Study of the Relation Between Intestinal Flora and Rheumatoid Arthritis (RA)

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Abstract

Background/ Aim: Rheumatoid arthritis (RA) is a chronic, systemic disease characterized by persistent inflammatory synovitis that typically involves peripheral joints in a symmetric distribution. The joint arthritis is due to synovitis, which is the inflammation of the synovial membrane that covers the joint. Synovitis leads to erosion of the joint surface, causing deformity and loss of function. The bacterial load from the intestinal flora as a whole may well be a trigger for autoimmune rheumatoid synovitis.

Study Design: Isolation and identification of some bacteria from normal flora was carried out in microbiological laboratory in NODCAR in year 2007. The biochemical markers for rheumatoid arthritis in patients were observed as CRP (33.391 ± 15.189 mg/dl), RF (49.565 ± 15.805 IU/ml), ANA were positive in 78.3 of patients (33.391 ± 15.189 IU/ml), and Anti-CCP2 (42.391 ± 7.590 U/ml). An aqueous suspension of outer membrane (OM) was isolated from the cell wall fragments (CFW) of bacterial flora isolates were prepared according to Severijnen, et al., 1990. Physical and chemical measurements of outer membrane as determined the molecular weight by SDS polyacrylamide gel electrophoresis and determination of the outer membrane total proteins. Arthritis was induced in rats with a single intraperitoneal injection of an aqueous suspension of outer membrane (OM) was isolated from the cell walls of isolates are found in the normal flora of arthritic and non-arthritic human intestines. Some of these rats leave for 30 days and others for 60 days after induction of arthritis.

Results: Biochemical and histological parameters were made for all groups of rats. CRP, RF, ANA and Anti-CCP2 were highly significant increase in positive control of OM-induced arthritic rats for 30-days (32.0 ± 8.76 mg/dl, 50.0 ± 10.0 U/ml, 50.0 ± 24.49 IU/ml and 33.833 ± 5.382 U/ml, p < 0.01 respectively), and for 60-days (37.3 ± 9.24 mg/dl, 53.3 ± 11.56 IU/ml, 53.3 ± 23.10 IU/ml and 37.833 ± 5.345 U/ml, p < 0.01 respectively) when compared to the control group. Also, there were significant increase in CRP, RF, ANA and Anti-CCP2 in OM-induced arthritic rats before treatment with Echinacea extract (Immunvita) (24.0 ± 6.753 mg/dl, 45.0 ± 10.00 IU/ml, 30.00 ± 11.55 IU/ml and 35.50 ± 4.203 U/ml, p < 0.05, respectively) when compared to that after treatment (10.67 ± 24.62 mg/dl, 33.34 ± 11.56 IU/ml, 16.67 ± 5.78 IU/ml and 16.667 ± 2.517 U/ml, p < 0.05 respectively) for 30-days, also to control group (p < 0.05). Similar results obtained before Glucosamine (Genuphil) treatment (26.0 ± 4.0 mg/dl, 45.0 ± 10.0 IU/ml, 22.5 ± 12.59 IU/ml and 35.00 ± 3.367 IU/ml, respectively) when compared to that after treatment (10.67 ± 24.62 mg/dl, 16.67 ± 5.78 IU/ml, 13.34 ± 5.78 IU/ml and 16.60 ± 1.673 U/ml, p < 0.05 respectively) for 30-days, also to control group (p < 0.05). Also, in 60 days OM-induced arthritic rats, CRP, RF, ANA and Anti-CCP2 were significance increase before treatment with Echinacea extract (Immunvita) (26.0 ± 4.53 mg/dl, 45.0 ± 10.00 IU/ml, 23.00 ± 12.55 IU/ml and 37.20 ± 4.438 U/ml, p < 0.05, respectively) when compared to that after treatment (12.00 ± 4.62 mg/dl, 35.00 ± 10.00 IU/ml, 15.00 ± 5.78 IU/ml and 19.80 ± 2.168 U/ml, p < 0.05 respectively), also to control group (p < 0.05) & with Glucosamine (Genuphil), before treatment (24.0 ± 6.0 mg/dl, 45.0 ± 10.0 IU/ml, 29.5 ± 11.59 IU/ml and 35.75 ± 4.992 U/ml, respectively) when compared to that after treatment (13.37 ± 4.63 mg/dl, 26.67 ± 11.56 IU/ml, 16.67 ± 5.78 IU/ml and 19.75 ± 2.986 U/ml, p < 0.05 respectively), also to control group. Histologically, the arthritis lesions produced by OM in the paw of the inspected animals were of mild degree.

