ORIGINAL RESEARCH PAPER
Volume - 10 | Issue - 06 | June - 2021 | PRINT ISSN No. 2277 - 8179 | DOI : 10.36106/ijsr
INTERNATIONAL JOURNAL OF SCIENTIFIC RESEARCH

EFFECTS OF THE COMBINATION OF KTTKS AND GEKG PEPTIDES ON AN EXPERIMENTAL RAT MODEL OF OSTEOARTHRITIS

Orthopaedics

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ABSTRACT

OBJECTIVE: The aim of this study was to analyze the therapeutic potential of the combination of two artificial synthetic peptides of KTTKS and GEKG in osteoarthritis (OA).

METHODS: The combination of KTTKS and GEKG (ICPPX) were injected into the intra-articular space in different dose (10 ppm, 50 ppm and 250 ppm). Grossly inspection of swollen status, running wheel test, pro-inflammatory markers of IL-1β and TNF-α were measured. Finally, histopathological examination of the knee joints was performed.

RESULTS: The swelling status were higher in the control group than treatment groups. However, the running wheel test showed no significant changes. Both pro-inflammatory markers of IL-1β and TNF-α were significantly lower in treatment groups. In the histopathological examination, the cartilage damage is most severe in the control group. Similar results were seen in the immunohistochemical stain of collagen type II staining.

CONCLUSIONS: The combination of KTTKS and GEKG peptides (ICPPX) injection ameliorates the joint inflammation and prevents further deterioration of the cartilage structure as manifested by the OARSI scoring system and collagen stain of the joint.

KEYWORDS

peptides; Osteoarthritis; KTTKS; GEKG; ICPPX

INTRODUCTION

Osteoarthritis (OA) is a most common form of degenerative joint disease affecting worldwide and mainly in elderly. It is estimated that approximately a tenth of the population aged over 50 years will be affected. [1] The knee is the principal joint affected resulting in not only pain bust also progressive loss of its function. [2] Articular cartilage is the mainly component in knee joint and it's an avascular, alymphatic, and aneural connective tissue. The articular cartilage composed of 65–80% of water and 10% of collagen, which provides tensile strength. Type II collagen is the major collagen in articular cartilage. It forms highly cross-linked interconnected network of collagen fibrils. The increased degradation of type II collagen fibrils was observed in OA patients. The degradation speed is faster than the synthesis rate in the cartilage of OA patients. Aggrecans diminish in parallel to the severity of OA, as there is a net loss of matrix due to the inability of chondrocytes to compensate for loss of proteoglycan. Although new aggrecan is synthesised to help with cartilage repair in the early stages of OA, it is lost to synovial fluid at later stages. [3] Moreover, the newly synthesised aggrecan may be different to the original in its composition, particularly in the content of keratan sulfate and chondroitin sulfate. [4] As a result of the overall effect on altered extracellular matrix network, the articular cartilage decreases the modulus of elasticity. The progression of cartilage degeneration would lead to synovitis. [5,6] Studies have demonstrated that chondrocytes, osteoblasts and inflamed synovium all produce and contribute to various proinflammatory cytokines. [7-10] The proinflammatory cytokine IL-1β has been implicated as the most important factor responsible for the catabolic process in OA. Other proinflammatory cytokines such as TNF-α, IL-6, and IL-8 have all been shown to contribute to the cartilage degradation process in OA. [7,11,12]

Short amino acid sequences, of less than 50 amino acids, can possess bioactivity and these include components or precursors of larger proteins such as collagens. [13] The most well-known peptide is KTTKS which is comprised of five amino acid residues. KTTKS is a subfragment of type I collagen propeptide and was discovered in 1993 with the ability to inhibiting collagenases activities and increasing extracellular matrix production [14,15] KTTKS was described as being the minimum sequence able to retain 80% of the original activity of the much larger parent peptide necessary for stimulating ECM synthesis, especially collagen types I and III and fibronectin expression. [15,16] It has been suggested that the stimulatory effect of KTTKS on collagen relates primarily to the biosynthetic pathway by maintaining the stability of mRNA in a process associated with the up-regulation of transforming growth factor-β expression, rather than the export or degradation pathways. [15,17] Despite the observation of promoting collagen synthesis, the mechanism of action of KTTKS has yet to be fully established.

