2pg/ml, moderate pain group 119.9 ± 31.5pg/ml, strong pain group 171.2 ± 37.3pg/ml and severe pain group 197.3 ± 41.9pg/ml). Meanwhile, according to Tomihisa Koshino’s scoring system, 100 KOA patients were divided into 3 groups: early stage group 30, middle stage group 53, advanced stage group 17. NPY of middle and advanced stage of KOA patients was significant higher than early stage of KOA patients (early stage group 96.4 ± 27.1pg/ml, middle stage group 153.3 ± 16.9pg/ml, advanced stage group 149.5 ± 36.7pg/ml ). NPY of advanced stage of KOA patients has no significant difference compare with middle stage of KOA patients.

**Conclusions:** This study demonstrated the presence and variation of NPY in the KOA joint fluid, suggesting a role for NPY as a putative regulator of pain transmission and perception in KOA pain.

**Key words:** Pathogenesis; Arthroplogosis; Synovia; Radioimmunoassay; Regulator

[2]. Methods:
9. Still extremely unclear as written and substantial revisions are necessary.
10. No need for inpatients and outpatients to be stated…they are all patients and there is no further analysis nor mention of in vs. out-patient.
11. “One hundred patients were recruited from the department of…” No need for 100 joints.
12. Bilateral assessment is still not clear. In patients with bilateral KOA, it appears that the MORE SEVERE SIDE (?) was assessed. However, who determined which was more severe, was this by the physician or patient? And was this objective or subjective?
13. Exclusion criteria needs to be re-written. It is confusing as written and may need more explanation for certain areas. For example, history of knee joint trauma – is this EVER in their lives? Also, NSAID use…is this ANY use in the last 4 weeks? Lastly, other musculoskeletal condition…is this anything? Self-reported?
14. Control group consisted of 20 healthy participants…recruited from where???How is the peak bone mass defined? No KOA or other diseases (All diseases???Self-reported???)

**Answer:**

One hundred KOA patients were recruited from the department of orthopedic surgery in People's Hospital of Hangzhou, fulfilling the American College of Rheumatology clinical criteria for the diagnosis of KOA [36]. In case of bilateral KOA, the more serious pain and/or edema and/or deformed side, which determined by the patient’s subjective judgment, were assessed. Exclusion criteria included knee joint trauma ever in their lives (periarticular fracture, meniscectomy, etc.), other arthritis (gout, rheumatoid arthritis, purulent arthritis, etc.), metabolic bone diseases (osteoporosis, Paget’s disease, osteopetrosis, etc.), malignancy, bone tumor (multiple myeloma, etc.), primary or secondary hyperparathyroidism, inflammatory arthropathy and surgical procedure of any knee during the last 6 months. And any use of anti-inflammation (oral NSAID, etc.), odynolysis and/or cortico-therapy within last 4 weeks.

20 healthy participants were recruited from the People’s Hospital of Hangzhou as control groups between 35 and 65 years of age without any diseases judged by the physician.

[3]. Arthrocentesis:
15. As listed….it appears that arthrocentesis was done prior to pain assessment and radiographs.
16. This section should not have a 1 sentence paragraph about positioning on stretcher. Fold into the 2nd paragraph
17. Unclear as to the location of the aspiration…“directly behind the patella” was their a superior/inferior or medial/lateral approach?
18. Sentence starting with “Upon insertion…” there are spacing issues and it is unclear with the “and after 20sthe synovial” Only 1 to 3 mL was aspirated? Was this as much as could be aspirated? Was it the same for each person?
How did you decide on the 1-3 if you put 3 mL of saline in???
19. Same sentence as comment 18, change centrifugal to centrifuged. And “until needed” to “until analyzed”.

