Original Article

Histological and Immunohistochemical Study of the Possible Curative Effect of Intra-articular Injection of N-Acetyl Phenylalanine Glucosamine on Surgically Induced Osteoarthritis in Knee Joint of Adult Male Albino Rat

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ABSTRACT

Introduction: Osteoarthritis (OA) is the most common degenerative joint disease for which there is no permanent treatment. The most affected structures are articular cartilage and subchondral bone. Knees are the most affected by OA, thus leading to severe disability. N-Acetyl phenylalanine glucosamine (NAPA) is a derivative of glucosamine (GlcN), which is one of the components of glycosaminoglycans (GAGs) of cartilage extracellular matrix (ECM).

Aim of the **Work:** This work aimed to study the possible curative effect of N-acetyl phenylalanine glucosamine on surgically induced osteoarthritis in the knee joint of the adult male albino rat.

Materials and Methods: This study was performed on 30 adult male albino rats. They were divided into three groups: group I included ten animals that were subdivided into two subgroups, IA (control) & IB (underwent Sham operation), group II included ten animals underwent destabilization of medial meniscus (DMM) surgery without receiving any treatment, group III included ten animals underwent DMM surgery then received a single intraarticular injection of NAPA after 4 weeks from the surgery and sacrificed 4 weeks later. At the end of the experiment, knee joints were obtained and processed for histological and immunohistochemical studies.

Results: Hematoxylin and Eosin-stained sections showed a moderate degree of OA in group II that was improved in group III. Mallory's trichrome stained sections revealed decreased collagen content in group II that was also improved in group III. The same results were found as regards the GAGs content of the cartilage matrix that was clearly decreased in group II and restored in group III.

Conclusion: From the present study, it could be concluded that the osteoarthritic changes induced by surgical DMM in adult male albino rats can be improved by subsequent intra-articular administration of NAPA.

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Key Words: DMM surgery, knee Joint, NAPA, osteoarthritis.

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INTRODUCTION

Osteoarthritis (OA) is the commonest degenerative joint disease that does not have a permanent cure. All joint tissues are involved in this degenerative process, especially the articular cartilage and the subchondral bone (SB). The knees are the most affected joint by OA and may result in significant disability.[1,2]

The process of osteoarthritis (OA) includes localized loss of cartilage, remodeling of adjacent bone, and subsequent inflammatory reaction. The initial degenerative event is opposed by a slow repair process that leads to structural alterations of the joint. Symptomatic osteoarthritis and subsequent joint failure occur because of significant degeneration and/or compromised repair process. The variability in the repair process may explain the remarkable variability in clinical presentation among patients, as well as at different joints in the same patient.[3]

Damage of the ECM leads to loss of the shock absorber function of the articular cartilage. Other joint tissues, including the subchondral bone and the synovial membrane, are also involved in this degenerative process. In addition, the articular cartilage has a pivotal role in the initiation and progression of OA. The articular cartilage resistance is due to the structure of the extracellular matrix so, degradation of the matrix leads to failure of the cartilage to bear cyclic loading leading to further degeneration.[4,5]

Osteoarthritis is also characterized by altered chondrocyte metabolism leading to reduced glycosaminoglycan content and increased water content. Matrix metalloproteinase (MMP) enzyme plays a significant role in the degradation of both proteoglycans (PGs) and collagen. Damage of the cartilage results in bone-to-bone contact with subsequent worsening of joint motion. Degenerated hyaline cartilage cannot be regenerated because adult chondrocytes cannot undergo mitosis.[6,7]

The articular cartilage regenerative process is very slow due to the well-established collagen network and the long half-life of its proteoglycan molecules. Furthermore, the MMP activity in healthy articular cartilage is low.[8,9]

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Glucosamine is an aminosaccharide constituent of some glycosaminoglycans such as hyaluronic acid and keratan sulfate, which are present in the articular cartilage and the synovial fluid. Multiple formulations of oral glucosamine have been tried, including glucosamine sulfate and glucosamine hydrochloride.[10]

N-Acetyl phenylalanine glucosamine (NAPA) is a derivative of glucosamine (GlcN), which is one of the glycosaminoglycans (GAGs) of the extracellular matrix of the cartilage (ECM).[11]

