AMLS Archives of Medical Laboratory Sciences

Original Article

The Effect of Ag-NPs-Menthol on the Expression of Metalloproteinase in A Model-Like Osteoarthritis

Maryam Ahmadi[1], Hossein Maghsoudi[2*], Gholamreza Bakhshi Khaniki[1], Reza Haj Hosseini[1], Parviz Ahangar[3]

1. Department of Biology, Payame Nour University, Tehran, Iran.
2. Department of Biotechnology, Payame Nour University, Tehran, Iran.
3. Department of Orthopedics, Baqiyatallah University of Medical Sciences, Tehran, Iran.

Received: May 18, 2021; Accepted: July 22, 2021

Abstract

Background and Aim: Osteoarthritis (OA) is a chronic inflammatory disease that causes dysfunction of cartilage and joints. Activation of the immune system and the development of inflammatory responses are among the main pathogenesis of the disease. Recently, it has been shown that the expression of Matrix metalloproteinase (MMPs) in patients is increased and leads to the destruction of cartilage in the joints of patients. The introduction of Nanoparticles (NPs) has transformed many fields like medicine, nutrition, and electronics. The usage of nanotechnology in medicine particularly for drug delivery is revealed to have numerous benefits.

Therefore, in this study, for the first time, we evaluated the use of Menthol and silver nanoparticles on the expression of MMPs type 1, 3, 9 and 13 in OA models.

Methods: BFLS cells were first incubated with LPS to induce inflammation. Then, the expression of MMPs type 1, 2, 3, and 9 was evaluated using RT-PCR, ELISA, and migration and invasion assay. This work, therefore, was aimed to synthesize Menthol-mediated silver nanoparticles and evaluate their anti-inflammatory activity. Menthol mediated silver nanoparticles were synthesized by short-term (1 day) interaction of Menthol (1 mL) with 2mM AgNO3 solution and centrifuged to obtain silver nanoparticles. Further, the menthol mediated AgNPs were evaluated for anti-inflammatory activity by in vitro method.

Results: The results showed that the expression of MMPs in LPS-treated cells and silver menthol nanoparticles was decreased compared to LPS-treated cells. In addition, it was shown that menthol silver nanoparticles had the greatest reduction in MMP9 expression and reduced their migration and invasion.

Conclusion: It can be said that the use of menthol silver nanoparticles can be used as a supplement along with corticosteroid and anti-inflammatory drugs and even to replace them for the treatment of OA patients, which requires further studies in the future.

Keywords: Metalloproteinase; Osteoarthritis; Menthol; Silver Nanoparticles.

[*]**Corresponding Author:** Hossein Maghsoudi; **Email:** h.maghsoudi@pnu.ac.ir; **ORCID iD:** 0000-0002-1524-6420

Please cite this article as: Ahmadi M, Maghsoudi H, Bakhshi Khaniki G, Haj Hosseini R. The Effect of Ag-NPs-Menthol on the Expression of Metalloproteinase in A Model-Like Osteoarthritis. Arch Med Lab Sci. 2021;7:1-13 (e11). https://doi.org/10.22037/amls.v7.34925

Introduction

Osteoarthritis (OA) is a chronic joint-related disease characterized by disability as well as disruption of joint tissue (1). According to global statistics, about 37% of OA patients in the United States are over 60 years old (2). The World Health Organization (WHO) also reports that by 2050, approximately 130 million people worldwide will have OA and about 40 million will be disabled due to the severity of the disease (3). Therefore, paying special attention to this disease and identifying the mechanisms of pathogenesis, as well as the use of preventive and therapeutic strategies can greatly reduce the social burden and treatment costs (3, 4). The main organs mainly involved in this disease are the knees, fingers, and spine. The clinical manifestations of this disease are not the same in all patients and the clinical symptoms are different in obese people, athletes, and patients with trauma (5, 6).

However, the most common clinical symptoms in patients with pain are joint and cartilage disorders

and deformity of the joints (7, 8). The pathogenesis of OA is not fully understood however inflammation is known to be the main cause of OA. Activation of immune system cells has been shown to lead to the production of inflammatory cytokines. Cytokines then destroy cartilage and joint matrices by increasing the expression of Matrix Metalloproteinase (MMPs) (9).

MMPs are a family of protease enzymes that break down the extracellular matrix. The expression of MMPs in the synovial fluid and cartilage of OA patients has been shown to increase. Among MMPs, MMPs of types 1, 3, 9, and 13 increase their expression compared to other MMPs in patients (10, 11).

These MMPs increase the breakdown of collagen in cartilage and ultimately lead to joint dysfunction. Although many drugs, including corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs), are used to treat OA, none of them prevent the disease from progressing and cartilage damage. In addition, some patients require surgery despite the use of anti-inflammatory drugs (10, 12). Although the use of anti-inflammatory drugs relieves patients, their side effects can exacerbate clinical symptoms and the severity of the disease.

Accordingly, today, the use of herbal medicines to treat patients due to low side effects has been considered (13, 14). Menthol is one of the herbal medicines derived from *Mentha piperita L.*

Menthol, also called peppermint camphor, is terpene alcohol with a strong minty, cooling odor and taste. It is obtained from peppermint oil or is produced synthetically by hydrogenation of thymol. Menthol is used medicinally in ointments, cough drops, and nasal inhalers. It is also used as a flavoring in foods, cigarettes, liqueurs, cosmetics, and perfumes. The menthol molecule can exist as one of two enantiomers (mirror-image isomers). The naturally occurring material is the levorotatory form (the compound that rotates the plane of polarized light to the left), called (-)-menthol (or l-menthol). Synthetic menthol is racemic, consisting of equal amounts (-)-menthol and (+)-menthol (or d-menthol), the latter being the isomer that rotates the plane of polarized light to the right (11).

when studying the effect of an electrical stimulus applied to human skeletal muscle tissue after the tissue was exposed to menthol. At various application strengths, inactivated sodium channels were measured to determine the effect on depolarization. It was demonstrated that the menthol blocked the alpha subunit of voltage-gated sodium channels, therefore causing hyperpolarization of the nervous membrane and a block in the signal of pain transduction (12).

On the other hand, the simultaneous use of nanoparticles for specific intra-compartmental analysis using the examples of delivery to malignant cancers, to the central nervous system, and across the gastrointestinal barriers has been shown to significantly reduce inflammation (13).

Different animal studies have reported the analgesic effects of M. spicata essential oil and its main abundant compounds such as carvone, limonene, and menthol, also, the efficacy and safety of spearmint oil in reducing pain severity were confirmed in osteoarthritis patients. Despite the beneficial effects of spearmint oil in reducing pain, other large clinical trials are required to confirm the efficacy and safety of M. spicata oil (14).