An in-silico approach was used to develop efficient peptides by identifying highly repetitive amino acid motifs in several extracellular matrix proteins. The resulting tetrapeptide GEKG was assessed and the sequence repeatedly occurs in type IV collagen. Besides KTTKS, GEKG have been identified as matrikines for its effects on the formation of extracellular matrix components. GEKG was usually applied to the skin to reduce wrinkles by promoting the formation of collagen and different extracellular matrix genes such as alpha-1 type I collagen (COL1A1), hyaluron synthase 1 (HAS1) or fibronectin 1 (FN1). [18]

The two specific peptides (KTTKS and GEKG) were artificial synthetic and were both proven to be benefit for OA animal model previously. The aim of this study was to analyze the therapeutic potential of the combination of KTTKS and GEKG peptides (ICPPX) injection as an alternative to OA treatment in rat model. In this study, we found that the combination of KTTKS and GEKG peptides (ICPPX) was able to provide a protective effect by inhibiting the process of articular damage. This may be part of anti-inflammatory effect and promoting collagen synthesis effect on the combination of two specific peptides.

METHODS

Animal

In the current study, totally 28 Sprague-Dawley male rats (aged 8~12 weeks, weight 400-450 g) were purchased from the BioLASCO (Taiwan). All the animals were kept in our animal facility for at least 1 week before use. According to the experiments, they were divided into 4 groups with OA experimental model. All rats were randomly allocated to groups. All procedures on animals were approved by the animal ethics committee and they were housed in optimum conditions according to animal facility regulations. Briefly, all animals were allowed free access of water and food, humidity and temperature was controlled between 30~70% and 19~25°C. Light periods were set as 12 hours in the day and 12 hours in the night.

Study design

For the development of OA in rats, intra-articular injection of MIA (Monosodium iodoacetate) was performed. The injection of 50 μL

MIA (60 mg/mL) in both knees to mimic OA clinically, then randomly grouped into vehicle or treatment groups after 1 week later. The intra-articular injection of either vehicle or different dosage of peptides were performed once a week, and last four weeks. Four groups are defined as the control group and the treatment groups of different peptide levels (10 ppm, 50 ppm, 250 ppm). Briefly, 50 μL of normal saline (control group) or peptides (10 ppm, 50 ppm, 250 ppm) were injected once a week, totally 4 times of injections. All animals were sacrificed at 5th weeks after randomly grouped at beginning. Running wheel tests, inflammation cytokines (IL-1β and TNF-α) tests in intra-articular fluid and histopathological analysis of cartilage tissues were performed at time of sacrifice. Healthy right knees of operated animals were used as healthy tissue control. Before sacrifice, running wheel test was performed.

Peptides

In the current study, there were two specific peptides were used. One is the KTTKS peptide and another is a GEKG peptide. The combination of KTTKS and GEKG peptides were given by Yusen biotechnology company (Taipei, Taiwan) with the commercial brand name of ICPPX. The original concentration of ICPPX was 5000 ppm and we diluted to be 10 ppm, 50 ppm and 250 ppm in different rat groups.

Intra-articular fluid analysis

All mice were sacrificed by carbon dioxide. Before sacrifice, intraarticular injection of 100 μL normal saline were performed in both knees. 50 μL fluid would be extracted for IL-1β and TNF-α analysis. Both IL-1β and TNF-α analysis were performed by Elisa kit.

Running wheel test

Experimental rats were placed into the running wheels (16 cm diameter, 7 cm width) at the 4th weeks. The speed was set as 5 rpm in the beginning and would speed up to 40 rpm. The time of rat sustained in the wheel before falling down was recorded. The longer time means the exercise activity is better.

Histopathological examination

The knee joints were removed and fixed in 10% formalin for 3 days, followed by decalcified solution based on EDTA disodium for 7 days. After decalcification, the joints were embedded in paraffin blocks, and histological coronal sections were obtained. The paraffin blocks were sectioned at 3-5 μm thickness, and stained with hematoxylin and eosin (H&E). H&E staining was used to examine morphological changes. In addition, immunohistochemical stain of collagen was performed to aid in evaluation of cartilage damage. The severity of articular cartilage damage was evaluated using the modified Osteoarthritis Research Society International (OARSI) scoring system. [19] The medial tibia cartilage degeneration score, total cartilage degeneration width (mm), significant cartilage degeneration width (mm), cartilage matrix loss (mm), zonal depth ratio of lesions and collagen score were evaluated.