Surgical instability models are the most used models of OA in experimental animals. Advantages of surgical models over natural spontaneous models include faster disease onset, minimal variability, and decreased effect of genetic predisposition. Smaller animal models are preferred for initial screening because of the high expense of maintaining larger species and their greater drug needs. Among other surgically induced OA models, DMM surgery can reproduce the slow process of natural disease.[12,13]

DMM model in the rat was also selected because it is easy and reproducible. It produces a mild to moderate OA, so the effects of disease alteration would not be covered by the biomechanic damage related to severe models. Furthermore, severe models of OA have greater iatrogenic damage, variability, and prominent regenerative changes such as osteophyte formation that can hinder the evaluation of cartilage degradation.[14,15]

In this work, DMM surgery was performed to produce an OA model in the rat knee joint to evaluate the curative effect of intra-articular NAPA injection as a possible treatment for that degenerative disease.

MATERIALS AND METHODS

I- Experimental animals

Thirty adult male albino rats, weighing 170-230 grams each, were utilized in this study. The rats were kept in plastic cages under good illumination and ventilation conditions with access to laboratory diet and water according to recommendations by the National Research Council of the National Academies.[16] Rats with gait disturbance or swollen joints were excluded. Rats were adapted to their environment one week before starting the experiments.

All experiments were performed at the animal house of the Medical Research Institute at Alexandria University. The processing, staining, and image acquisition of the specimens were done at Histology department of Tanta Faculty of Medicine.

The animals were divided into three main groups

Group I: Consisted of ten rats used for histological study of the normal knee joint tissue. Those were further divided into two subgroups: subgroup (IA) consisted of five rats as control, and subgroup (IB) consisted of five rats which underwent Sham surgery. They were all sacrificed after six weeks from the start of the experiment.

Group II: Consisted of ten rats which underwent DMM surgery without receiving any treatment and they were sacrificed after four weeks.

Group III: Consisted of ten rats which underwent DMM surgery, and after four weeks, they received a single intra-articular injection of NAPA and they were sacrificed four weeks later.

II- The experimental procedures

1- Surgical induction of osteoarthritis by destabilization of the medial meniscus (DMM) surgery

The rats were anesthetized by intramuscular injection of Ketamine (100 mg/kg) and Xylazine (5 mg/kg), then the hair over the right knee joint was shaved, and the skin was sterilized with betadine.[17] A longitudinal incision over the distal patella to the proximal tibial plateau was performed. The joint capsule medial to the patellar tendon was incised with a blade, and the joint capsule was opened with scissors. The intercondylar region was exposed by blunt dissection of the overlying fat pad to identify the meniscotibial ligament of the medial meniscus that was sectioned with the blade directed proximolaterally.[18] Cefotaxime (250 mg/ml) and Ropivacaine hydrochloride (5 mg/ml) were used locally at the wound at a dose of 0.01 ml.[17] After replacement of the extensor muscles, the skin was closed with unabsorbable silk sutures. The rats were then allowed to unrestricted activity with free access to food and water.[17]

2- Sham operation

Sham operation was performed using the same approach as DMM surgery without MML transection. It was performed to exclude the local response following the surgical incision from the inflammatory effect of the DMM on the articular cartilage and the subchondral bone.[18,19]

3- Intra-articular Glucosamine injection

After anesthesia with Ketamine and Xylazine, the hair over the right knee was shaved. The skin was sterilized with betadine. The knee joint was fully flexed with the rat on its back. 0.1 ml of NAPA at a concentration of 2.5 mM was injected by an insulin syringe.[11] NAPA was purchased from Sigma-Aldrich Company, Cairo (Egypt), in the form of white powder with a molecular weight 221.21 in a glass bottle containing 10 mg. To prepare the concentration of 2.5 mM of NAPA, 10 mg was dissolved in 18 ml of distilled water.[20]

III - Processing of the knee specimens

Rats were anesthetized by diethyl ether inhalation then sacrificed and the knee joints were excised and immediately fixed.[21] Disodium EDTA solution was utilized for decalcification of specimens.[22] The decalcified knee joints were dehydrated then cleared. The specimen was then Impregnated in pure soft paraffin and finally embedded in hard paraffin. The blocks were cleaved longitudinally in a sagittal plane along the central portion.

