Author's response to reviews

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

Authors:

Lei Wang (wldzzs@163.com)
William Weijia Lu (jw.lv@siat.ac.cn)
Haobo Pan (hb.pan@siat.ac.cn)
Songlin Peng (songlin824@gmail.com)

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

We have substantially revised our manuscript after reading the comments provided by the two reviewers. We invited Prof. Hala Zreiqat (NH&MRC Senior Research Fellow, the Head of Tissue Engineering & Biomaterials Research Unit, University of Sydney, Australia) to polish our manuscript.

We also expanded part of the experiment, providing details in the current version. We would like to make some minor modifications to our list of authors to reflect their separate contributions.

Lei Wang

Answers to reviewers:
First, we would like to thank the reviewers and the editor for the positive and constructive comments and suggestions.

Reviewer 1: Nicole Cattano

[1]. Title:
- Spell out NPY in title

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

[2]. Abstract:
- Clarify methods (120 total participants)
- Correct capitalization/spacing issues throughout.
- First line of background Knee osteoarthritis (K doesn’t need to be capitalized).
- Last sentence “effects of neuropeptide Y (NPY) need space in between end of Y and the abbreviation.
- Space after period “advanced stage group 17. And”
- These are just some examples of the multiple issues throughout the abstract and the entire manuscript.
- Results indicated a significant increase in NPY concentrations. Increase does not appear to be the correct word; it implicates an entirely different methodology.
- Select keywords that are NOT found in the title.

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 outpatients and inpatients (100 joints) and twenty healthy participants (volunteers) (20 joints) as control group were recruited. According to the two different standards of KOA, 100 KOA patients were divided into different groups. 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. According to Tomihisa Koshino’s scoring system of KOA, 100 KOA patients were divided into 3 groups: Early stage group 30, Middle stage group 53, Advanced stage group 17. Radioimmunoassay was used to examine the concentration of NPY in synovial fluid of knee.

**Results:** According to Hideo Watanabe’s pain score, NPY in synovial fluid demonstrated a significant elevation in KOA patients (124.7±33.4 pg/ml) compared with Healthy participants (64.8±26.3 pg/ml). Levels of NPY renders a significant upward trend with KOA 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±37.3 pg/ml and Severe pain group:197.3±41.9 pg/ml). According to Tomihisa Koshino’s scoring system, NPY in synovial fluid demonstrated a significant elevation in KOA patients (124.7±33.4 pg/ml) compared with Healthy participants (64.8±26.3 pg/ml). 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 and has a downward trend compare with Middle stage of KOA patients.

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

**Key words:** Osteoarthritis; Pain; Synovie; Radioimmunoassay; Neuropeptide Y

[3]. Background:

-Knee Osteoarthritis (KOA)- O should not be capitalized

**Answer:**

Knee osteoarthritis (KOA) is a chronic degenerative joint disorder that affects a large proportion of the population, particularly in elderly people [1-6].

-KOA Patients’ – P should not be capitalized

**Answer:**

KOA patients’ major clinical manifestation is chronic pain that typically worsens as a result of weight bearing, activity or movement of the affected joint [8].

-Line starting with “Many studies have been performed to unravel…” This idea is very unclear and confusing. Please clarify what you mean by stating probably include…

**Answer:**

Synovial inflammation, raised intra-osseous pressure and mechanical stresses on intra-articular and peri-articular ligaments and tendons [9-11] are potential contributors to the chronic pain encountered.

-End of this 1st paragraph-the sentence stating that there is no known effective treatment is confusing and detracts from the primary purpose of this specific study.

**Answer:**

However, the precise aetiology of KOA pain remains highly controversial, which limit the progress in developing effective treatment for KOA pain [12].

-Consider 2nd paragraph rewording to active voice.