**Answer:**

**KOA pain assessment**

Pain was assessed by the physician based on patient’s medical history according to Hideo Watanabe’s knee scoring system-related pain score [37], patients with KOA were divided into 5 groups: no pain group, mild pain group, moderate pain group, strong pain group and severe pain group. (Table 1)

<table>
<thead>
<tr>
<th><strong>Group</strong></th>
<th><strong>Standard</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>No pain group</td>
<td>Occasionally feeling fatigue or heaviness, but no pain at any time</td>
</tr>
<tr>
<td>Mild pain group</td>
<td>Pain at starting time of various activities or occasionally during</td>
</tr>
<tr>
<td></td>
<td>long-distance walking, but no pain at rest</td>
</tr>
<tr>
<td>Moderate pain group</td>
<td>Pain usually on walking, but pain gradually subside after a brief rest</td>
</tr>
<tr>
<td>Strong pain group</td>
<td>Persistent pain on walking, but pain gradually mitigate after a rest,</td>
</tr>
<tr>
<td></td>
<td>usually associate with spontaneous pain</td>
</tr>
<tr>
<td>Severe pain group</td>
<td>Persistent pain at any time, including walking and rest</td>
</tr>
</tbody>
</table>

**KOA radiographic grade**

Full-extension radiographs (X-ray) of the knees were obtained. The degree of radiographic KOA in individual joints was graded (0 to 5) using the Tomihisa Koshino’s scoring system[38] where grade 1 was considered as an early stage, grade 2-3 as middle stage and grade 4-5 as advanced stage. It was assessed by the physician. (Table 2)

<table>
<thead>
<tr>
<th><strong>Stage</strong></th>
<th><strong>Grade</strong></th>
<th><strong>Standing x-ray</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stage</td>
<td>1</td>
<td>Bone sclerosis or osteophyte formation</td>
</tr>
<tr>
<td>2</td>
<td>Narrowing of joint space (≤3mm)</td>
<td></td>
</tr>
</tbody>
</table>
Obliteration of joint space or subluxation
Defect of tibial plateau (<5mm)
Defect of tibial plateau (≥5mm)

---

3. Obliteration of joint space or subluxation
4. Defect of tibial plateau (<5mm)
5. Defect of tibial plateau (≥5mm)

*a* An anteroposterior and weight-bearing radiograph taken in a standing position was used for grading.

*b* “Subluxation” indicates the condition in which the medial edge of the medial tibial plateau shows a lateral shift by more than 5mm against the medial edge of articular surface of the medial femoral condyle without including osteophyte.

**Arthrocentesis & Joint fluid sampling**

All participants were supine position on a stretcher. The same entry site was demarcated with a skin-marking pen. The skin was prepared with povidone-iodine. A sterile drape was placed around the site. Then the region was anesthetized by placing a wheal of lidocaine, using a small (25-gauge) needle. And intermittently the plunger was pulled back during the injection of the anesthetic to exclude intravascular placement.

A large syringe was used. An 18-gauge needle was used directly behind the patella into the synovial cavity with the lateral approach. Upon insertion into the articular cavity, 3 ml of 0.9% saline was injected slowly into the joint and after 20s the synovial fluid 3ml was aspirated and immediately centrifuged (2000rpm,10min) at 4°C and stored at -70°C until analyzed.

Synovial fluid from healthy individuals samples were collected, frozen, and stored in the same way as the material from the KOA patients.

[4]. KOA Pain assessment:
20. Pain was assessed according to Hideo….
21. I am still unclear as to WHO categorized patients…did patients select this, did physicians assign based on a history?

**Answer:**

**KOA pain assessment**

Pain was assessed by the physician based on patient’s medical history according to Hideo Watanabe’s knee scoring system-related pain score [37], patients with KOA were divided into 5 groups: no pain group, mild pain group, moderate pain group,
strong pain group and severe pain group.

[5]. KOA radiographic assessment:
22. Eliminate first sentence (unless a decision was made AFTER radiograph was taken).
23. Radiographs were weight-bearing, please include in 2nd sentence.
24. You have a capital The…consider having this a stand alone sentence instead of a compound sentence
25. This Grading seems like a modification of Kellgren Lawrence (which is fine), and it seems that the grades come from Koshino, however, who decided on the defining of the Stages?
26. Unclear as to WHO assessed, was it one physician? Radiologist? Study investigator?

Answer:

KOA radiographic grade

Full-extension radiographs (X-ray) of the knees were obtained and assessed by the physician. The degree of radiographic KOA in individual joints was graded (0 to 5) using the Tomihisa Koshino’s scoring system[38] where grade 1 was considered as an early stage, grade 2-3 as middle stage and grade 4-5 as advanced stage by the study investigator. (Table 2)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Grade</th>
<th>Standing x-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stage</td>
<td>1</td>
<td>Bone sclerosis or osteophyte formation</td>
</tr>
<tr>
<td>Middle stage</td>
<td>2</td>
<td>Narrowing of joint space (≤3mm)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Obliteration of joint space or subluxation$^b$</td>
</tr>
<tr>
<td>Advanced stage</td>
<td>4</td>
<td>Defect of tibial plateau (&lt;5mm)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Defect of tibial plateau ($≥$5mm)</td>
</tr>
</tbody>
</table>

$^a$ An anteroposterior and weight-bearing radiograph taken in a standing position was used for grading.