Serial sections were subjected to either Hematoxylin & Eosin, Mallory's Trichrome, or Safranin O/ Fast Green staining. Immunohistochemical staining using anti MMP-3 antibodies was also performed, negative control sections were done without addition of the primary antibody and the positive control was from placenta.[21]

Olympus light microscopy (Tokyo, Japan) coupled with Olympus digital camera (DXC-1850p, Tokyo, Japan) were used for image acquisition, and then Image J software was used to measure the thickness of articular cartilage in the center of each knee joint in (×400) H&E-stained sections, area percentage of collagen fibers (%) in Mallory's trichrome stained sections (×400), color intensity in Safranin O\Fast Green stained sections (×400) and the proportion of cells expressing MMP-3 in immune-stained section. The data were statistically analyzed using the SPSS program and were represented as mean ± standard deviation (SD).

RESULTS

A) histological light microscopic results

The two subgroups of group I had a similar microscopic picture. Hematoxylin and eosin-stained sections showed a histological structure similar to the normal knee joint that consisted of two opposing regular smooth articular cartilages of the hyaline type with no perichondrium. Four poorly demarcated zones were identified: a superficial zone, an intermediate zone, a radial zone, and a calcified zone. The superficial tangential zone contained flat elongated chondrocytes, the intermediate transitional zone showed rounded randomly distributed chondrocytes, the deep radial zone demonstrated rounded chondrocytes arranged in short columns perpendicular to the surface, while the calcified cartilage zone was separating the radial zone from the underlying subchondral bone with small scattered chondrocytes; also a deep basophilic line (tidemark) was seen separating the radial uncalcified zone from the calcified zone. The subchondral bone was composed of a subchondral bone plate (compact bone) and subchondral trabecular bone (cancellous bone) (Figure 1A).

In Mallory's trichrome stained sections, both subgroups illustrated normal arrangement of collagen fibers (blue color) in the territorial and the interterritorial matrix of the uncalcified zones and in the territorial matrix of the calcified zone of the articular cartilage (Figure 1B). The Safranin O/ fast green stained sections demonstrated homogenous red staining of extracellular matrices of the articular cartilages of both subgroups, mainly in the non-calcified regions and to less extent in the calcified regions (Figure 1C).

In group II, the two articular cartilages of the joint were the most affected structures and demonstrated different degrees of damage in H&E-stained sections. There was a significant reduction of the thickness up to a focal loss of the cartilage. The cartilage surface demonstrated a wide range of irregularities from focal to diffuse areas of superficial erosions. Loss of the normal zonal layers arrangement with the superficial layer showing cells with pycnotic nuclei and vacuolated cytoplasm or completely lost. Furthermore, focal disappearance of the calcified zone and the tidemark were seen, with cartilage replacement by the underlying subchondral bone. The tidemark showed either focal fainting and discontinuity or complete absence (Figure 2A).

In Mallory's trichrome stained sections, marked reduction in the collagen fibers was observed in the uncalcified zones of the articular cartilage. Moreover, a complete absence of the collagen fibers in the interterritorial matrix was noticed in many areas of these zones. However, there were preserved collagen fibers of the territorial matrix (Figure 2B). Safranin O/ fast green stained sections showed fainting of the red color up to complete absence was observed due to reduction or depletion of the ground substance, especially in the destroyed areas (Figure 2C).

Different degrees of improvement were observed in group III (The NAPA treated group) with decreased, arrested, or improved osteoarthritic changes. This group revealed incomplete recovery compared with the control group I and relative improvement compared with group II that appeared in H&E-stained sections. There was restored cartilage thickness and zonal layers arrangement, but the tidemark was still faint (Figure 3A).

Examination of Mallory's trichrome stained sections in this group revealed increased collagen fibers in the matrix of the uncalcified zones of the articular cartilage, especially in the territorial matrix (Figure 3B). There were few areas with absent collagen fibers, mostly in the interterritorial matrix. Safranin O/ fast green stained sections revealed moderate to deep staining of the ground substance of the articular cartilage (Figure 3C).

B) Immunohistochemical Reaction For Detection Of MMP-3

Immunohistochemically stained sections of group I showed very weak or faint MMP-3 positive immunoreactivity in a few chondrocytes of the articular cartilage of both control and sham rats (Figure 4A), while a large number of MMP-3 positive chondrocytes were seen in group II, especially in the non-calcified zones (Figure 4B). Immunoreactivity was still present in group III with minimal difference between its two subgroups. However, decreased intensity than the previous group was noticed (Figure 4C).