**Answer:**

Neuropeptide Y (NPY), a 36 amino acid peptide, is one of the most widely distributed neuropeptides in the nervous system [13,14]. It has diverse
and complex biological functions, such as capacity to influence cardiovascular performance, food intake and pain processing [15-17]. In addition, pathophysiological role of NPY in infection and inflammation, as well as in autoimmunity, has been suggested [18,19]. The up-regulation of NPY in dorsal root ganglia and spinal cord has been shown in various models of inflammatory and neuropathic pain [20-23] and around blood vessels in the capsule of the joint [24-26]. NPY and its Y1 and Y2 receptors are located at key pain signaling centers throughout the nervous systems [27-32]. Previous work also suggested that joint pain results from the activation of primary afferent nerve fibres by neuropeptides at the joint [33-35]. Due to the known or suggested important effects of NPY on pain, we hypothesized that NPY may be involved in the pathogenesis of KOA pain. Therefore, the aim of this study was to assess the relationship between the concentration of NPY in the synovial fluid of knee and its effect on KOA pain.

[4]. Methods:
- Spell out January in the Patients section

Answer:

This 1-year study was conducted in People's Hospital of Hangzhou, Nanjing Medical University from January 2009 to January 2010.

- Need to better define the inclusion and exclusion criteria.
- Need to better state how patients were recruited...was it from an office visit for pain or some other type of treatment?
- Virtually no information is provided regarding the control participant recruitment

Answer:

One hundred KOA outpatients and inpatients (100 joints) 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, serious pain and/or edema and/or deformed side were assessed. Control groups were twenty healthy participants (volunteers) (20 joints) between 35 and 65 years of age, who had reached peak bone mass and
were without KOA and other diseases.

- In case of bilateral KOA, how as “the serious side” defined to be assessed?

**Answer:**

In case of bilateral KOA, serious pain and/or edema and/or deformed side were assessed.

- Exclusion criteria seems to contradict the previous statement. It appears that you permitted bilateral KOA patients, however, the exclusion criteria included another symptomatic OA joint. Is this actually other than the knee???

- Within the exclusion criteria-the use of e.g., implies a list, therefore “on and on” can be removed.

- Last sentence of exclusion criteria regarding anti-inflammation therapy…This sentence is a fragment, please revise. Also, it is unclear as to whether oral NSAID use was permitted or not. It appears possibly this would be an exclusion criteria???

**Answer:**

Exclusion criteria included history of knee joint trauma (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, and anti-inflammation (oral NSAID, etc.), odynolysis and cortico-therapy within last 4 weeks, inflammatory arthropathy or any other musculo-skeletal condition and surgical procedure of any knee during the last 6 months.

- Arthrocentesis section: All needs to be written in the past tense. This entire 2 paragraph needs to be re-written to provide clarity.

**Answer:**

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.

An large syringe was used. An 18-gauge needle was used directly behind the patella into the synovial cavity. Upon insertion into the articular cavity, 3 ml of 0.9% saline was injected slowly into the joint and after 20sthe synovial fluid 1-3ml was aspirated and immediately centrifugal (2000rmin,10min) at 4 ℃ and stored at -70 ℃ until needed.

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

-KOA pain assessment: this section is extremely vague and does not allow a reader to understand HOW this assessment was done.

**Answer:**

<table>
<thead>
<tr>
<th>Group</th>
<th>Standard</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 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, 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: Same as above…when were radiographs taken in relationship to arthrocentesis? Who read them and assigned grade? Did all have radiographs done at time of visit?

**Answer:**

Serious pain and/or edema and/or deformed side were assessed in the bilateral KOA. Full-extension radiographs(X-ray) of the knees were obtained and The degree of radiographic KOA in individual joints was graded(0 to 5) using the Tomihisa Koshino’s scoring system[38] where Grade1 was considered as an Early stage, Grade2-3 as Middle stage and
Grade 4-5 as Advanced stage. (Table 2)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Grade</th>
<th>Standing x-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<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 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.

- **NPY in synovial fluid:** Reword first sentence: Radioimmunoassay was performed to determine NPY concentration in KOA synovial fluid.

  **Answer:**
  
  Radio-immunoassay was performed to determine NPY concentrations in KOA synovial fluid. The concentrations of NPY in joint fluid was determined by commercially available radioimmunoassay kits (Iodine\(^{125}\) Neuropeptide Radioimmunoassay kit, Institute of RIA, Chinese PLA General Hospital, China) in accordance with the standard protocols included in the kits. The sensitivity of the assay was < 33pg / ml.