$^b$ “Subluxation” indicates the condition in which the medial edge of the medial tibial plateau shows a lateral shift by more than 5mm against the medial edge of articular surface of the medial femoral condyle without including osteophyte.
Statistical Analysis:
27. This section needs to be re-written with drastic revisions.
28. STILL did not address what statistical package was utilized for analyses.
29. First sentence seems like a fragment...is there a table that you want to reference with this?
30. ANOVA is not typically done for homogeneity of variances
31. State that group t-test was used to compare the means of KOA group with healthy control group....but OF WHAT DEPENDENT VARIABLES?
32. Still question whether analyses were correct. ANOVA, Pearson’s correlation, and t-tests are definitely appropriate. But perhaps a linear regression for the pain scale sub-analysis?

Answer:

Statistical analysis

All analyses were performed using SPSS version 13.0. Data presented as mean ± SD. ANOVA was executed first to identify the homogeneity of variances. Group t-test was used to compare the mean of NPY concentrations in synovial fluid of KOA Group with Healthy control group. Dunnett-t test was used to compare the mean of NPY concentrations in synovial fluid of each subgroup of KOA Group with Healthy control group. And the Student-Newman-Keuls (SNK) test was used to compare the mean of NPY concentrations in synovial fluid of each subgroup of KOA Group. Linear regression was used for the correlation between NPY concentrations in synovial fluid and the pain of KOA patients. A p value less than 0.05 was considered to be statistically significant.

\[ y = 25.665x + 28.97 \]
\[ R^2 = 0.7553 \]
Figure 2 The correlation between synovial fluid NPY levels and pain of KOA patients
(1=Healthy control group, 2=No pain group, 3=Mild pain group, 4=Moderate pain group, 5=Strong pain group, 6=Severe pain group)

[7]. Results:
33. KOA pain and NPY: subdivisions no pain group (n = 12)….use parentheses and maybe put an n.
34. Page 7 under figure 1: how is there a positive correlation with no correlations ran in the analysis section? This is possibly where a linear regression will be more appropriate.
35. Same page/paragraph, 2nd sentence starting with “NPY concentrations demonstrated…” reword to NPY concentrations were significantly higher in KOA patients than in healthy participants” Take out or SUBSET the subcategories into the final sentence of that paragraph.
36. KOA radiographic grade and NPY: 1st sentence...revise to 100 KOS patients were divided into 3 stage groups according to Tomihisa scoring system: Early, middle, and advanced.
37. Page 8 under figure 2…spacing issues in 1st sentence. Also, same issues as comment #35.

Answer:

KOA pain and NPY

KOA patients (n=100) were divided into 5 groups according to Hideo Watanabe’s pain score: no pain group (n=12), mild pain group (n=25), moderate pain group (n=37), strong pain group (n=19) and severe pain group (n=7). (Figure 1, 2)
In all KOA groups tested there was a positive correlation to NPY concentrations and the level of pain was significantly higher. NPY concentrations led to a significant increase in pain in each subgroup tested. NPY concentrations were significantly higher in KOA patients (124.7 ± 33.4pg/ml) than in healthy participants (64.8 ± 26.3pg/ml). Within KOA group, significantly higher NPY concentrations were found in each subgroup as pain increased (no pain group 81.4 ± 11.7pg/ml, mild pain group 99.1 ± 23.2pg/ml, moderate pain group 119.9 ± 31.5pg/ml, strong pain group 171.2 ± 37.3pg/ml and severe pain group 197.3±41.9pg/ml).

KOA radiographic grade and NPY

100 KOA patients were divided into 3 stage groups according to Tomihisa Koshino’s scoring system: early (n=30), middle (n=53) and advanced (n=17). (Figure3)
NPY concentrations were significantly higher in KOA patients (124.7 ± 33.4 pg/ml) than in healthy participants (64.8 ± 26.3 pg/ml). NPY concentrations in synovial fluid of middle and advanced stage of KOA patients (153.3 ± 16.9 pg/ml, 149.5 ± 36.7 pg/ml) was significant higher than early stage of KOA patients (96.4 ± 27.1 pg/ml). NPY concentrations in synovial fluid of advanced stage of KOA patients have no significant difference to that for the middle stage of KOA patients.