C) Quantitative Assessment and Statistical Results

1. Measurement of articular cartilage thickness

Statistical analysis of the data collected by the image analysis software program (Image J, version 1.48) revealed that there was a statistically significant difference in both femoral and tibial cartilage thickness between the three groups ($p=0.009$). Paired-wise comparisons between different groups showed that group II had statistically significant lower thickness than group I. Group III had

greater thickness than group II, but the difference was not statically significant. Group III had a lower thickness than group I, but the difference was not statistically significant. The mean (±SD) femoral cartilage thickness for group I was 291.72 ± 20.71 and for group II was 209.43 ± 33.60 and for group III was 276.86 ± 16.45. The mean (±SD) tibial cartilage thickness for group I was 148.01 ± 8.34 and for group II was 111.43 ± 8.53 and for group III was 137.99 ± 9.12.

2. Evaluation of collagen fiber content

Mallory's trichrome stained sections (×400) from all groups were used to evaluate the mean area percentage of collagen fiber content. The data were collected and then statistically analyzed. There was a statistically significant difference in the area percentage of collagen fibers between the three groups (p =0.001). Paired-wise comparisons between different groups showed that group II had a statistically significant lower area percentage than group I. Group III had a statistically significant greater area than group II and a statistically significant lower area than group I. The mean (±SD) area percentage of collagen fiber content in group I was 45.23 ± 0.81 and for group II was 43.02 ± 1.19 and for group III was 177.25 ± 1.25.

3. Evaluation of proteoglycan and glycosaminoglycans content

Safranin O\Fast Green stained sections (×400) were used to assess the red color intensity that stains proteoglycan and glycosaminoglycans of the cartilage matrix. There was a statistically significant difference in the mean color intensity between the three groups (p =0.002). Paired-wise comparisons between different groups showed that group II had statistically significant lower color intensity than group I. Group III had statistically significant greater color intensity than group II and lower intensity than group I. The mean (±SD) color intensity in group I was 182.61 ±7.8 and for group II was 43.02 ± 1.19 and for group III was 177.25 ± 1.25.

4. Counting the number of MMP-3 positive chondrocytes

The total number of chondrocytes and positive immunostained chondrocytes were counted in three different fields (×400) of each slide, and the mean number of these cells was used to determine the proportion of cells expressing MMP-3. There was a statistically significant difference in the ratio of the positive MMP-3 chondrocytes between the three groups (p =0.001). Paired-wise comparisons between different groups showed that group II had a statistically significant higher ratio of positive MMP-3 chondrocytes than group I. Group III had a statistically significant lower ratio than group II and a higher ratio than group I. The mean (±SD) ratio of positive MMP-3 chondrocytes in group I was 10.99 ± 5 and for group II was 81.19 ± 3.36 and for group III was 42.93 ± 7.46.

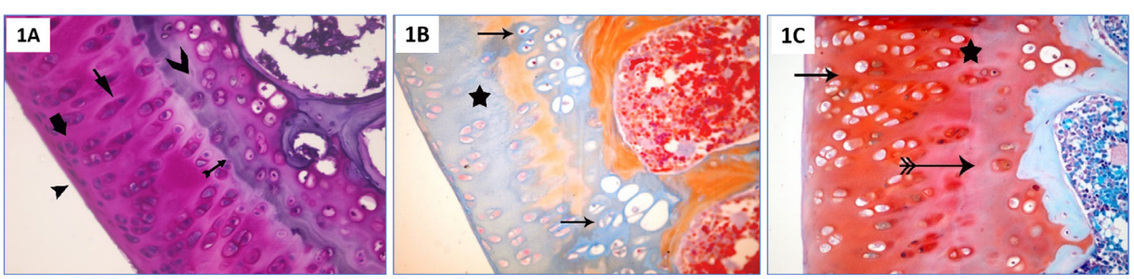

Fig. 1: A photomicrograph of articular cartilage of group I: (1A) showing the four zones of the articular cartilage: the superficial (arrowhead), the intermediate (thick arrow), the radial (thin arrow), and the calcified (bifid arrowhead). Notice the tidemark (bifid arrow) (H&E x400). (1B) showing normal distribution of the collagen fibers in the territorial and the interterritorial matrix of the uncalcified zones (star) and in the territorial matrix of the calcified zone (arrows) (Mallory's trichrome x400). (1C) showing homogenous deep red color of the articular cartilage matrix (star). The red color is deeper in the non-calcified (arrow) than in the calcified zone of the articular cartilage (double-bifid arrow) (Safranin O/ fast green x400).

