The Effect of Citrullus Colocynthis on the Reduction of Inflammatory Agents in Osteoarthritis

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Abstract

Nowadays, the use of medicinal plants has been very common. Colocynth, Scientific name is Citrullus colocynthis in traditional medicine used to relieve pain and inflammation. The aim of this study is to evaluate the effects of ethanol extract of Colocynth root on pro-inflammatory cytokine COX-2, iNOS, IL-1β, TNF-α and NO, PGE2 on Inflammatory cells, similar osteoarthritis in chondrocyte cells and monocytes/macrophages and then treating them. At first the ethanol extract of the Citrullus colocynthis plant was prepared from the Iranian biological resource center. Then chondrocyte cells and THP-1 monocyte/macrophage (5×10^5 cells/well) were incubated at a humidity of 90%, 5% CO2, 37°C for 72 h with control media alone or Citrullus colocynthis at concentrations of 70 μg/ml. One set of cells was activated by 20 μl LPS 20, then active cells are exposed to ethanol extract. And finally, cells tested with two control, cells and inflamed cells with LPS20, then results were evaluated Real Time PCR. Our review of the three levels of normal cells and inflamed cells and cells treated with ethanol extract of roots of Citrullus colocynthis plant with 20 ng/ml LPS20 showed that plant can reduce the expression of inflammatory cytokine and pro-inflammatory cytokine COX-2, iNOS, TNF-α in Chondrocyte cells and reduced expression levels of TNF-α in THP-1 monocytes /macrophages and was reduced production of NO, PGE2. Our observations indicated that ethanol extract of root Citrullus colocynthis can reduce expression levels of pro-inflammatory cytokines in inflamed cells caused by situation same osteoarthritis, and we can use this plant for the treatment osteoarthritis in the future.

Keywords: Osteoarthritis; Citrullus colocynthis; Monocytes /macrophages; Chondrocyte

Abbreviations: ADAMT: A Disintegrin and Metalloprotease (ADAM) and ADAMTS (ADAM with Thrombospondin Motifs); ATP: Adenosine 3'-Phosphate; CCT: Citrullus colocynthis; COX-2: Cyclooxygenase-2; IL-1β: Interleukin-1β; iNOS: Inducible NOS; JNK: c-Jun NH2-Terminal Kinase; MAPK: Mitogen-Activated Protein Kinase; MMP: Matrix Metalloproteinase; NF-κB: Nuclear Factor Kappa B; NO: Nitric Oxide; OA: Osteoarthritis; PGE2: Prostaglandin E2; TGFβ: Transforming Growth Factor-Beta; TNF-α: Tumor Necrosis Factors Alpha

Introduction

Osteoarthritis is a common joint disease in old ages. It has very advanced destructive activities and is identified by the metabolic changes and the changes on the restructure of cartilage, bone and joint surfaces [1]. The disease can occur in the joints of the hands, feet, knees, spine and other parts of the joint. Osteoarthritis is characterized by narrowing or complete loss of cartilage, bone stimulation of cartilage and bone formation in bony bumps at the edges of the bone called osteophytes, and sclerosis (thickening evolve) is the bone under the cartilage. Cyst of bone also appears beneath the cartilage.

Osteoarthritis patients complain of pain and stiffness that interferes with daily activities appropriate to knee pain, back pain, neck pain, joint pain is small. Some patients with pain and swelling in the knee and small joints of the hands, which are the cause of inflammatory exacerbation is a worsening [2]. Inflammatory degenerative changes in the joints by a series of biochemical events such as overproduction of proinflammatory cytokine IL-1β, tumor necrosis alpha TNFα [3]. Among the pro-inflammatory cytokines involved in the development of osteoarthritis, TNF-α is considered as the main inflammation agent. TNF-α also stimulates the production of a number of inflammatory mediators in the osteoarthritis disease. For example, the expression of iNOS and COX-2 genes increases with the addition of TNF-α to chondrocytes, and consequently, the amount of NO and PGE2 increases. Previous molecular studies performed on medicinal herbs with the anti-inflammatory effects, such as avocado, have indicated that the consumption of these plants reduces the expression of inflammatory cytokine IL-1β, TNF-α, PGE2 and induced NO [4]. The osteoarthritis treatment goals includes: reducing swelling and pain in suffering joint and preventing the progression in the destruction of articular cartilage. The current therapies are not often effective since are associated with some side effects such as peptic ulcers and gastrointestinal bleeding. Because of the degree of inflammation associated with pain in the patients with osteoarthritis, research on the plants that have anti-inflammatory properties, although low, in addition to the analgesic effect, can make a substantial contribution to the patients with osteoarthritis, because the side effects caused by steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs) are not induced by them [5].