Immunocytochemistry, and Scoring

The paraffin-embedded blocks were cut in to 5-mm-thick sections to perform immunohistochemical stain (IHC). IHC staining was performed using the Ventana BenchMark XT automated stainer (Ventana, Tucson, AZ). The primary antibody was carried out by collagen II (1:100; Calbiochem, San Diego, CA, US). Each run included phosphate buffered solution used as the primary antibody for the negative controls.

STATISTICALANALYSIS

All data are presented as mean ± SEM (standard error of mean). The significance of differences between groups was determined with Student's t-test (two-tailed) and one-way analyses of variance (ANOVA) with Bonferroni posttest. Differences were considered significant at $p < 0.05$.

RESULTS

The gross inspection of knee joint and running wheel test In figure 1 (A) and (B) showed grouping and experiment design. Briefly, rats were divided into four groups, control group, treatment with ICPPX 10 ppm, ICPPX 50 ppm and ICPPX 250 ppm, all groups were injected with MIA to create OA experiment model of rat. The swelling status of the control group and untreated side of different dosage treatment group showed the model grossly success. When compared with control group, treatment group showed decrease swelling ratio of the bilateral knee. However, no significant changes between different dosage within 4 weeks (figure 2A). We further test the usage of the knee in the running wheel. Surprisingly, no difference between control group and any treatment groups. This may be due to the individual difference and muscle endurance difference (figure 2B).

Inflammatory markers analysis in the intra-articular fluid

To further analysis the inflammatory status of the knee joint, intra-articular fluid was tested for pro-inflammatory markers of IL-1β and TNF-α. The level of IL-1β showed 24.57 ± 3.64 (pg/mL) in control group, 16.79 ± 7.30 (pg/mL) in 10 ICPPX treatment group, 14.10 ± 3.73 (pg/mL) in 50 ICPPX treatment group, and 10.67 ± 5.63 (pg/mL) in 250 ICPPX treatment group. The level of TNF-α showed 71.22 ± 16.26 (pg/mL) in control group, 25.83 ± 13.00 (pg/mL) in 10 ICPPX treatment group, 17.58 ± 8.73 (pg/mL) in 50 ICPPX treatment group, and 14.48 ± 2.50 (pg/mL) in 250 ICPPX treatment group. Both IL-1β and TNF-α were lower in the treatment groups with dose dependence effect (figure 2C and 2D). Moreover, the treatment effect on IL-1β was weaker with dominant dose dependence. The treatment effect on TNF-α was stronger which ambiguous the dose dependence effect than IL-1β.

Histopathological examination

To see the morphological changes and to quantify them, the scoring criteria was use according to the previous literature. [19] In figure 1C also showed the scoring criteria of the cartilage degeneration. Each of the cartilage degeneration result showed significant better in the treatment group including the medial tibia cartilage degeneration score (figure 3A), total cartilage degeneration width (mm) (figure 3B), significant cartilage degeneration width (mm) (figure 3C), cartilage matrix loss (mm) (figure 3D), zonal depth ratio of lesions (figure 3E), and collagen score (figure 3F). The overall results showed effective in the treatment group of each dosage when compared with control group. Although there was a trend to be dose dependence, no significant was seen in the different dosage group. In the H&E stain, the MIA injected knee cartilage damage is obvious in all group when compared with MIA uninjected knee cartilage which provide the strong evidence of model success. When compared with control group, all treatment groups show mild cartilage damage in the left knee joint. The cartilage damage is most severe in the control group and followed by peptide 10 group, peptide 50 group and peptide 250 group. The mildest cartilage damage is in the peptide 250 group which shows the dose effect of the treatment in this experiment (figure 4A~D). Similar results were seen in the immunohistochemical stain of collagen type II staining. The surface of the cartilage was discontinued and bookend in the control group. The linine showed more continuous and more intact in the treatment group of ICPPX 250 ppm, followed by 50 ppm and 10 ppm (figure 4E~H).

DISCUSSION

This study aimed to evaluate the protective effect of the combination of KTTKS and GEKG peptides (ICPPX) in an experimental model of knee OA in rats. It is the first study to evaluates the effect of the combination of KTTKS and GEKG peptides (ICPPX) in knee joint of OA rats. During this study, we successfully established an animal model of knee osteoarthritis, evident as significantly different OARSI scores between right and left knee in groups receiving peptides treatment. After 4 times peptides treatment, histopathological analysis of the synovial membrane and articular cartilage showed encouraging results, suggesting the possible efficacy of the combination of KTTKS and GEKG peptides (ICPPX) in the treatment of OA is partial dose dependent. By inflammatory markers analysis in intra-articular fluid, both IL-1β and TNF-α level were decreased when compared with the vehicle group. In addition, the reduction level showed dose dependent. These findings may lead us to hypothesis that the reduction of inflammatory cytokines and further reduce cartilage damage.