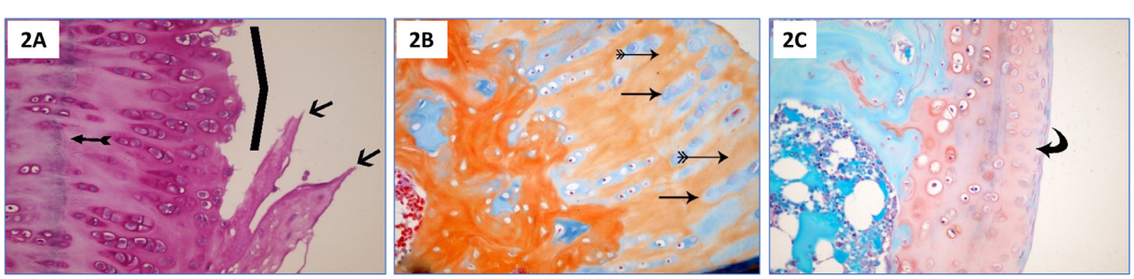

Fig. 2: A photomicrograph of articular cartilage of group II: (2A) showing loss of the superficial layer of the articular cartilage (kinked line) with irregularities (arrows). There is fainting of the tidemark (bifid arrow) (H&E x400). (2B) showing complete absence of the collagen fibers in the interterritorial matrix was noticed with preservation of the collagen fibers in the territorial matrix (arrows) (Mallory's trichrome x400). (2C) showing marked depletion of the ground substance (heterogenous fading of the red color) of the articular cartilage (curved arrow) (Safranin O/ fast green x400).

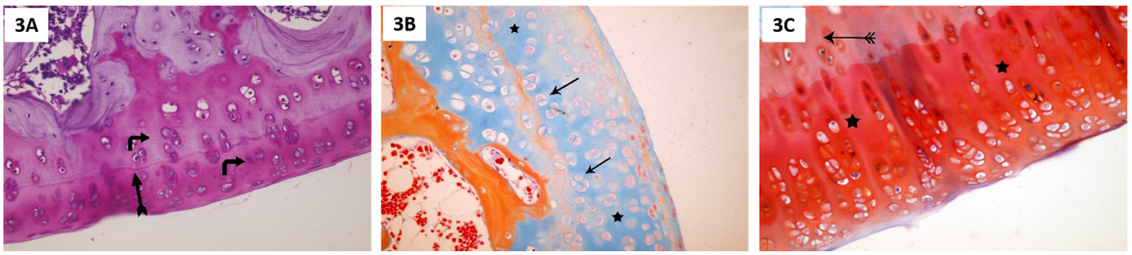

Fig. 3: A photomicrograph of articular cartilage of group III: (3A) showing restoration of the normal zonal organization (curved arrows) and faint tidemark (bifid arrow) (H&E x400). (3B) showing an increase in the collagen fibers of the interterritorial (stars) and the territorial matrix (arrows) in almost all cartilage (Mallory's trichrome x400). (3C) showing restoration of the red color (stars) with a lighter calcified area (double-bifid arrow) (Safranin O/ fast green x400).

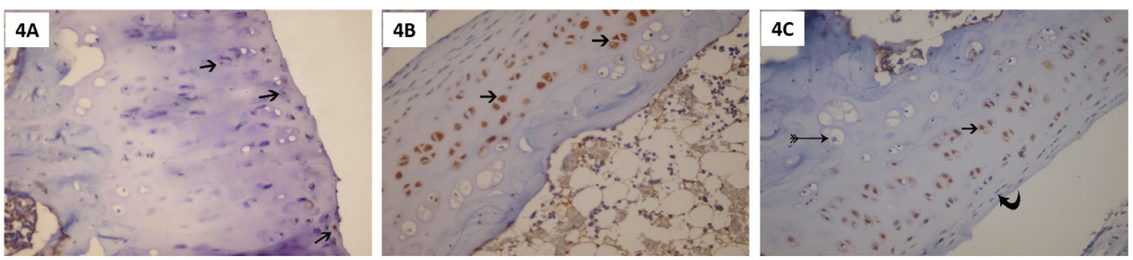

Fig. 4: (4A) A photomicrograph of articular cartilage from group I showing a faint positive cytoplasmic and nuclear MMP-3 immunoreactivity in a few cells of the articular cartilage (arrows), (4B) showing a group II photomicrograph with strong positive cytoplasmic and nuclear MMP-3 immunoreactivity in many cells (arrows), (4C) from group III showing moderate positive cytoplasmic and nuclear MMP-3 immunoreactivity in the intermediate and radial zone (arrows) with a negative reaction in the superficial (curved arrow) and the deep layer (bifid arrow) (MMP-3 immunostaining counterstained with Hx, × 400).