Citrullus colocynthis is a perennial herbaceous creeping plant, with angular and rough stems. It is geographically distributed in the desert of North Africa, Southern Europe and Asia [6]. A number of metabolites including cucurbitacins, flavonoids, caffeic acid derivatives and terpenoids isoscoparin, isovitexin, isoorientin 3'-O-methyl ether, 2-O- β-D-glucopyranosyl-cucurbitacin I, 2-O- β-D-glucopyranosyl-cucurbitacin L, and the antioxidant [7]. Research carried out on

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the plant antioxidant effect and reducing cytokine [8], reducing fat and Hypolipidemic [9], antimicrobial effect and bacterial [10]. And anti-inflammatory properties, during the study Marzouk and their colleagues used this extract on rats carried out in the treatment of rheumatism arthritis analgesic effect and anti-inflammatory and loss without any side effects observed in rats and effectiveness of the plant as a medicine proved. [11]. The aim of this experiment is survey effect of ethanol extract of plant roots in decreased expression Citrullus colocynthis inflammatory cytokine TNF-α and iNOS and IL1β and COX-2 and PGE2, NO at the molecular level on chondrocytes and monocytes/macrophages.

**Method**

All steps of research were proceeding in Rey’s PNU biotechnology laboratory in Tehran.

Citrullus colocynthis was initially obtained from Iran’s center of genetic resources, and then its aqueous extraction was prepared. On the other hand, chondrocyte cells were obtained from the healthy radiocarpal joint cartilage of an 8-month-old Holstein cow from Asia Meat Industrial Complex and monocytes/macrophages (THP-1) cells were obtained from the Pasteur Institute of Iran, and were amplified in a sterile medium to the extent necessary. The next steps were accomplished completely identical and separately in the two groups of chondrocyte cells and monocytes/macrophages cells. Following the investigation of Citrullus colocynthis’s root extract in different concentrations on the cell samples, the amount of LC50 was determined. Then the cells were treated with LPS to cause inflammatory conditions. Then, RNA was isolated and RNA concentration was determined. In the following, isolated RNA was employed to produce cDNA using RT-PCR method, PCR was used to amplify cDNA and finally Real Time PCR was used to determine the expression levels of IL-1β, TNF-α, PGE2 and NO genes by specific primers. ELISA system was used to determine the amount of prostaglandins in the required cells. The production of nitric oxide was determined using biochemical methods.

**Chemicals needed**

Absolute Ethanol, collagenase II (SIGMA), ascorbic acid L, HCI, RPMI 1-1640 medium, DMEM-F12 medium, HBSS solution, citric acid, dextrose, sodium bicarbonate, sodium pyruvate, B mercaptoethanol, HEPEs solution, Trypan blue, Tris Base, agarse, EDTA, PBS, Ethidium bromide, NaOH, NaCl, kCl, potassium dihydrogen phosphate(KH2PO4), monosodium dihydrogen phosphate, sodium dodecysulfate, SDS, Xylene Sylanid bromophenolblue, lipopolysaccharide LPS, regulatory buffers (pH=4,7), antibiotics including penicillin, streptomycin, amphotericin B, and gentamicin, Dnase I, Taq DNA Polymerase, 1M HCl, methanol, hexane, ethyl acetate, RNA extraction Kit (Takapouzist).