[8]. Discussion:
38. First 2 sentences unnecessary. Eliminate.
39. Sentence starting with “Due to the important role” NPY has already been previously defined.
40. 2nd paragraph, Primary finding is that MPY concentrations were significantly higher in KOA patients compared to healthy controls. Eliminate according to watanbe’s pain score until the next sentence.
41. Consider new paragraph starting with “As pain increased we found significantly higher NPY concentrations....”
42. Bottom of page 8 “which matched the reported clinical observations where...” this sentence is a run on and extremely confusing...rewrite.
43. Page 9: Sentence that starts with Moreover... You already dated this is a primary finding. I would start this paragraph with “NPY concentrations in synovial fluid of middle and advanced KOA stages were significantly higher than early KOA stage.”
44. “We further demonstrated a positive correlation” Still no evidence of a correlation being run.
45. Sentence starting with “But NPY in synovial fluid of advanced stage KOA...” this sentence is very confusing. Re-write or eliminate.
46. I like the notion of lack of agreement between objective x-rays and
subjective pain reporting by patients. This is a strength of your study that you highlight other research finding as well. BUT did they use any of the same subscales of classifications as you did?

47. Last Paragraph that goes into pro-inflammatory mediators is unnecessary and I would eliminate altogether.

**Answer:**

Despite the widespread prevalence of KOA in the adult population, very little is known about the causes of KOA pain or the chemical mediators involved in the initiation of painful stimuli in KOA joints [39]. Due to the important role played by NPY in pain [20-23, 27-32], we sought to study the effect of varying concentrations of NPY in KOA joint fluid and its association with the pain encountered. To our knowledge, this is the first study specifically designed to evaluate the relationship between NPY and KOA pain.

Primary finding was that NPY concentrations were significantly higher in KOA patients compared to healthy controls. The results of our study were in agreement with the available clinical information, where NPY was found in significantly higher concentrations in the synovial fluid of patients with arthritis of the knee (crystal induced arthritis, chronic polyarthritis, post-infectious arthritis, rheumatoid arthritis), who were admitted due to acute joint pain and swelling, compared to the controls with non-inflammatory joint disorders (lateral meniscus injury, medial meniscus injury, cruciate ligament injury), otherwise healthy and without joint disease, admitted for arthroscopy [40, 41].

As pain increased we found significantly higher NPY concentrations in each subgroup of KOA patients tested. It matched that the pain gradually developed from the initial mild pain into a long period of severe pain during the pathological process of KOA [9-12]. These indicate that levels of NPY have significant relevance to the joint pain in patients with KOA.

NPY concentrations in synovial fluid of middle and advanced KOA stages were significantly higher than early KOA stage. But NPY concentrations in synovial fluid of advanced stage of KOA patients have no significant difference to compare with middle stage of KOA patients. These results contradict the notion that NPY has
significant relevance to joint pain in patients with KOA. It means a lack of agreement between X-rays evidence of KOA and patients’ report of pain at that site base on the result of our studies, which are the first study specifically designed to evaluate the relationship between NPY and KOA pain. Meanwhile, the orthopedic community has been plagued for years by this discordance. Many researchers [42-46] have found evidence for a substantial discordance between pain and observed radiographic evidence of KOA. In a 2008 systematic review of population studies, Bedson and Croft quantitatively described the problem for KOA: “In those with radiographic KOA the proportion with pain ranged from 15% to 81%” [47]. The discordance between pain and radiographic KOA points to the need for further investigation of this phenomenon.