DISCUSSION

Knee osteoarthritis (OA) is the commonest degenerative disease of the joints. However, there is still no effective treatment for it. The experimental chemical and surgical models of OA have been developed to evaluate the pathogenesis of OA and to investigate the curative effect of different therapeutic modalities. At later phases, these models display characteristics similar to established OA in humans. However, the early pathogenesis might differ between the models and humans.[23,24]

In this study, DMM was performed to induce an OA-like model in adult male rats to discover the efficacy of NAPA in counteracting the surgically stimulated inflammatory changes. Veronesi *et al* cleared that DMM surgery has multiple advantages over other models, including the high reproducibility, generation of slowly progressive moderate OA, and minimal iatrogenic damage. Furthermore, DMM initiates changes similar to OA that occur with aging and is more reliable than other surgical techniques.[20]

These changes were observed in the hematoxylin and eosin-stained sections in the form of a wide range of surface abnormalities of both articular cartilages, including erosions, irregularities with loss of smoothness (fibrillations), or focal loss with decreased thickness, especially in weight-bearing areas and dipping of the uncalcified cartilage into the subchondral bone up to focal absence of the calcified cartilage in these areas. Tidemark line displayed disruption in the form of fainting, discontinuity, or absence. The chondrocytes revealed loss of their normal zonal organization, degeneration, apparently reduced number (hypocellularity). All these microscopic alterations were also described by.[20,25]

Both tibial and femoral cartilages width were assessed by the micro ruler using Image J program. Statistical analysis revealed that this group had statistically significant lower thickness than the control group.

These changes were associated with decreased collagen content of the matrix, as observed in Mallory's trichrome stained sections. It appeared as decreased blue color affinity in the articular cartilage, heterogeneous staining, or complete absence and replacement. These results were also noticed by Szychlinskaand coworkers[26]. This was confirmed by quantitative assessment of the percentage of the area of collagen fiber content stained by the blue color of the Mallory's trichrome stain using Image J program in different sections (×400). Statistical analysis cleared that this group had a statistically significant lower area percentage than the control group.

Safranin O/Fast Green was used to distinguish ground substance contents, especially proteoglycans and glycosaminoglycans.[27] The DMM-only group (group II) showed a marked decrease in the red staining affinity, and this finding was the same as Iijima *et al* and Hu *et al* finding.[18,28] For more confirmation, the color intensity was measured using Image J. The data was then statistically analyzed and cleared that this group had statistically significant lower color intensity than the control group. All these findings ensured the arthritic changes following the DMM surgery and suggested that it can be taken as a model for OA.

Iijima *et al* demonstrated that DMM results in an increase of contact stress in the medial compartment of the knee and alters the distribution of mechanical loading in menisci-covered and uncovered cartilage.[18] This

was also described by Bendele who reported rapidly progressive degenerative changes in the cartilage 3-6 weeks after iatrogenic unilateral medial meniscal tear.[29] Early changes after surgery occurred as a result of matrix proteases production due to mechanical stress.[30] In trying to understand the mechanism of the osteoarthritic changes following DMM, Aktas *et al* and Hwang & Kim stated that series of mechanical and biochemical processes happened after the surgical destabilization and resulted in chondrocytes death, as well as an imbalance between synthesis and degradation of articular cartilage matrix.[31,32] Although the initial trigger in this degenerative process is mechanical, Rutgers *et al* reported that the inflammatory events are important as the degenerative events. Matrix metalloproteinases (MMPs) and cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-alpha (TNF- α) play a pivotal role in this inflammatory process[33]

The role of MMPs, especially MMP-3, was also illustrated by Aktas *et al* who demonstrated an increased level of these enzymes after mechanical stress, thus leading to damage of the cartilage matrix.[31] IL-1 and TNF- α are involved in the progressive cartilage damage due to stimulation of MMP secretion from chondrocytes and synovial tissues. The authors also used anti MMP-3 antibodies to confirm the proposed role of the matrix metalloproteinase enzyme. The chondrocytes of the DMM group showed greater uptake of the DAB brown color, and this was quantitively assessed by counting the positive cells using the Image J software and calculating the ratio.