**List of primers**

Nucleotide sequences, the melting Temperature of primers and the size of PCR products are as follows:

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Melting Temperature</th>
<th>PCR Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Specific primers for bovine – COX2</td>
<td>Reverse: 5´ -TAA CAA GCC GGT AGC CCA CG-5´</td>
<td></td>
</tr>
<tr>
<td>Forward: 3´ -CTC TCT CTC TCT GTC CTG AT-5´</td>
<td>Tm forward: 52/9ºC, Tm reverse: 52/6ºC, PCR product size: 100 bp</td>
<td></td>
</tr>
<tr>
<td>2-Specific primers for bovine TNF-α</td>
<td>Forward: 3´-GAA GTA CCT GAG TCA GGC AGA GG-5´</td>
<td></td>
</tr>
<tr>
<td>Reverse: 5´ -GAG TGA CAA GCC TGT AGC CCA TGT GYTAGC -5´</td>
<td>Tm forward: 67/1ºC, Tm reverse: 69/5ºC, PCR product size: 100 bp</td>
<td></td>
</tr>
<tr>
<td>3-Specific primers for bovine IL-1β</td>
<td>Reverse: 5´ -GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC TCC CGCAT -3´</td>
<td></td>
</tr>
<tr>
<td>Forward: 3´ -GCA GAT CCA CCC ACG GCA AGT T -5´</td>
<td>Tm forward: 69/5ºC, Tm reverse: 70/4ºC, PCR product size: 100 bp</td>
<td></td>
</tr>
<tr>
<td>4-Specific primers for bovine Glyceraldehyde 3-phosphate (GAPDH)</td>
<td>Reverse: 5´ -GTA GCA ATG TAT AGC GGA GCA -5´</td>
<td></td>
</tr>
<tr>
<td>Forward: 3´ -GTA GCA ATG TAT AGC GGA GCA -5´</td>
<td>Tm forward: 69/5ºC, Tm reverse: 70/4ºC, PCR product size: 100 bp</td>
<td></td>
</tr>
<tr>
<td>5-Specific primers for bovine Glyceraldehyde 3-phosphate (GAPDH)</td>
<td>Reverse: 5´ -GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC TCC CGCAT -3´</td>
<td></td>
</tr>
<tr>
<td>Forward: 3´ -GCA GAT CCA CCC ACG GCA AGT T -5´</td>
<td>Tm forward: 69/5ºC, Tm reverse: 70/4ºC, PCR product size: 100 bp</td>
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<tr>
<td>6-Specific primers for Human TNF-α</td>
<td>Reverse: 5´ -GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC TCC CGCAT -3´</td>
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</tr>
<tr>
<td>Forward: 3´ -GCA GAT CCA CCC ACG GCA AGT T -5´</td>
<td>Tm forward: 69/5ºC, Tm reverse: 70/4ºC, PCR product size: 100 bp</td>
<td></td>
</tr>
<tr>
<td>7-Specific primers for Human Glyceraldehyde 3-phosphate (GAPDH)</td>
<td>Reverse: 5´ -GTA GCA ATG TAT AGC GGA GCA -5´</td>
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</tr>
<tr>
<td>Forward: 3´ -GTA GCA ATG TAT AGC GGA GCA -5´</td>
<td>Tm forward: 69/5ºC, Tm reverse: 70/4ºC, PCR product size: 100 bp</td>
<td></td>
</tr>
<tr>
<td>8-Specific primers for Human Glyceraldehyde 3-phosphate (GAPDH)</td>
<td>Reverse: 5´ -GTA GCA ATG TAT AGC GGA GCA -5´</td>
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</tr>
<tr>
<td>Forward: 3´ -GTA GCA ATG TAT AGC GGA GCA -5´</td>
<td>Tm forward: 69/5ºC, Tm reverse: 70/4ºC, PCR product size: 100 bp</td>
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**Isolation, culture and proliferation of chondrocyte cells**