KTTKS promotes the synthesis of type I collagen by maintaining the stability of mRNA in a process associated with the up-regulation of transforming growth factor-b (TGF-b) expression. [17] In addition, the steady-state levels of the messenger RNAs encoding type I procollagen was not measurably by treatment with KTTKS, which lead us to think that KTTKS was active during the post-transcriptional phase. [15,20] This can be supported by no changes were observed either in the ratio of secreted/cell-associated collagen or in the rate of intracellular degradation. Proline content may directly reflect collagen synthesis primarily because of its co-translational conversion to hydroxyproline which constitutes a relatively high proportion of the total amino acid composition of collagens. [21] GEKG has strong interaction potential as it exhibits 7 proton donors and 9 acceptors for hydrogen bond formation. [22] This peptide is less addressed in the

literature and mostly use in the cosmetic industry. The efficacy of the peptide to significantly induce collagen production of the protein level and mRNA level has been demonstrated. The effect of GEKG on facial wrinkles was also been shown to be effective. [18] Therefore, the effect of both KTTKS and GEKG on collagen production can be consistent with our result and showing benefit in OA model of rat.

In general, OA treatment is based on drugs that control the pain and inflammation associated with synovitis instead of reduce the destruction of the articular cartilage. [23] Therefore, a treatment option with the potential cartilage or synovial membrane protection are needed in clinical. Thus, the possible protective effect of the combination of KTTKS and GEKG peptides (ICPPX) on the cartilage demonstrated in the present study may justify the translation of its use for this purpose. The reduction of the IL-1β and TNF-α in synovial fluid may be associated with a reduction in the inflammatory infiltrate in the synovial membrane and, consequently, reduction of edema and cartilage damage. The supporting evidence in the histopathological observation was seen which is compatible with previous knowledge that synovial inflammation in OA is usually found next to pathologically damaged areas with bone and cartilage, releasing proteinases and cytokines capable of accelerating joint destruction. [24]

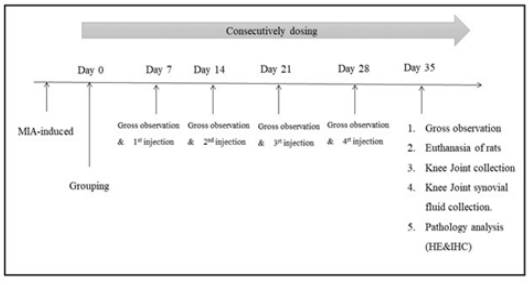
The effects of the combination of KTTKS and GEKG peptides (ICPPX) on intra-articular fluid analysis and histological observation in OA presented in the study are impressive, however, this work had some limitations that need to be highlighted. No further underlying mechanism exploration such as anti-inflammation pathway or the involvement of signaling pathway induce collagen synthesis. Also, in future studies, a centrally acting medication could be used as a positive control, in addition to evaluating different doses and their possible effects on the inhibition of inflammation and synthesis of collagen.

In summary, the present study concludes that the combination of KTTKS and GEKG peptides (ICPPX) was able to provide a protective effect by inhibiting the process of articular damage. This may be part of anti-inflammatory effect and promoting collagen synthesis effect on the combination of two specific peptides. Future research investigating the signal pathway and underlying mechanism are required to establish the definite effect in this model.

(A)

Group	Treatment	Route	N	Frequency
A	Control	-	3	-
B	Peptide ICPPX 10 ppm	intra-articular injection	8	Once weekly (for three weeks)
C	Peptide ICPPX 50 ppm	intra-articular injection	8	Once weekly (for three weeks)
D	Peptide ICPPX 250 ppm	intra-articular injection	8	Once weekly (for three weeks)

(B)



(C)

Cartilage degeneration score	
Grade	Description
0	No degeneration
1	Minimal degeneration; 5-10% of the total projected cartilage area affected by matrix or chondrocyte loss
2	Mild degeneration; 11-25% affected
3	Moderate degeneration; 26-50% affected
4	Marked degeneration; 51-75% affected
5	Severe degeneration; greater than 75% affected

Adapt from Osteoarthritis Cartilage. 2010; 18 Suppl 3:S24–34.