Many topical ointments and pain-relieving gels such as Biofreeze, Icy Hot, and BenGay use menthol as an active ingredient (15). Menthol has antinociceptive and counterirritant properties and it has also the ability to elicit cold sensations (16). Used menthol-containing topical gels create a cold sensation by stimulating the potential family of transient receptors potential through ion channels (TRPs).

TRPs are found throughout the body, but the transient cation channel receptor subfamily M member 8 (TRPM8), also known as the cold and menthol receptor 1 (CMR1), is a protein that in humans is encoded by the TRPM8 gene (17, 18). The TRPM8 channel is the primary molecular transducer of cold somatosensation in humans (19). In addition, mints can desensitize a region through the activation of TRPM8 receptors (the 'cold'/menthol receptor) (20). TRPM8 contributes a neuronal sensor of cold temperatures and is fundamental for innocuous cool and noxious cold sensations (21).

Silver nanoparticles have unique optical, electrical, and thermal properties and are being incorporated into products that range from photovoltaics to biological and chemical sensors. Examples include conductive inks, pastes and fillers which utilize silver nanoparticles for their high electrical conductivity, stability, and low sintering temperatures.

Additional applications include molecular diagnostics and photonic devices, which take advantage of the novel optical properties of these nanomaterials. An increasingly common application is the use of silver nanoparticles for antimicrobial coatings, and many textiles, keyboards, wound dressings, and biomedical devices now contain silver nanoparticles that continuously release a low level of silver ions to protect against bacteria (22). There are several methods for synthesizing nanoparticles. However, the synthesis of nanoparticles by chemical or physical methods is detrimental to the production of hazardous products and is not environmentally friendly (23). In this scenario, a safe method for NP synthesis is required. The synthesis of NPs using plants is more beneficial than those synthesized using chemical products. Plant-derived nanoparticles are well-suited for medical applications due to the absence of toxic chemicals in the production process (24, 25).

Therefore, in this study, for the first time, we evaluated the use of Menthol and silver nanoparticles on the expression of MMPs type 1, 3, 9 and 13 in OA models.

Methods

Reagents

Silver nitrate, Menthol Crystal, Ascorbic acid, Lipopolysaccharides (LPS), ELISA kit, Migration and invasion kit, vimentin antibody, were purchased from Sigma-Aldrich. Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 and fetal bovine serum, were purchased from GIBCO (GIBCO®, Grand Island, NY, USA). Penicillin and streptomycin, Trypsin EDTA 0.5%, were purchased from Bioidea (BIO-IDEA, Tehran, Iran), Gentamycin from Daropskhsh (Iran), Amphotericin B (Cipla, Mumbai, INDIA), Dimethyl sulfoxide, RNA isolation kit, RT-PCR and all of the primers were purchased from CinaClone (CinnaGen, Tehran, Iran).

Preparation of menthol silver nanoparticles

First, add 1.69 g of silver nitrate powder to a container containing 100 mL of sterile distilled water. It was then placed on a shaker for 10 minutes. Then 50 mL of menthol was added to 100 mL of silver nitrate solution. It was then placed on a shaker for 24 hours at 37°C. The supernatant was then discarded after centrifugation and added to the remaining 1 mL of a distilled precipitate. It was centrifuged again and placed in an incubator to dry the solution (15).

Cell Culture

Isolation and Cultivation of Synovial Cell: Synovial cells were isolated from either biopsy of synovial membrane or samples of radiocarpal joints fluid from 8 months old Holstein Friesians cattle from a slaughterhouse. Tissue biopsies were minced into pieces with a length of 3mm along their longest axis.

The amount of joint fluid that had been taken, ranging from 0.5 to 50 mL, was centrifuged at RCF 500×g at 20°C, and the sediment was transferred to a T-25 tissue culture flask control media containing: Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 (GIBCO®, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO®, Grand Island, NY, USA), 50 mg/mL ascorbic acid (Sigma-Aldrich, UK), and 50 mg/mL Gentamycin (Daropakhsh, Tehran, Iran), Penicillin 100 U/mL, Streptomycin 100 µ/mL (BIO IDEA, Tehran, Iran), Amphotericin B 0.25 µg/mL (Cipla, Mumbai, India).

The flasks were placed in a humidified atmosphere with 5% carbon dioxide at 37°C, and the medium was entirely replaced with a fresh medium with intervals of seven days. The adherent cells were harvested after detachment with Trypsin EDTA 0.5% when the culture had reached 75% confluence, frozen in FBS with 10% dimethylsulfoxide at a cooling rate of 1 C/min, and stored in liquid nitrogen. For the experiments, cryopreserved cell isolates that had been passed 1 or 2 times before they were frozen were selected. These cryo-preserved cell isolates were rapidly

thawed and gently mixed with the previously mentioned medium, grown to 75% confluence, detached, split, replated, and again grown until a minimum of 150 cm^2 flask bottom area was covered with a 90% Confluent layer of cells with good fibroblast morphology, corresponding to minimum 1.5×10^7 cells. This propagation required four to six passages (16).

Confirmation of BFLS cells

The marker selected to confirm BFLS cells was vimentin (28). For this purpose, 5×10^5 cells were prepared and lysed using the appropriate buffer. The proteins were then isolated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose membrane. Then, to identify the protein, the nitrocellulose membrane was incubated with vimentin-specific antibodies according to the manufacturer's protocol. The relative expression of each protein was determined by densitometry.

Treating cells with lipopolysaccharides

LPS was used to induce inflammation (expression of CD14 and Toll-like receptor (TLR) and expression of MMPs (29). For this purpose, 6×10^6 cells were cultured. After 72 hours, 100 ng LPS was added to the culture medium. The plates were then incubated in a CO_2-containing incubation.

RNA isolation and cDNA synthesis

To extract RNA, mononuclear cells were isolated using the Ficoll-Hypaque method. Then RNA was extracted based on the related kit and its protocol. The extracted RNA was evaluated using nanodrop and 260 nm light absorption. Then cDNA synthesis was performed using the related kit protocol. Temperature cycle conditions for cDNA synthesis are included 5 min at 70 C, 1 hour at 42 C, and finally 5 min at 94 C. Materials used for cDNA synthesis included 1000 ng total RNA, 0.5 µL Oligo (dT) primer, 2 µL first strand buffer, 1 µL dNTP, 1 µL M-Mlv, 0.5 µL Rnase inhibitor, and Up to 10 µL Diethyl pyrocarbonate (DEPC) water.

MTT Assay

Metabolic activity can be evaluated by measuring the activity of the mitochondrial enzyme succinate dehydrogenase using 3-(4, 5-dimethylthiazol-2-yl) −2, 5-diphenyltetrazolium bromide (MTT test). This test is widely used in in vitro evaluation of the toxicity of plant extracts. We applied the MTT test to evaluate the potentially toxic effects of menthol extract on cells from the human monocyte cell line THP-1.