Samples were taken from the radiocarpal joint cartilage of an 8-month-old Holstein cow. In the laboratory, in sterile conditions, the cartilages were taken from the internal and external condyle of the lower surface of metacarpal joint, according to what is shown in the figure. A layer with the thickness of about 1 mm was isolated and washed out three times by 1M PBS buffer (pH=7.4) containing a combination of antibiotics, including 50 μg/ml gentamicin and 100 unit/mlpenicillin, 100 μg/ml streptomycin (PHARMATEX ITALIA) and 0.25 μg/ml Amphotericin B (KimiaDaru-Tehran). Then, it was incubated in 220 μl/ml collagenase at 37ºC for 24 h. After 18 h, the content of test tube was mixed for 5 min and was filtered through 1mm Wire Strainer Screen, which is sterilized, and the wastes resulted from the impact of...
the collagenase type II were isolated from chondrocyte cells. The tube was centrifuged for 3 min, the supernatant was discarded and the pellet cells were washed four times with HBSS. The supernatant was removed with a pipette and finally deposited cells were incubated in the medium containing DMEM-F12 supplemented with FBS, 50 μg/ml ascorbic acid, 100 μg/ml penicillin, and 25 μg/ml streptomycin, with a density of 5 × 10^5 cell in the 22.2 cm plates at a temperature of 37°C, the humidity of 90% and5% CO₂ to reach cell density of 80-85%.

**Culture and proliferation of monocytes/macrophages (Human THP-1)***

A flask containing 60 million monocytes and 60 ml enriched cell culture medium was prepared from Pasteur Institute and incubated at 37°C and 5% CO₂ for 4 to 5 days to reach cell density above 80%.

The cells were resuspended slowly over 3 to 4 times in RPMI-1640 medium supplemented with L-gutatin, 10 mM HEPES, 0.1 mM sodium pyruvate, 0.05 mM B-mercaptoethanol, and a series of antibiotics including 50 μg/ml gentamic, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotripcin B. The Lc50 for chondrocyte cells was 50 μg/ml, and the mean was calculated 25 μg/ml. The Lc50 for monocyte cells was 30 μg/ml.

**cDNA Production:** A strand of mRNA was synthesized as template using reverse transcriptase and DNA polymerase enzyme, in the following, cDNA was amplified using RT-PCR standard method.

**Real-Time PCR:**

Real-Time PCR method quantitatively measures the copy number of templates such as DNA or cDNA. Therefore, it is able to eliminate the defect of PCR standard method which is End point method.

**Results**

**LC50 at concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 micrograms per milliliter for chondrocytes 70 μg/ml and median equivalent35 μg/ml was measured. By increasing the concentration ethanol extract increased the percentage of non-living cells and living cells is reduced (Figure 1).

LC50 at concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80 and 90 micrograms per milliliter for chondrocytes 30 μg/ml and median equivalent 15 μg/ml was measured. By increasing the concentration ethanol extract increased the percentage of non-living cells and living cells is reduced (Figure 2).

To measure the effectiveness of drug CCT (ethanolic extract plant root *Citrullus colocynthis*) in the treatment of osteoarthritis, the cells into three groups: The first group controls the cell with the same FBS normal conditions inside the cell, the second group inflamed cells with LPS 20 ng for an hour (as a control of the patient samples) and the third group cell with LPS 20 ng and ethanol extract the root of Citrullus colocynthis (to evaluate the efficacy of the drug in inflamed cells) Inflammatory cytokine for IL1β, TNF-α, iNOS, COX2 and prostaglandin and nitric oxide (NO) Chondrocyte cell extracted from calf Holstein 8 months and the cells monocytes / macrophages to control human samples were evaluated results are as follows. Compare the quantitative analysis of the effect of ethanol extract of the root of Citrullus colocynthis in cell cytokine TNF-α gene expression in inflamed the cartilage cells, reduce the level gene expression showed 94.29% (Tables 1-4).