Studies have shown that during arthritis, pro-inflammatory mediators are released into the joint [48] which sensitize joint afferent nerves such that previously innocuous physicochemical stimuli can activate these fibres leading to the sensation of joint pain [49,50]. One important family of agents known to be involved in the peripheral sensitization of joint afferents is the inflammatory neuropeptides including NPY [30–32, 51]. NPY, belonging to the pancreatic polypeptide family, was first isolated from pig brain by Tatemoto [24]. It’s produced together with noradrenaline in certain sympathetic nerve fibres [25] has a strong and long-standing vasoconstrictive effect on both arterial and venous vessels. In the rat, this neuropeptide was found around blood vessels in the capsule of the joint, but not in the disc or cartilaginous joint surfaces [26]. This potent neuromodulator is stored in the terminal branches of Aδ and C fibres where it release into the joint lowers the activation threshold of nociceptive nerve endings hat is likely to contribute to chronic, sensitised pain responses [52]. Based on current study, the presence and variation of NPY in KOA joint fluid strongly point to a role for NPY as a regulator of pain transmission and perception in KOA pain. Possible mechanisms by which NPY can modulate pain processing. NPY can lower membrane Ca 2+ conductance in dorsal root ganglion neurones and inhibit substance P release from central terminals of primary afferent fibers [14,53,54]. Furthermore, the observation that peripheral
inflammation increases both NPY and its Y1 and Y2 receptor synthesis in the spinal dorsal horn reinforce the concept that spinal NPY participates in the processing of nociception [12]. Noradrenergic neurons of the locus coeruleus and A1 noradrenergic cell groups also constitute a major system concerned with the modulation of nociception [55] and NPY is co-localized with noradrenaline in a subpopulation of the neurones [25,56,57]. In the locus coeruleus, NPY depresses the spontaneous firing rate of these neurones and potentiates the hyperpolarizing effect of $\alpha_2$-agonists through stimulation of its Y2 receptor subtype [58].

Study limitations

This study is limited by a small sample size and the different subscales of classifications for pain and grade of KOA. And the underlying molecular and cellular mechanisms of the role of NPY in KOA pain remain poorly understood. Hopefully, future studies will provide answers to this question.

[9]. Conclusion:
48. Study demonstrated a positive correlation? Still no evidence of this anywhere. What you found was biochemical distinction of NPY concentrations in KOA vs. healthy patients.
49. This paragraph should be re-written as I do not think it articulates the summary of your findings. Therapeutic interventions would not USE NPY, but would perhaps target NPY.
50. The last sentence of the conclusion should be what the paragraph is built around, perhaps move this to your 2nd sentence.

Answer:

Conclusion

This study demonstrated the biochemical distinction of NPY concentrations between KOA patients and healthy controls, suggesting a role for NPY as a putative regulator of pain transmission and perception in KOA pain. At the same time, NPY as a biochemical indicator can reflect the pathological progressing and severity of KOA. The precise roles of NPY in the pathogenesis of KOA pain require further investigation. However, our results have contributed to a better understanding of
the molecular processes underlying KOA pain and, in addition, foster the option of local therapeutic intervention targeting NPY. The understanding of the role of NPY in KOA pain is a prerequisite to developing such novel therapeutic options for the treatment of KOA pain and restoration of tissue function.

**Minor Essential Revisions**

1. Background: correction spacing issues in last sentence.
2. Background: consider changing “concentration of NPY” to “NPY concentrations” throughout document

**Answer:**
Therefore, the aim of this study was to assess the relationship between NPY concentrations in the synovial fluid of knee and in turn its effect on KOA pain.

3. Patients: “change control groups were twenty” to control group was 20 healthy participants

**Answer:**
20 healthy participants were recruited from the People's Hospital of Hangzhou as control groups between 35 and 65 years of age without any diseases judged by the physician.

4. Results: space after 56 before the sd.
5. Results; 2nd sentence, which was not significantly different (Drop the e typo at the end of the word)
6. Results same sentence, \( P = \) should be lower case \( p \) that is italicized

**Answer:**
In total, 100 KOA patients and 20 healthy individuals participated in this study. The mean age of KOA patients was 56 ± 6.9 years, which was not significantly different \( (p = 0.347) \) from the mean age of 48 ± 8.1 years for the healthy participants. The gender distribution between the two groups was similar with 39% of KOA patients being male, compared with 50% of healthy participants.
Reviewer 2: Guang-hua Lei

[1].
Please add figure to the correlation between synovial fluid NPY levels and pain scores of KOA

Answer:

![Figure 2: The correlation between synovial fluid NPY levels and pain of KOA patients](image)

**Figure 2** The correlation between synovial fluid NPY levels and pain of KOA patients

(1=Healthy control group, 2=No pain group, 3=Mild pain group, 4=Moderate pain group, 5=Strong pain group, 6=Severe pain group)