Conclusion: The OM prepared from the selected strains from human intestinal flora induced acute and chronic arthritis in rat models. Serum concentrations of antibodies against cyclic citrullinated peptide (anti-CCP) seem to be just as sensitive and more specific than rheumatoid factor for diagnosing rheumatoid arthritis and predicting its progression. The two treatments of Echinacea and glucosamine showed reduction in the inflammatory cell influx and joint damage.

Key Words: Rheumatoid arthritis – Synovitis – Bacteria – Intestinal flora – Anti-cyclic citrullinated peptide – C-reactive Protein – Rheumatoid Factor – Anti-nuclear antibodies – Echinacea extract – Glucosamine.

Introduction

RHEUMATOID arthritis occurs when body’s immune system attacks and destroys the tissues that make up joints. Rheumatoid arthritis is a
common autoimmune disease characterized by chronic, progressive and erosive synovitis [1]. The synovial histopathological features include, a marked villous hypertrophy, proliferation of superficial synovial cells, and a marked infiltration of chronic inflammatory cells with a tendency to form lymphoid nodules. The chronic progressive of this synovitis commonly induced the erosion of cartilage and bone, and finally destroyed the joints. The joints become swollen, stiff and painful. The affected joints typically include hands, fingers, wrists, ankles, feet, elbows and knees. Extra-articular (outside the joints) manifestations occur in about 15% of individuals with rheumatoid arthritis [2]. Genes may play a role in the development of rheumatoid arthritis [3]. It is also possible that a change in the body, such as infection or hormonal shift, can trigger its development [4,5]. There are an aetiologic role for intestinal bacteria in rheumatoid arthritis [6-8]. The bowel bacteria like, Salmonella spp, Shigella spp, Campylobacter spp and Yersinia spp are involved in the reactive arthritis [9-11] and Crohn’s disease [12]. Klebsiella spp was related to ankylosing spondylitis and Reiter’s disease [13]. Cell wall fragments (CWF) from two strains, isolated from a rheumatoid arthritic patient, induce chronic polyarthritis in rats after a single intraperitoneal injection [14]. These findings prompted us to investigate the composition of the intestinal flora in rheumatoid arthritic patients. Several authors proposed a role for micro-organisms in rheumatoid arthritis [6-8,15,16]. Some infectious organisms suspected of triggering rheumatoid arthritis include Mycoplasma, Erysipelothrix, Parovirus B19 and Rubella [17]. Also some studies have confirmed a potential association between RA and two Herpes virus infections such as Epstein-Barr virus (EBV) and Human Herpes Virus 6 (HHV-6) [18]. The bacterial load from the intestinal flora, as a whole, may well be a trigger for autoimmune rheumatoid synovitis [19]. The joint arthritis of rheumatoid arthritis is due to synovitis, which is inflammation of the synovial membrane that covers the joint. Synovitis leads to erosion of the joint surface, causing deformity and loss of function [1].

Rheumatoid Factor (RF) is an autoantibody, antibody direct against an organism’s own tissues most relevant in rheumatoid arthritis [20]. It is an antibody against the Fc portion of the IgG, which is itself an antibody [21]. Not all people with rheumatoid arthritis have detectable rheumatoid factor (called seronegative). Also, there is positive results can occur due to causes other than arthritis, including, chronic hepatitis, leukemia, dermatomyositis, infectious mononucleosis, Scleroderma and systemic lupus erythematosus (SLE) and negative results do not rule out disease. But in combination with signs, symptoms, and other diagnostic tests, it can be diagnostic and prognostic [22]. The higher levels of RF, the higher the possibility of a more destructive articular disease [21].