**The effect of Citrullus colocynthis on prostaglandin production in the ethanol extracts stem cells from monocytes**

The first group of monocyte cells were cultured for 72 hours alone compared to cells stimulated with lipopolysaccharide PEG2 production level is lower.

The second group of monocyte cells stimulated with LPS and treated with 95 ml of 20 nanograms per milliliter root extract *Citrullus colocynthis* compared to monocyte cells stimulated with lipopolysaccharide without roots *Citrullus colocynthis* showed lower the level of production PEG2. The third group monocyte cells stimulated with 20 nanograms per milliliter for 1 hour LPS showed an increase in the production of PEG2.
The third group monocyte cells stimulated with 20 nanograms per milliliter for 1 hour lipopolysaccharide in the production of NO was positive control is intended that maximum amount of nitric oxide is nitrite oxide is zero and monocyte cells stimulated with LPS as a positive control.

In this study, cells were cultured monocytes alone at OD=0 the nitrate oxide is zero and monocyte cells stimulated with LPS as a positive control is intended that maximum amount of nitric oxide is equal to 1.59.

And monocyte cells stimulated with lipopolysaccharide with
root extract *Citrullus colocynthis* has optical absorption of 0.036 was obtained in accordance with the following formula of nitric oxide.

\[
\text{Dilution factor} \times \text{OD Test} - \text{OD Blank}
\]

\[
\text{OD St} - \text{OD Blank} = 0.036 \quad \text{OD Blank}=0.01 \quad \text{OD St}=0.084
\]

Dilution factor=2 (Figure 4)

**Discussion**

Osteoarthritis is a progressive and debilitating disease that factors such as age, genetics, trauma, obesity, increased biomechanical stress on the joints and it involved. Increase bone density in the hip and it led to a waterfall of catabolic imbalance at the molecular level in the physiology of the disease. The most common form of arthritis (Osteoarthritis) is a progressive degeneration of the articular cartilage in the joint space narrowing and Which leads to pain, loss of motion, instability and disability, resulting in disruption of life that involved joints including joints, fingers, toes, joints, neck and lower back and hips and femur [12] OA is a degenerative joint disease and joint pain common trait of the disease around the world cartilage damaged if left untreated, can lead to the development of this disease. OA are affected about 18% of women and 10% of men aged 60-64 years. Pain and loss of function and joint damage in OA in older age lead to osteoarthritis will be presented in 2020 as the leading causes of disability [13].

Inflammatory cytokine primarily destructive effects on the articular cartilage. This impacts not only induce cell aging and apoptosis of cartilage, it also reduces the synthesis of the key components such as proteoglycans, aggrecan and type II collagen. In addition to increased inflammatory cytokine synthesis and release of proteolytic enzymes including MMP and ADAMTS many of the metallocproteinase family, leading to the disintegration articular cartilage. And in addition its effect on cartilage, synovial cell and synovial tissue surrounding the affected joint and leading to inflammation. They can also increase their synthesis process and would be pushing the inflammatory process [14].

During observations in OA, IL-1β and TNF-α intracellular signals such as: P53, MAPKs, JNK; NF-κB the catabolic response and ultimately cause destruction of the cartilage are activated [15].

As one of OA pathogenesis cytokine engaged in inflammatory and catabolic effect and reactions on the cartilage. Chondrocyte exposed to IL-1β and TNF-α is, cells quickly forward to inducing apoptosis. Activation of TNF-α and IL-1β and other inflammatory cytokines inhibit TGF-β signaling pathway involved in the growth path and inhibit protein TGF-β type II receptor SAMAD7 and inhibit synthesis of cartilage cells, The effects on the release of enzymes and mediators involved in the pathophysiology of OA with the compounds including iNOS; Which leads to the production of NO, phospholipase A2, cyclooxygenase 2 (COX-2), prostaglandin synthetase to produce 2 prostaglandin E2 (PGE2) [16,17].

TNF-α from same cells are responsible for synthesis of IL-1β in the joint secreted and increased expression of IL-1β in the same tissues such as synovial fluid, synovial membrane, cartilage and subchondral bone layer has been found, TNF-α in the process pathogenesis of OA is important [18-21].