Cells were exposed to increasing concentration (1×10^{-3}, 9×10^{-3}, 1×10^{-2}, 9×10^{-2}, 1×10^{-1}, 9×10^{-1}, 1, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 pg/mL) of Ag-NPS-Menthol in the presence of LPS (1 µg/mL) or incubated with LPS (1 µg/mL) alone for 24 hours.

The cells were incubated with 20 µl MTT and placed in a 37°C incubator for 4 hours. Then, cell culture suspension was removed and 100 µL of isobutyl alcohol was added to the microplate to dissolve the formazan substance. The absorbance of the solution at 570 nm was measured by a microplate reader. There was no evidence of a negative effect after treatment with concentrations up to $1x10^{-3}$ pg/mL. Concentrations higher than 3 pg/mL caused a significant reduction in cell viability (Figure 1).

Table 1. Summary of primers sequences.

Primer Name	Forward 5´ to 3´	Reverse 5´ to 3´	Tm	
			Forward	Reverse
B.MMP-1	3´-ACACCCCAGACCTGTCAAGA-5´	3´-CGACTGAGCGACTAACACGA-5´	60.40	59.83
B.MMP-2	3´-CGAATAGAATAGCCCCTCGG-5´	3´-AGGCTCGGTGCGTGTG-5	57.34	59.63
B.MMP-3	3´-GAGGAGGCGACTCCACTTAC-5´	3´-GGCTGATGCATGTGACCAGA-5´	59.54	60.25
B.MMP-9	3´-CGCTATGGCTACACTCCTGG-5´	3´-TTGTCCTGGTCGTAGTTGGC-5´	60.00	55.00
B.GAPDH	3´-GAGAACGGGAAGCTCGTCAT-5´	3´-TTGATGGTACACAAGGCAGGG-5´	55.00	60.27
CD14	5′-GAC GAC GAT TTC CGT TGT GT-3′	5´-TGC GTA GCG CTA GAT ATT GGA-3′	57.34	59.63
BTLR4	5′-AAC CAC CTC TCC ACC TTG ATA CTG-3	5′-CCA GCC AGA CCT TGA ATA CAG G-3	59.54	60.25

RT-PCR

Real-time polymerase chain reaction (RT-PCR) was used to evaluate gene expression. The GAPDH gene was used as an internal control.

Temperature cycle conditions for RT-PCR included initial denaturation for 2 min at 94℃, (20s at 94℃ and 20s at 50℃) with 35 cycles in 20 µL of PCR master mix. The primers used for RT-PCR reactions are listed in Table 1.

In vitro migration and invasion assay

Cell migration was measured by 6.5 mm thick Transwell culture chambers and 8 mm diameter pores. 10^5 BFLS cells treated with silver menthol nanoparticles were cultured in the upper part of the Transwell plate.

A serum-free culture medium with or without LPS was added to the bottom of the plate and incubated for 24 hours. The non-migrating cells were then collected at the top by sterile swab and stained with violet crystal. Non-migrating cells were counted with a microscope in 5 fields.

To determine the extent of the invasion, the pores at the top of the plate were first covered with Matrigel. 5×10^4 cells were then transferred to a culture medium containing FBS and then to a Transwell previously coated with Matrigel, and after 24 hours the cells were counted (30).

Enzyme-linked Immunosorbent Assay (ELISA)

Serum-free conditioned media samples were collected and centrifuged at 10000 g for 5 min to remove particulates. The concentrations of MMP-9 were measured using an ELISA kit (Sigma-Aldrich, UK) according to the manufacturer's instructions.

Imaging of menthol silver nanoparticles

After the preparation of menthol silver nanoparticles, FESEM, EDAX, TEM, and XRD imaging were performed. An electron beam is used in TEM and FESEM imaging, while an X-ray is used in XRD and EDAX imaging.

Statistical analysis

Error limits cited and error bars plotted to represent the simple standard deviation of the mean. When different samples were compared, the results were considered statistically different when $p<0.05$ or $p<0.001$ (Student's t-test, ANOVA test, and REST test) for the unpaired samples. The IC50 values were calculated by scatter plot in an Excel graph.

Results

Confirmation of BFLS cells and induced inflammation by LPS

we analyzed the expression of two members of the LPS-receptor complex, TLR4 and CD14 by RT-PCR. Using primers designed for the bovine TLR4 and CD14, mRNA transcripts for both were detected in BFLs treated with Ag-NPs-Menthol and untreated cells, indicating that BFLS possess the required LPS-receptor complex to respond to LPS. Stimulation of BFLSs with LPS resulted in the up-regulation of TLR4, CD14, and COX-2 the enzyme required for the conversion of AA to PGs, which suggested the initiation of a pro-inflammatory response. Since LPS-stimulated BFLSs produced high levels of PGE2, an anti-inflammatory molecule, down-regulation of TLR4 may prevent the cells from further recognizing LPS and producing PGE2, thereby preventing suppression of an immune response(17).

Evaluation of MMPs, CD14, and TLR genes expression by RT-PCR

As Ag-NPs-Menthol inhibited BFLS migration and invasion, we examined the effect of Ag-NPs-Menthol on MMPs gene expression. As shown in Figure 1, LPS induced MMP-9, MMP-2, and MMP-1 mRNA expression in BFLS, whereas treatment with Ag-NPs-Menthol suppressed LPS-induced MMP-9 expression in a dose-dependent manner. However, MMP-1, MMP-2, and MMP3 mRNA expression were not affected by Ag-NPs-Menthol treatment. The treatment of BFLS with Ag-NPs-Menthol suppressed LPS-induced MMP-9 secretion and activity in a dose-dependent manner. These results indicated that Ag-NPs-Menthol selectively inhibited LPS-induced MMP-9 expression in gene expression (Figure 2 and 3).

In addition, the results showed that treatment of BFLS cells with menthol silver nanoparticles reduced the expression of CD14 and TLR4, which was statistically significant with untreated) (Figure 4).