**Statistical Analysis:**

- Please state what statistical procedures were done and on what statistical package.
- In order to run Post Hoc testing, should have run an ANOVA first??
  Also-Why not Tukey’s?

  **Answer:**
  
  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 KOA Group with Healthy control group. Dunnett-t test was used to
compare the mean of each subgroup of KOA Group with Healthy control group. And the Student-Newman-Keuls (SNK) test was used to compare the mean of each subgroup of KOA Group. A \( p \) value less than 0.05 was considered to be statistically significant.

[5]. Results:
- Perhaps present results information on the primary aim first, and consider the demographics for each subcategory (age and SD?)
- Doesn’t appear to have controlled for body weight?

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 differente from the mean age of 48 ± 8.1 years for the healthy participants (\( P = 0.347 \)). The gender distribution between the two groups was similar with 39% of KOA patients being male, compared with 50% of healthy participants.

-Same information is presented in Table 3 and Figure 1…perhaps pick which you feel better presents information.

Answer:

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-“NPY in synovial fluid demonstrated a significant increase in KOA patients…compared with healthy participants.” This sentence is confusing. NPY concentrations were significantly higher…
-Within KOA group, significantly higher NPY concentrations were found in each subgroup as pain increased?

Answer:
In all KOA groups tested there was a positive correlation to NPY concentrations and the level of pain was significantly higher NPY
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concentrations led to a significant increase in pain in each subgroup tested. NPY concentrations demonstrated a significant higher in KOA patients (124.7±33.4 pg/ml) (No pain group 81.4±11.7 pg/ml, Mild pain group 99.1±23.2 pg/ml, Moderate pain group 119.9±31.5 pg/ml, Strong pain group 171.2±37.3 pg/ml and Severe pain group 197.3±41.9 pg/ml) than in Healthy participants (64.8±26.3 pg/ml). Within KOA group, significantly higher NPY concentrations were found in each subgroup as pain increased.

-KOA radiographic grade and NPY section....same concerns as above.
-Were you appropriately powered to look at these subgroups?
Answer:

![NPY concentrations chart](chart.png)

NPY concentrations were significantly higher in KOA patients (124.7±33.4 pg/ml) (Early stage group 96.4±27.1 pg/ml, Middle stage group 153.3±16.9 pg/ml, Advanced stage group 149.5±36.7 pg/ml) than in Healthy participants (64.8±26.3 pg/ml). NPY in synovial fluid of Middle and Advanced stage of KOA patients was significant higher than Early stage of KOA patients. NPY in synovial fluid of Advanced stage of KOA patients has no significant difference to that for the Middle stage of KOA patients.

Discussion:
-The entire first paragraph of this section is redundant with previous information. I do think there is value with the last 2 sentences to lead into the discussion.
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]. Therefore, the exact pathophysiology of KOA pain remains to be delineated completely. Due to the important role played by neuropeptide Y (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.

-2nd paragraph states that this study is aimed at evaluating the effects of NPY in the development of pain in patients with KOA. This seems inappropriately worded as previously it was more the association between pain and NPY concentrations. This purpose should be clarified and consistent throughout the entire document.

- The authors do not get to their specific study results until the 3rd paragraph. This should be incorporated earlier.

Answer:

Using the Hideo Watanabe’s pain score, we demonstrated that NPY concentrations was significantly higher in KOA patients compared to the Healthy participants.
- 3rd paragraph when stated that “NPY concentrations were found in significantly higher concentrations in the synovial fluid of patients with arthritis of the knee than in controls with non-inflammatory joint disorders.” Seems that your study reinforces this finding. However, it is unclear as to what kind of arthritis of the knee from this previous study (e.g., rheumatoid or osteo-) as well as what non-inflammatory joint disorder the controls may have had.

**Answer:**

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].

-Sentence starting with “And as pain intensified…” is very confusing and needs to be clarified.

**Answer:**

As pain increased we found significantly higher NPY concentrations in each subgroup of KOA patients tested, which matched the reported clinical observations where KOA pain gradually developed from the initial mild pain into a long period of severe pain [9-12], indicating that levels of NPY has significant relevance to the joint pain in patients with KOA.

- Within 3rd and 4th paragraphs the use of down/upward trends or increases in concentrations is confusing and misleading.