IL-1β and TNF-α secretion are usually at the same time and increased secretion of cell signaling pathway in addition to impact on the joints tissue and increased catabolism of inflammation, in reducing efficiency and reducing respiratory chain ATP in the mitochondria of cartilage cell sand thus decreased mitochondrial membrane potential, as well as synthesis of PGE₂, iNOS; NO; COX-2 is induced [22-25].

CCT (*Citrullus Colocynthis*) is a member of the Cucurbitaceae family or juice watermelon extract *Citrullus colocynthis* are powerful antibiotics against some microbes. Including against *Salmonella typhi*, *Coryne bacterium diphtheriae*, *Escherichia coli* and Staphylococcus aureus were a little less effective (and is highly effective for combating *Salmonella Paratyphi*) [26]. Colocynthin and Colocynthinin has been used on the plant for several diseases such as ascites, cancer, hepatitis, leukemia and tumors. Colocynthis anticancer the drug substance as is named cucurbitacin. Beta-sitosterol as well as anti-tumor agent - glucoside also be available in case of Colocynthis [27,28].

One of the proposed mechanisms of anti-tumor *Citrullus colocynthis* extract and many plant extracts is the mechanism of apoptosis or programmed cell death, the mechanism of apoptosis is the activation of several factors, most important of which, endonuclease enzyme activation, induction of p53 and activation of protease caspase3. There are two types of cucurbitacin B and E in the plant that the antitumor effect of cucurbitacin of type E is more effective [29,30].

In traditional Chinese medicine for the treatment of leukemia and tumors of the liver and spleen used *Citrullus colocynthis* extract [31]. Research on histological changes induced by different doses of watermelon fruit Colocynthis on diabetic and normal rats such as inflammation and necrosis in the liver, but a large group of diabetic that *Citrullus colocynthis* had received less visible effects of inflammation and necrosis. During the study Marzouk and his colleagues extract *Citrullus colocynthis* on rats conducted for the treatment of rheumatoid arthritis, they observed anti-inflammatory effect and reduce pain and
swelling extract of the plant without any adverse events in rats with rheumatoid arthritis, Marzouk and his colleagues believe that the Citrullus colocythis affects in the inhibition of endogenous substances such as serotonin, histamine, prostaglandins and arachidonic acid metabolism through cyclooxygenase Barad’re 18 and 19, and thus cytokines substances in the plant reduces IL-6; IL-1β; COX-2 anti-inflammatory cytokine IL-4 and raising reduce pain and swelling and inflammation. [11]

Conclusion

During the study was conducted on the pathophysiology of OA that increased expression of inflammatory cytokines in this disease, including IL-1β and TNF-α PGE2, NO, INOS, COX-2, catabolic pathways that degradation joint cartilage and thus inducing apoptosis and activate the immune system. The best way to prevent symptoms is to reduce the synthesis of the cytokine. Nowadays, there are synthetic drugs with adverse side effects for reducing inflammation and arthritis pain for arthritis patients. For reducing side effects of chemical drugs can be as effective drugs with little side effects for medicinal plants for the treatment this disease. One of those plants is Citrullus colocythis. Studies were conducted in the past on Citrullus colocythis analgetic was observed the effect anti-inflammation and reduction of apoptosis and necrosis. Our research examined on the effect of ethanol extract of plant roots Citrullus colocythis inflammatory cytokine expression in inflamed cells and monocytes with LPS20 both cartilage cells / macrophage. Our tests showed that ethanol extract of this plant reduce the amount of IL-1β and it can affect very high levels of expression TNF-α, PGE2, NO, INOS, COX-2 and it can reduce the expression of cartilage cells and monocytes / macrophage. We suggest that the future of this plant as a medicine for reducing the expression of inflammatory cytokines and reduce inflammation and joint pain and swelling caused by the expression of these cytokines used in people with osteoarthritis.

References