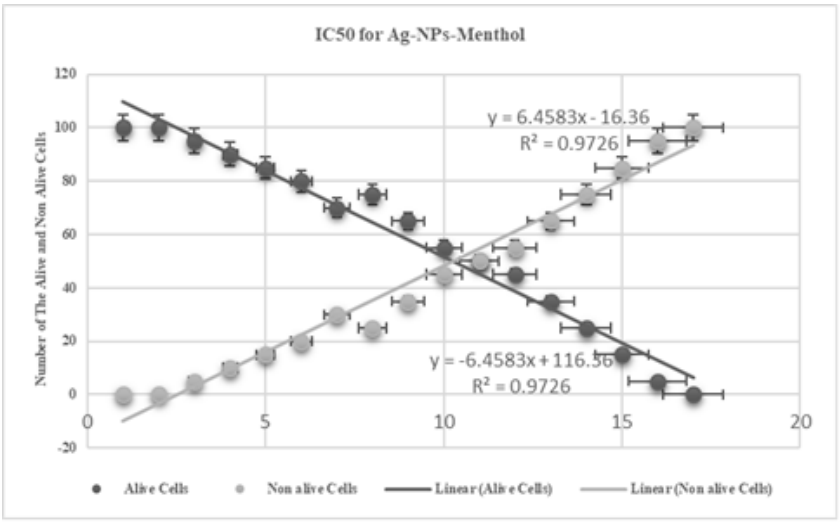

Figure 1. Graphs used for IC50 value determinations of Ag-NPs-Menthol against BFLS. Dose-response data points represent the mean value of 3 trials.

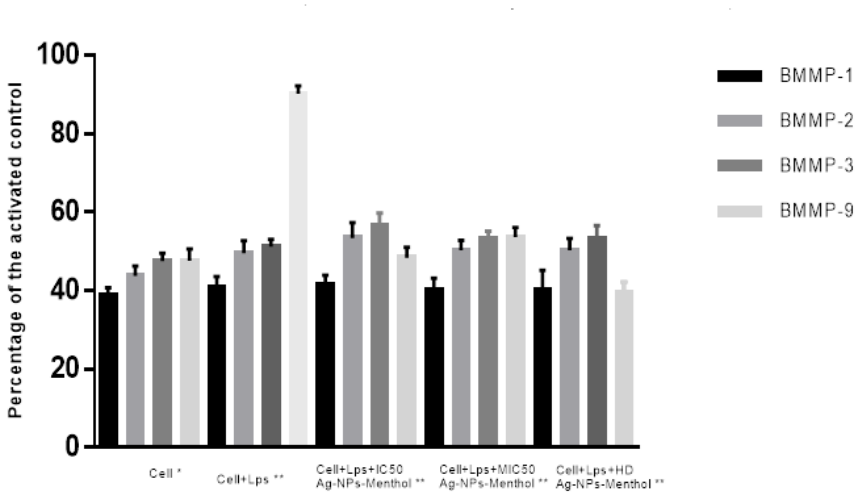

Figure 2. The effect of Ag-NPs-menthol on the expression of MMPs. Gene expression was examined in groups treated with different doses of Ag-NPs-menthol and compared with cells treated with LPS. There was a significant difference in gene expression between the groups. Statistical significance between cell and cell+LPS groups and other groups were analyzed using the student Newman-Keuls test (mean±1 SD, n±3, $p<0.05$ and $p<0.001$**).
Abbreviation: LPS: Lipopolysaccharides, IC50: Inhibitory concentration 50%, MIC50: median Inhibitory concentration 50%, HD: High Dosage. MMP: Bovine metalloproteinase; Ag-NPs-Menthol: Silver Nitrite Nanoparticle's-Menthol.

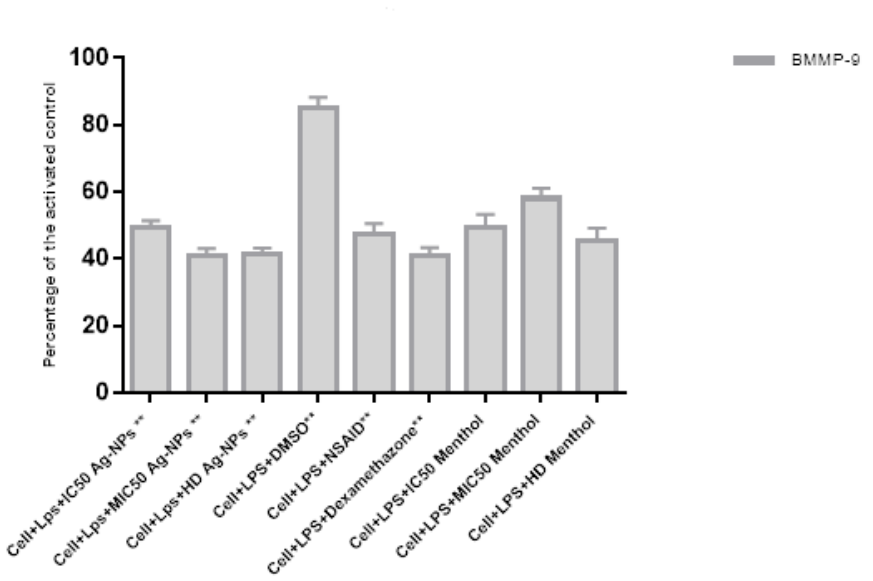

Figure 3. The effect of the different compounds on MMP-9 expression. The vertical axis shows the expression of MMP-9 and the horizontal axis shows the different compound and their concentrations. The results show that the use of Ag-NP with intermediate concentration reduced the expression of MMP-9 compared to other compounds. There was a significant difference in gene expression between the groups. Statistical significance between cell and cell+LPS groups and other groups were analyzed using the student newmane keuls test (mean ±1 SD, n ±3, $p<0.05$ and $p<0.001$**).

Abbreviations: DMSO: Dimethyl sulfoxide (DMSO); NSAID: Non-steroidal anti-inflammatory drugs (NSAIDs); LPS: Lipopolysaccharides; IC50: Inhibitory concentration 50%; MIC50: median Inhibitory concentration 50%; HD: High Dosage; MMP: Bovine metalloproteinase; Ag-NPs-Menthol: Silver Nitrite Nanoparticle's-Menthol.

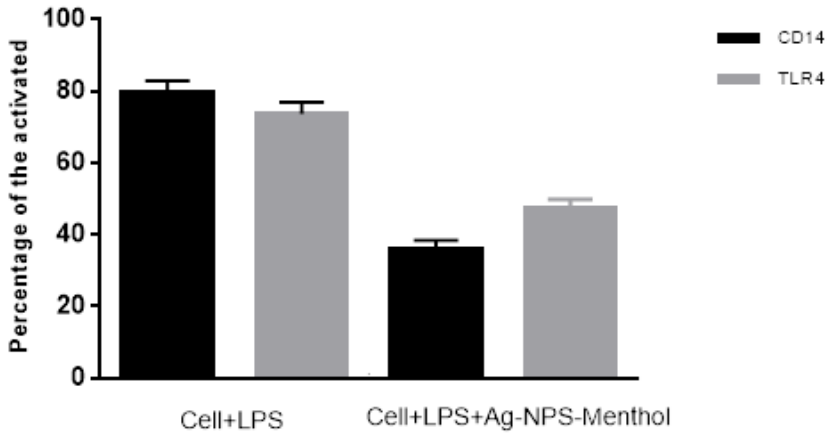

Figure 4. The expression of TLR4 and CD14 in BFLS cells. The results show that the use of Ag-NPs-menthol reduced the expression of CD14 and TLR4. However, it was found that the use of this combination further reduced the expression of CD14 compared to TLR4. There was a significant difference in gene expression between the groups. Statistical significance between cell and cell+LPS groups and other groups were analyzed using the student Newman-Keuls test (mean ±1 SD, n ±3, $p<0.05$ and $p<0.001$**). The column is Percentage of the Activated.