Figure 1 Study design of the current experiment

In figure 1 (A) and (B) showed grouping and experiment design. In figure 1(C) showed the scoring criteria of the cartilage degeneration.

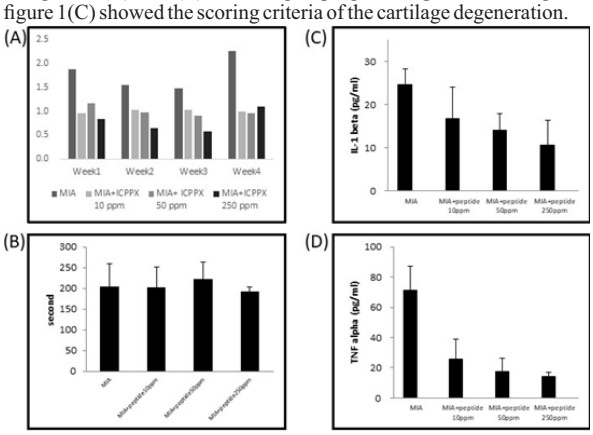


Figure 2 The quantitative results of the pro-inflammatory marker

In the figure 2 (A), the swelling status of the bilateral knee showed highest ratio in t control group and lesser in treatment groups of different dosage. The running wheel test was not seen significant differences between groups (B). However, both IL-1β and TNF-α were lower in the treatment groups with dose dependence effect (C and D).

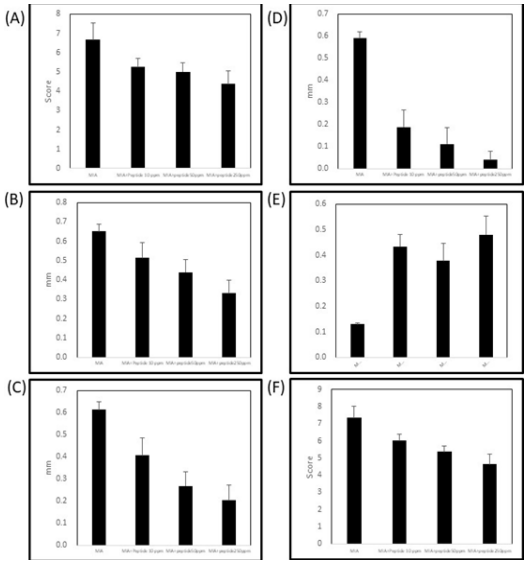


Figure 3 The quantitative results of the cartilage degeneration

Each of the cartilage degeneration result showed significant better in the treatment group including (A) the medial tibia cartilage degeneration score, (B) total cartilage degeneration width (mm), (C) significant cartilage degeneration width (mm), (D) cartilage matrix loss (mm), (E) zonal depth ratio of lesions, and (F) collagen score.

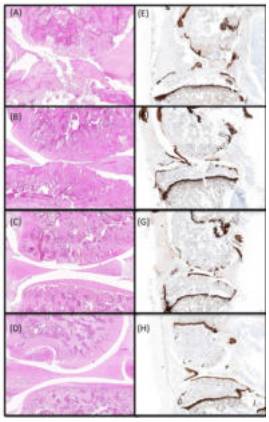


Figure 4 Representative figures of H&E and collagen type II immunohistochemical stain

In the figure 4 (A) ~ (D) showed representative H&E figures and (E) ~ (H) showed representative figures of collagen type II immunohistochemical stain in each group. In the control group, H&E (A) showed destructive cartilage and similar finding in the collagen type II immunohistochemical stain (E). As the treatment dose increased, 10 ppm ICPPX in (B) and (F); 50 ppm ICPPX in (C) and (G); 250 ppm ICPPX in (D) and (H), the morphology of cartilage destruction became better, which was consistent with the findings in collagen type II immunohistochemical stain.

CONCLUSION

The combination of KTTKS and GEKG peptides (ICPPX) injection ameliorates the joint inflammation and prevents further deterioration of the cartilage structure as manifested by the OARSI scoring system and collagen stain of the joint, which lead us to the conclusion that the combination of KTTKS and GEKG peptides (ICPPX) has beneficial role in treating OA. It is a potential disease-modifying treatment option in OA.

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