During the past few years, oral glucosamine was commonly used as a symptomatic drug for OA treatment. However, the therapeutic effect is limited as regards disease modification.[34] Here in this project, the glucosamine derivative NAPA was used locally as an intra-articular injection, and the following results were observed. Group III showed a noticeable recovery of the changes in comparison with the DMM-only group. As regards to H&E-stained sections from this group, the tibial and femoral cartilage thickness appeared to be regained, this was confirmed by quantitative assessment of the cartilage thickness. This improvement in the thickness was associated with improvement of the matrix collagen fibers, as observed in Mallory's trichrome stained sections. It appeared as increased blue color affinity and more homogenous staining of the articular cartilage, and this was also described by Wang & Cai.[35] Measurement of the collagen area percentage using Image J software and subsequent statistical analysis confirmed the progression.

Safranin O/Fast Green sections demonstrated better red staining affinity in all zones suggesting regained proteoglycans and GAGs content of the matrix. These results were also described by Scotto d`Abusco *et al* and Veronesi *et al*, who reported that NAPA resulted in more homogeneous chondrocyte cellularity, absence of fragmentation, and more intense staining of the matrix with Alcian Blue compared to those treated with saline solution.[11,20]

Studies by Scotto d`Abusco *et al* proposed that NAPA could play an important disease-modifying role in OA by its anti-catabolic and anti-inflammatory activity on chondrocytes.[11] It down-regulates TNF-α, which causes cartilage matrix degradation, inhibits extracellular matrix production, and induces expression of MMPs and IL-1 in chondrocytes, and IL-6, which has a pivotal role in the host defense response to injury.

In this work, MMP-3 positive chondrocytes in immunostained sections of group III were counted, and the statistical analysis of the calculated ratio showed that group III had a statistically significant lower ratio than group II. Group III had a statistically significant higher ratio than group I. These findings are in line with Aktas *et al*, who found that the histomorphometric improvement of the osteoarthritis was associated with decreased expression of MMP-3.[31]

CONCLUSION

As described previously, it could be concluded that the osteoarthritic changes induced by surgical DMM in adult male albino rats can be improved by subsequent intra-articular administration of NAPA.

CONFLICT OF INTERSTS

There are no conflicts of interest.

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الملخص العربى

دراسة هستولوجية وهستوكيميائية مناعية للتأثير العلاجي المحتمل لحقن ان اسيتيل فينيل الانين جلوكوزامين مفصليا على خشونة الركبة المستحدثة جراحيا في ذكر الجرذ الأبيض البالغ

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مقدمة: يعد مفصل الركبة هو الأكثر تأثرا بالإلتهاب المفصلي العظمي الغضروفي وهو سبب رئيسي مهم للإعاقة الجسدية طويلة الأمد وحتي الآن لا يوجد علاج فعال له.

الهدف من هذه الدراسة: هو تقييم التأثير العلاجي المحتمل لـإن أسيتيل فينيل ألانين جلوكوزاميين على الإلتهاب المستحدث جراحيًا بزعزعة استقرار الغضروف الإنسي في مفصل الركبة لدى ذكور الجردان البيضاء البالغة.

المواد والطرق: تم تقسيم ثلاثون جرذا إلى ثلاث مجموعات رئيسية على النحو التالي, مجموعة ضابطة ومجموعة خضعت للجراحة بدون علاج وأخري خضعت للجراحة ثم تلقت العلاج. في نهاية التجربة تم أخذ عينات مفصل الركبة وإزالة الكالسيوم منها وصباغتها وتم الفحص باستخدام المجهر الضوئي.

النتائج: ظهرت تغيرات في المجموعة الثانية ترجح حدوث الالتهاب المفصلي وتحسنت في المجموعة الثالثة.

الخلاصة: من هذه الدراسة يمكن استنتاج أن التغيرات العظمية الناتجة عن جراحة زعزعة استقرار الغضروف المفصلي الإنسي يمكن تحسنها عن طريق الحقن اللاحق لإن لأسيتيل فينيل ألانين مفصليا