Evaluation of migration and invasion of BFLS cell

The results showed that treat of BFLS cells with LPS and silver menthol nanoparticles significantly reduced cell invasion and migration compared to cells treated with LPS alone. These results indicated that menthol silver nanoparticles reduced the number of BFLS cells and lost them. The best dose for BFLS cells was median IC50 (Figure.4). As shown in Fig. 4 the in vitro migration ability of BFLS was increased 2-fold when stimulated with LPS for 24 h. Similarly, data obtained from the invasion assay showed that LPS increased cell invasion 2.5-fold in comparison with the control group. However, LPS-induced cell migration and invasion were inhibited by AG-NPs-Menthol in a dose-dependent manner. These results suggest that non-toxic concentrations of Ag-NPs-Menthol ranging from 6.2 pg/mL inhibit BFLS migration and invasion induced by LPS in vitro (Figure 5).

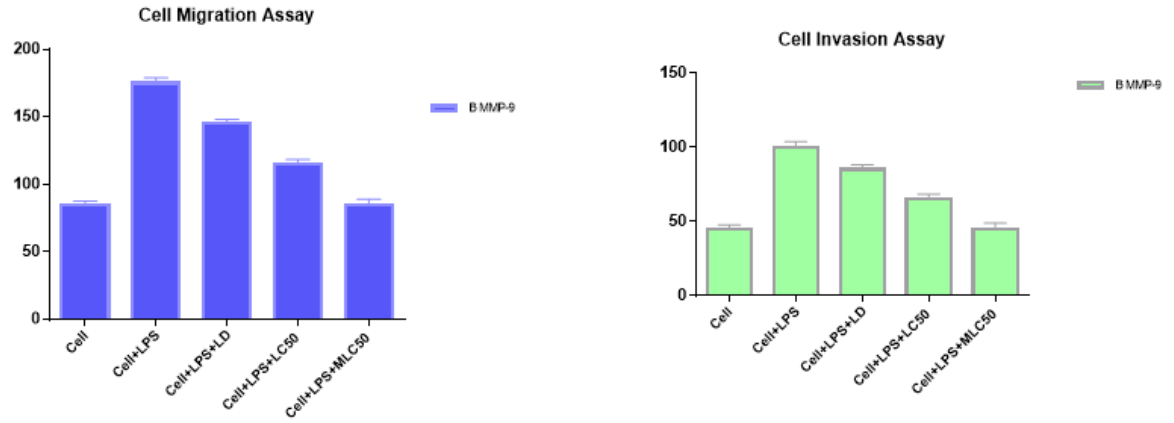

Figure 5. The effect of menthol silver nanoparticles on the invasion and migration of LPS-treated BFLS cells. The results showed that increasing the concentration of Ag-NPs-Menthol reduces the invasion and migration of cells. There was a significant difference in gene expression between the groups. Statistical significance between cell and cell+LPS groups and other groups were analyzed using the student Newman-Keuls test (mean±1 SD, n ±3, $p<0.05$ and $p<0.001$**). The column is percentage of the Activated.

Evaluation of MMP-9 expression by ELISA

Based on the evaluation of the data, it was shown that the best dose to reduce the expression of MMP-9 was LC-50. For this purpose, after measuring the amount of MMP-9 by ELISA, it was shown that LC-50 from menthol silver nanoparticles is suitable to prevent the expression of MMP-9 (Figure 6).

Imaging of silver nanoparticle menthol

Photographs of silver menthol nanoparticles were performed at the University of Tehran using TEM, FESEM, EDAX, and XRD microscopes. Different images of menthol silver nanoparticles are shown in the following figures. The TEM results show two phases with darker and lighter colors, the dark phase is related to heavy silver particles and the light phase is related to the organic phase of menthol (Figure 7).

The XRD analysis report of biosynthesized silver menthol nanoparticles were shown that each of these peaks corresponds to a specific plane of the sample, and the angle of each peak depends on the distance between the plane and the intensity of the peak related to the arrangement of atoms in the plates. As a result, each material has a unique X-ray pattern that can be used as a fingerprint to identify it. The XRD diagram also shows that the material is composed of amorphous and crystalline phases, the amorphous phase is related to the organic phase and the crystalline phase is related to AgCl. For a more detailed study, the percentage of each phase was calculated using Origin software (Figure 9).

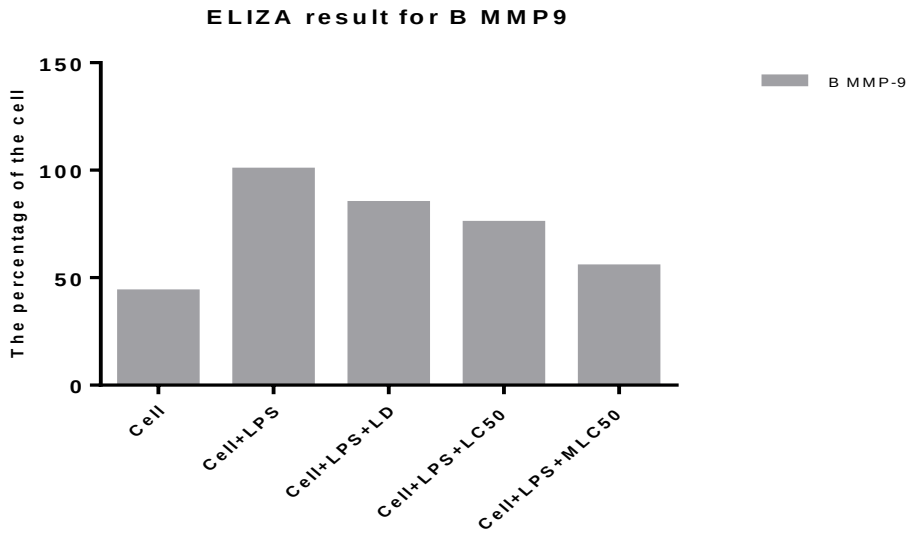

Figure 6. Examination of MMP-9 expression by ELISA. The results showed that the higher the concentration of Ag-NPs-Menthol cause reduced the expression of MMP-9 in cells. There was a significant difference in gene expression between the groups. Statistical significance between cell and cell+LPS groups and other groups were analyzed using the student Newman-Keuls test (mean ±1 SD, n ±3, $p<0.05$ and $p<0.001$**). Abbreviation: LPS: Lipopolysaccharides; IC50: Inhibitory concentration 50%; MIC50: median Inhibitory concentration 50%; HD: High Dosage; MMP: Bovine metalloproteinase; Ag-NPs-Menthol: Silver Nitrite Nanoparticle's-Menthol.