Antinuclear antibodies (ANA) are a unique group of autoantibodies that have the ability to attack structures in the nucleus of cells. Antinuclear antibodies are antibodies directed against the cell nucleus. They are raised in autoimmune conditions where the immune system makes antibodies to fight its own body. There are subsets of the ANA use to determine the specific autoimmune disease e.g. anti-dsDNA, anti-sm, Sjogren’s syndrome antigen (SSA, SSB), ScI-70 antibodies, anti-centromere, anti-histone and anti-RN.

The ANA test is complex, but the results (Positive, Negative, Titer or Pattern) and possible subset test results can give clinician valuable diagnostic information [21].

C-reactive protein (CRP) is a protein produced by the liver macrophages [23] and adipocytes [24], and found in the blood. The function of CRP, is can initiate opsonization, phagocytosis and lysis of invading cells, as a response of the inflammatory reaction. It has been recognized as one the most sensitive of the acute phase reactant proteins (APRPs). CRP levels in plasma can rise dramatically after myocardial infarction, stress, trauma, infection, inflammation, surgery, or neoplastic proliferation. Its increase occurs within 6h, and beaks at 48h, and its half-life is constant [23]. Determination of CRP is clinically useful for screening for organic disease, for assessing the activity of an inflammatory disease such as rheumatoid arthritis, for detecting intercurrent infections in systemic lupus erythematosus (SLE), and in leukemia [25]. It is used mainly as a marker of inflammation [23]. CRP is normally present in plasma at a mean concentration of less than 800 g/dl by using sensitive immunochemical methods [25].

In the late 1990’s antibodies directed against citrullinated peptides were discovered. When cells grow old, some of the structural proteins undergo citrullination under the direction of cellular enzymes. Arginine residues undergo deamination to form the non-standard amino acid citrulline. Citrullinated peptides fit better into the HLA-DR4 molecules that are strongly associated with rheumatoid arthritis development, severity and prognosis. Many types of citrullinated peptides are present.
in the body, both in and outside joints. Sera from individual rheumatoid arthritis patients contain antibodies that react against different citrullinated peptides, but these each individual antibodies do not react against all possible citrullinated peptides. Thus to improve the sensitivity of the citrullinated peptide assays, cyclic citrullinated peptides (CCP) have been artificially generated to mimic a range of conformational epitopes present in vivo. It is these artificial peptides that are used in the second generation anti-CCP assays [26]. CCP antibodies alone give a sensitivity of around 66% in early rheumatoid arthritis, similar to RF though they have a much higher specificity of >95% (compared with around 80% for RF). The combined use of anti-CCP and RF tests is now considered to be the Gold standard in the early detection of the RA [27-31]. Anti-CCP2 autoantibodies are specific-IgG autoantibodies against second generation cyclic citrullinated peptides (CCP2) in sera, Anti-CCP2 autoantibodies occur early in RA, this is particularly valuable in aiding diagnosis when clinical symptoms may not be easily distinguished from other conditions [29,32-34].

There is no known cure for rheumatoid arthritis, but many different types of treatment can alleviate symptoms and/or modify the disease process. The goal of treatment is two-folds; alleviating the current symptoms, and preventing the future destruction of the joints. Pharmacological treatment of rheumatoid arthritis can be divided into disease-modifying antirheumatic drugs, anti-inflammatory agents and analgesics [35,36]. The herb, *Echinacea Purpurea*, is a powerful immune system stimulators [37], it may have antiviral, antioxidant, anti-inflammatory properties [38], and it inhibits edema [39].