- Within 4th paragraph….try to avoid the use of direct quotations

**Answer:**

Moreover, using the Tomihisa Koshino’s scoring system of KOA radiographic grade, we demonstrated significant higher NPY concentration in the synovial fluid in KOA patients compared to the Healthy participants. NPY in synovial fluid of Middle and Advanced stage of KOA patients was significant higher than that for the Early stage of KOA patients. We further demonstrated a positive correlation between
the NPY levels in the synovial fluid and the level of degradation of the knee joint of KOA patients. But NPY in synovial fluid of Advanced stage of KOA patients has no significant difference to that for the Middle stage of KOA patients. These results contradicts 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 a patient’s report of pain at that site base on the result of our studies. 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.

-5th paragraph-based on CURRENT STUDY (instead of our studies...)
-“NPY as a putative regulator of pain transmission...” Putative does not accurately add value to this statement. I would consider removing.
-5th paragraph could be teased out throughout the discussion more. Seems to be where the bulk amount of the literature is found and reinforced or refuted.

Answer:

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 α2-agonists through stimulation of its Y2 receptor subtype [58].

[7]. Conclusion
- First sentence is not accurate. Change of NPY is not what this study looked at. Cannot made deductions based on current study to this level. The way this sentence is written it appears that an increase in NPY leads to increased knee pain. Since this is a cross-sectional study you cannot make this assumption.

Answer:
This study demonstrated a positive correlation between NPY concentration in the KOA joint fluid and KOA pain, suggesting a role for NPY as a putative regulator of pain transmission and perception in KOA pain.

[8]. The authors do not clearly acknowledge any work that they are building on.  

Answer:  
Authors’ contributions  
LW was the grant holder of the trial. This work was jointly conceived, planned, and written up by LW and WL. The analytical KOA pain assessment and KOA radiographic grade were performed by HP and SP. LZ, ML, ZM using the Radio-immunoassay to identify the NPY concentrations. All authors read and approved the final manuscript.

Reviewer 2: Guang-hua Lei  
[1]. Results: NPY of Advanced stage of KOA patients has no significant difference. The sentence "has a downward trend compare with Middle stage of KOA patients” should be deleted.  
Answer:  
NPY in synovial fluid of Advanced stage of KOA patients has no significant difference to that for the Middle stage of KOA patients

[2]. Perhaps you could measure NPY expression in serum, urine, cartilage, synovium. If author has no original data, some discussion on this point may be added.  
Answer:  
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].

[3]. Needless duplication of the results in Tables and figures should be avoided (Table 3 and Figure 1, Table 4 and Figure 2).
Answer:

[4]. Pearson's correlation / Spearman's correlation and linear regression should be applied to determine the correlation between the levels of NPY and pain scores of KOA, and between the levels of NPY and radiographic grade).

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

Answer:

KOA pain assessment

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 1 Hideo Watanabe’s knee scoring system-related pain score
<table>
<thead>
<tr>
<th>Group</th>
<th>Standard</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**

Serious pain and/or edema and/or deformed side were assessed in the bilateral KOA. Full-extension radiographs (X-ray) of the knees were obtained and 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. (Table 2)

**Table 2** Tomihisa Koshino’s radiographic grading for osteoarthritic knees in a standing position

<table>
<thead>
<tr>
<th>Stage</th>
<th>Grade</th>
<th>Standing x-ray</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td></td>
</tr>
<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 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**

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 KOA Group with Healthy control group. Dunnett-t test was used to compare the mean of each subgroup of KOA Group with Healthy control group. And the Student-Newman-Keuls (SNK) test was used to compare the mean of each subgroup of KOA Group. A $p$ value less than 0.05 was considered to be statistically significant.

7. Discussion: The first paragraph of discussion; reads more like the end of introduction. The first paragraph should be deleted.

**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]. Therefore, the exact pathophysiology of KOA pain remains to be delineated completely. Due to the important role played by neuropeptide Y (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.

8. Discussion: Potential source of synovial fluid NPY should be discussed.

9. Discussion: This statement needs some discussion e.g. what possible role NPY plays in the pathogenesis of OA and OA pain?

**Answer:**

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 α₂-agonists through stimulation of its Y2 receptor subtype [58].