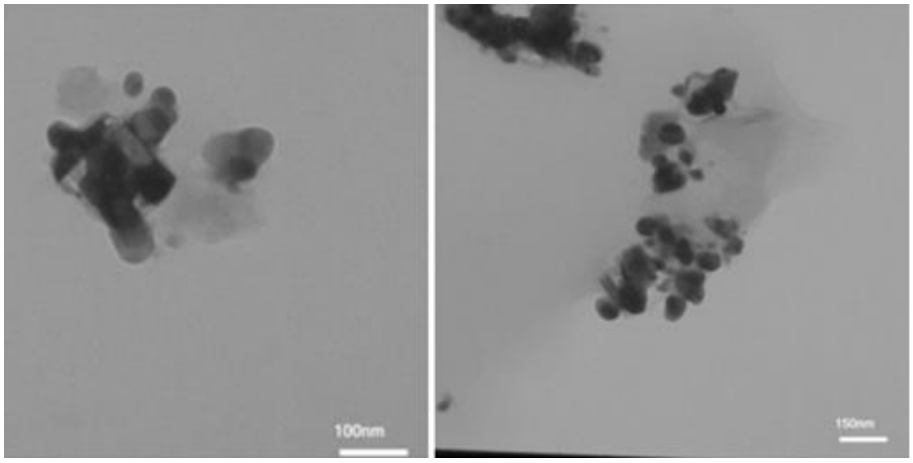

Figure 7. TEM photos of menthol silver nanoparticles (magnification 100 to 150-250 nm. Darkdots indicate the presence of silver).

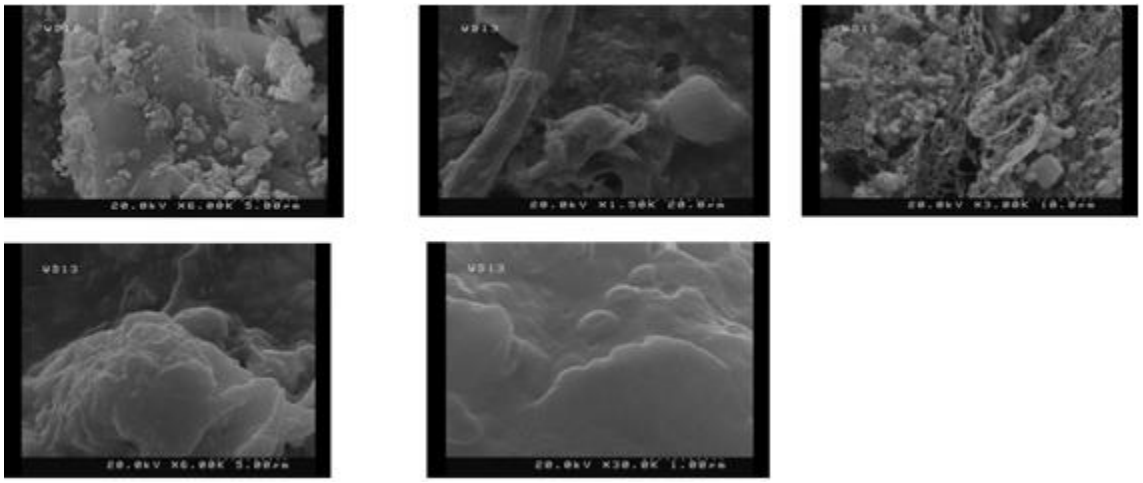

Figure 8. FESEM photos silver menthol nanoparticle's electron energy gives a porous state to organic matter, but silver does not become porous. As can be seen, the particle size in FESEM was about 700 nm, which according to the results of XRD analysis, there is a discrepancy in particle size. This may be due to the agglomeration of nanosilver particles in FESEM images, hence, it looks bigger.

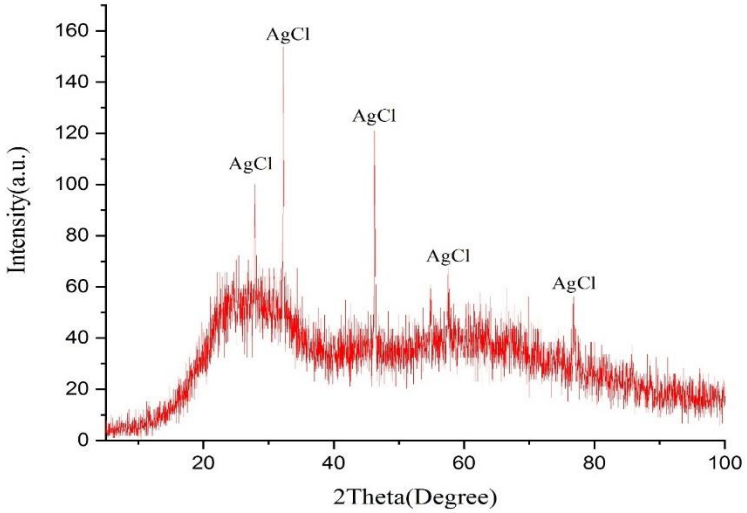

Figure 9. XRD analysis

Discussion

OA is a chronic inflammatory disease characterized by dysfunction of the cartilage and joints. This disease gradually leads to the inability of the person to walk (18, 19). So far, many factors have been identified in connection with the pathogenesis of this disease (20). Stimulation of immune cells and inflammation is one of the main pathogens of the disease. To this end, activation of inflammatory responses has been shown to stimulate MMPs. MMPs are one of the body's main cells and are present in many tissues and extracellular matrices (21). These cells are mostly involved in remodeling the extracellular matrix and producing inflammatory cytokines and growth factors. Previous studies have shown that the number of these cells in OA patients is higher than in normal individuals (21). In addition, activation of MMPs has been shown to increase the secretion of inflammatory mediators, which ultimately leads to

cartilage and joint destruction. Given that inflammation is one of the main pathways of OA pathogenesis, therapeutic approaches to treat this disease have shifted to the use of anti-inflammatory drugs (22). However, although the use of these drugs prevents the progression of the disease and improves the clinical course of patients, long-term use of them can be associated with complications in patients that can reduce their survival. To prevent complications in patients, the use of herbal medicines to treat patients, especially OA patients, is increasing (23, 24).

Menthol is one of the herbal remedies used for many diseases. Due to its anti-inflammatory properties, this drug can prevent inflammation and stimulation of immune cells (25). Previous studies have shown that the use of menthol in OA patients can reduce inflammation and improve patients. Although the use of menthol in OA has been shown to reduce inflammation, concomitant use of menthol and silver nanoparticles has not been performed in OA patients (26). Therefore, in this study, for the first time, we evaluated the use of menthol silver nanoparticles on the expression of MMPs and the incidence of inflammation in OA samples. A study by Robert et al. Showed that pain and inflammation were reduced in patients receiving menthol compared to other patients receiving placebo, a difference that was statistically significant (27).

Numerous studies have been showing that menthol gels have been used with limited empirical support to relieve pain and improve functioning among individuals with OA (41, 42, 43). In the present study, the results showed that the use of menthol silver nanoparticles led to a significant reduction in MMPs. On the other hand, it was found that the best dose used was IC50. In addition, it was found that the expression of MMPs (types 1, 2, 3, and 9) in LPS-treated BFLS cells and silver menthol nanoparticles was lower compared to LPS-treated cells, which was statistically significant.

The study by Huebner et al. Also showed that the use of dexamethasone in osteoarthritis patients reduced inflammation by inhibiting the secretion of inflammatory cytokines (28). The study by Zhao et al. Also showed that the use of dexamethasone reduces the incidence of inflammation in patients (29). In this study, the effect of dexamethasone on the expression of MMPs and the incidence of inflammation was evaluated. The results showed that the use of dexamethasone like silver menthol reduced the expression of MMP-9. However, it did not affect reducing the expression of other MMPs. Considering that dexamethasone and menthol have been shown to regulate the expression of TIMP-1 (inhibitor of MMP-9) and have no effect on other TIMPs, it can be said that the use of dexamethasone and menthol reduces MMP-9 (30, 44-46).

Conclusion

Given that the use of menthol silver nanoparticles reduces the expression of MMP and ultimately prevents inflammation, it can be said that the use of these compounds can be effective in the future for the treatment of patients with Osteoarthritis (OA) and because they are herbal medicines. It can be associated with reduced complications in patients. On the other hand, it has been shown that the use of nanoparticles, especially silver nanoparticles, can reduce inflammation.

Acknowledgment

This study is part of the MSc thesis of Maryam Ahmadi. Special thanks to Payame Nour University, Tehran, Iran (TH99010).

Funding/Support

The authors declare that no funding receives for conducting this project.

Conflict of Interest

The authors declared that they have no conflict of interest.

References

1. Roman-Blas JA, Castañeda S, Largo R, Herrero-Beaumont G. Osteoarthritis associated with estrogen deficiency. Arthritis research & therapy. 2009;11(5):1-14.

2. Lawrence RC, Felson DT, Helmick CG, Arnold LM, Choi H, Deyo RA, et al. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States: Part II. Arthritis & Rheumatism. 2008;58(1):26-35.

3. Mobasheri A, Saarakkala S, Finnilä M, Karsdal MA, Bay-Jensen A-C, van Spil WE. Recent advances in understanding the phenotypes of osteoarthritis. F1000Research. 2019;8.

4. Carey RM, Muntner P, Bosworth HB, Whelton PK. Prevention and control of hypertension: JACC health promotion series. Journal of the American College of Cardiology. 2018 Sep 11;72(11):1278-93. PMID: 30190007. PMCID: PMC6481176.

5. Cooper C, Adachi JD, Bardin T, Berenbaum F, Flamion B, Jonsson H, et al. How to define responders in osteoarthritis. Current medical research and opinion. 2013;29(6):719-29.

6. Bruyère O, Cooper C, Arden N, Branco J, Brandi ML, Herrero-Beaumont G, et al. Erratum to: can we identify patients with high risk of osteoarthritis progression who will respond to treatment? A focus on epidemiology and phenotype of osteoarthritis. Drugs & aging. 2017;34(5):411-.

7. Vina ER, Kwoh CK. Epidemiology of osteoarthritis: literature update. Current opinion in rheumatology. 2018 Mar;30(2):160.

8. Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, Feige U, Poole AR. Role of interleukin-1 and tumor necrosis factor α in matrix degradation of human osteoarthritic cartilage. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2005 Jan;52(1):128-35.

9. Ran J, Ma C, Xu K, Xu L, He Y, Moqbel SAA, et al. Schisandrin B ameliorated chondrocytes inflammation and osteoarthritis via suppression of NF-κB and MAPKsignal pathways. Drug design, development and therapy. 2018;12:1195.

10. Lazzaroni M, Bianchi Porro G. Gastrointestinal side-effects of traditional non-steroidal anti-inflammatory drugs and new formulations. Alimentary pharmacology & therapeutics. 2004;20:48-58.

11. Wade, L. G. (Invalid Date). Menthol. Encyclopedia Britannica.

12. Topp R, Brosky JA, Pieschel D. The effect of either topical menthol or a placebo on functioning and knee pain among patients with knee OA. Journal of geriatric physical therapy. 2013 Apr 1;36(2):92-9.

13. Emerich DF, Thanos CG. Targeted nanoparticle-based drug delivery and diagnosis. Journal of drug targeting. 2007 Jan 1;15(3):163-83.

14. Mahboubi M. Mentha spicata as natural analgesia for treatment of pain in osteoarthritis patients. Complementary therapies in clinical practice. 2017 Feb 1;26:1-4.

15. Macpherson LJ, Hwang SW, Miyamoto T, Dubin AE, Patapoutian A, Story GM. More than cool: promiscuous relationships of menthol and other sensory compounds. Molecular and Cellular Neuroscience. 2006 Aug 1;32(4):335-43.

16. Kraemer WJ, Ratamess NA, Maresh CM, Anderson JA, Volek JS, Tiberio DP, Joyce ME, Messinger BN, French DN, Sharman MJ, Rubin MR. Acetylated fatty acid topical cream with menthol reduces pain and improves functional performance in individuals with arthritis. The Journal of Strength & Conditioning Research. 2005 May 1;19(2):475-80

17. Andersen HH, Olsen RV, Møller HG, Eskelund PW, Gazerani P, Arendt-Nielsen L. A review of topical high-concentration L-menthol as a translational model of cold allodynia and hyperalgesia. European journal of pain. 2014 Mar;18(3):315-25.

18. Clapham DE, Julius D, Montell C, Schultz G. International Union of Pharmacology. XLIX. Nomenclature and structure-function relationships of transient receptor potential channels. Pharmacological reviews. 2005 Dec 1;57(4):427-50.

19. Olsen RV, Andersen HH, Møller HG, Eskelund PW, Arendt-Nielsen L. Somatosensory and vasomotor manifestations of individual and combined stimulation of TRPM 8 and TRPA 1 using topical L-menthol and trans-cinnamaldehyde in healthy volunteers. European Journal of Pain. 2014 Oct;18(9):1333-42.

20. Werkheiser JL, Rawls SM, Cowan A. Mu and kappa opioid receptor agonists antagonize icilin-induced wet-dog shaking in rats. European journal of pharmacology. 2006 Oct 10;547(1-3):101-105.

21. Xiao R, Xu XS. Temperature Sensation: From Molecular Thermosensors to Neural Circuits and Coding Principles. Annual Review of Physiology. 2020 Oct 21;83.

22. Roy A, Bulut O, Some S, Mandal AK, Yilmaz MD. Green synthesis of silver nanoparticles: biomolecule-nanoparticle organizations targeting antimicrobial activity. RSC advances. 2019;9(5):2673-702.

23. Y. Zhang, H. Peng, W. Huang, Y. Zhou, D. Yan, Facile preparation and characterization of highly antimicrobial colloid Ag or Au nanoparticles, J. Colloid Interface Sci. 325 (2008) 371–376.

24. K. Vijayraghavan, S.P. Nalini, Biotemplates in the green synthesis of silver nanoparticles, Biotechnol. J. 5 (2010) 1098–1110.

25. R.M. Crooks, B.I. Lemon, L. Sun, L.K. Yeung, M. Zhao, Dendrimer encapsulated metals and semiconductors: synthesis, characterization, and applications, Top. Curr. Chem. 212 (2001) 82–135.

26. Mokhtari N, Daneshpajouh S, Seyedbagheri S, Atashdehghan R, Abdi K, Sarkar S, Minaian S, Shahverdi HR, Shahverdi AR. Biological synthesis of very small silver nanoparticles by culture supernatant of Klebsiella pneumonia: The effects of visible-light irradiation and the liquid mixing process. Materials research bulletin. 2009 Jun 3;44(6):1415-21.

27. Maghsoudi H, Hallajzadeh J, Rezaeipour M. Evaluation of the effect of polyphenol of escin compared

with ibuprofen and dexamethasone in synoviocyte model for osteoarthritis: an in vitro study. Clinical rheumatology. 2018 Sep;37(9):2471-8.

28. Etienne-Manneville S. Cytoplasmic intermediate filaments in cell biology. Annual review of cell and developmental biology. 2018 Oct 6; 34:1-28.

29. Kumar S, Wang J, Shanmukhappa SK, Gandhi CR. Toll-like receptor 4–independent carbon tetrachloride–induced fibrosis and lipopolysaccharide-induced acute liver injury in mice: role of hepatic stellate cells. The American journal of pathology. 2017 Jun 1;187(6):1356-67.

30. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nature protocols. 2007 Feb;2(2):329..

31. Ichise Y, Saegusa J, Tanaka-Natsui S, Naka I, Hayashi S, Kuroda R, et al. Soluble CD14 Induces Pro-Inflammatory Cytokines in Rheumatoid Arthritis Fibroblast-Like Synovial Cells via Toll-Like Receptor 4. Cells. 2020;9(7):1689.

32. Castañeda S, Vicente EF. Osteoarthritis: More than Cartilage Degeneration. Clinical Reviews in Bone and Mineral Metabolism. 2017 Jun 1;15(2):69-81.

33. Goldring MB, Otero M. Inflammation in osteoarthritis. Current opinion in rheumatology. 2011;23(5):471.

34. Sokolove J, Lepus CM. Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. Therapeutic advances in musculoskeletal disease. 2013;5(2):77-94.

35. Robinson WH, Lepus CM, Wang Q, Raghu H, Mao R, Lindstrom TM, Sokolove J. Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis. Nature Reviews Rheumatology. 2016 Oct;12(10):580.

36. Barcella CA, Lamberts M, McGettigan P, Fosbøl EL, Lindhardsen J, Torp-Pedersen C, Gislason GH, Olsen AM. Differences in cardiovascular safety with non-steroidal anti-inflammatory drug therapy—A nationwide study in patients with osteoarthritis. Basic & clinical pharmacology & toxicology. 2019 May;124(5):629-41.

37. Silverstein FE, Faich G, Goldstein JL, Simon LS, Pincus T, Whelton A, Makuch R, Eisen G, Agrawal NM, Stenson WF, Burr AM. Gastrointestinal toxicity with celecoxib vs nonsteroidal anti-inflammatory drugs for osteoarthritis and rheumatoid arthritis: the CLASS study: a randomized controlled trial. Jama. 2000 Sep 13;284(10):1247-55.

38. Long L, Soeken K, Ernst E. Herbal medicines for the treatment of osteoarthritis: a systematic review. Rheumatology. 2001 Jul 1;40(7):779-93.

39. Rodriguez-Merchan E. Topical therapies for knee osteoarthritis. Postgraduate Medicine. 2018;130(7):607-12.

40. Topp R, Brosky JA, Pieschel D. The effect of either topical menthol or a placebo on functioning and knee pain among patients with knee OA. Journal of geriatric physical therapy. 2013;36(2):92-9.

41. Altis K, Schmidtko A, Angioni C, Kuczka K, Schmidt H, Geisslinger G, Lötsch J, Tegeder I. Analgesic efficacy of tramadol, pregabalin and ibuprofen in menthol-evoked cold hyperalgesia. PAIN®. 2009 Dec 15;147(1-3):116-21.

42. Patel R, Gonçalves L, Leveridge M, Mack SR, Hendrick A, Brice NL, Dickenson AH. Anti-hyperalgesic effects of a novel TRPM8 agonist in neuropathic rats: a comparison with topical menthol. PAIN®. 2014 Oct 1;155(10):2097-107.

43. Stevens CJ, Best R. Menthol: a fresh ergogenic aid for athletic performance. Sports medicine. 2017 Jun;47(6):1035-42. D

44. Pouladzadeh M, Safdarian M, Choghakabodi PM, Amini F, Sokooti A. Validation of red cell distribution width as a COVID-19 severity screening tool. Future Science OA. 2021 Mar(0):FSO712.

45. Pouladzadeh M, Safdarian M, Eshghi P, Abolghasemi H, Sheibani B, Choghakabodi PM, Feghhi A, Boroujerdnia MG, Forouzan A, Far MA, Kaydani GA. A randomized clinical trial evaluating the immunomodulatory effect of convalescent plasma on COVID-19-related cytokine storm. Internal and emergency medicine. 2021 Apr 10:1-1.

46. Bian F, Pelegrino FS, Henriksson JT, Pflugfelder SC, Volpe EA, Li D-Q, et al. Differential effects of dexamethasone and doxycycline on inflammation and MMP production in murine alkali-burned corneas associated with dry eye. The ocular surface. 2016;14(2):242-54.