Glucosamine is an amino monosaccharide composed of glucose, with a bound amino group, it is present in several tissues including cartilage [40]. Complex sugars include glucosaminoglycans is a major component in articular cartilage. Its effects include stimulation of physiologic proteoglycan synthesis and decrease in the activity of catabolic enzymes such as metalloproteases [41]. It is also a common constituent of cartilage matrix and synovial fluid [42]. Dietary supplements as glucosaminoh-chondritin combination are commonly used products for osteoarthritis and rheumatoid arthritis. Exogenous glucosamine is derived from marine exoskeletons or produced synthetically [43]. Exogenous glucosamine may have anti-inflammatory effects and is thought to stimulate metabolism of chondrocytes [44].

**Aim of the work:**

The aim of this work to investigate the role of anaerobic feacal flora isolates from arthritic and non-arthritic patients to induce arthritis in animal models, and to evaluate the isolated bacterial species which have arthropathic properties. Also, investigate of some biochemical markers in arthritic humans and animal models in a trial to early laboratory diagnose of arthritis and follow-up after treatment with some drugs.

**Material and Methods**

**Selection of patients:**

Prior to enrolment in this study we obtained written informed consent from the patients according to the declaration of Imbaba Fever Hospital. Twenty-three RA patients who fulfilled the revised criteria prescribed by the American Rheumatism Association were enrolled in this study [45] as following:

- Morning stiffness for at least 6 weeks.
- Arthritis and soft-tissue swelling of more than 3 of 14 joints/joint groups, present for at least 6 weeks.
- Arthritis of hand joints, present for at least 6 weeks.
- Symmetric arthritis, present for at least 6 weeks.
- Subcutaneous nodules in specific places.
- Rheumatoid factor at a level above the 95th percentile.
- Radiological changes suggestive of joint erosion.

At least four criteria have to be met for classification as RA.

**Bacterial isolations:**

Twenty three rheumatoid arthritic patients were studied, aged from 35-65 years. From each subject, two stool specimens obtained on different days were investigated. The control group comprised 10 healthy subjects, aged from 30-55 years. The Control group, who were apparently healthy individuals, had no joint symptoms or complaints and did not receive any medications during the period of stool culture. All groups were laboratory investigated by Anti-CCP2, C-reactive protein (CRP), rheumatoid factor (RF), and Anti-nuclear antibodies (ANA). Stool samples of 2g were suspended in anaerobic and aerobic broth. Three serial 10-fold dilutions were prepared from the suspensions. A 0.2 ml was taken from the final dilution of the stool sample and inoculated on a nutrient agar and
incubated at 37°C for 48 hours. Gram stain for all colonies were made, and the number of isolates was multiplied by the dilution factor to give numbers per gram faeces wet weight. The composition of the intestinal flora was determined [46]. The microorganisms were separated into groups on the basis of morphology and gram stain as; Gram-negative bacilli and Gram-positive cocci and cocacobacilli.

**Preparation of bacterial outer membranes (OM):**

Two isolates from the intestinal flora of RA patients and four isolates from the normal flora of the healthy individuals were isolated. Pure cultures were identified according to the "Bergey’s Manual of Systematic Bacteriology". Bacterial outer membrane (OM) were prepared [47] followed by the differential centrifugation procedure [48]. Briefly, cells were harvested, washed and subsequent fragmented with glass beads in a brown shaker. OM were collected by 5000 g for 15 min at 4°C centrifugation, treated with ribonuclease and trypsin, washed and sonicated for 75 min. After sedimentation of debris, the sonicated suspension was centrifuged at 10 000 g for 30 min; the 10 000 g supernatant was centrifuged twice at 100 000 g for 60 min. Both 100 000 g pellets were collected, resuspended in PBS and then passage through a 0.45 g millipore filter and subsequent control for sterility [47,48] and used for intraperitoneal injection of the animal model.

**Determination of lysozomal activity:**

The lysozomal activity were determined as; OM suspensions were diluted in 0.1 M sodium acetate buffer pH 5.0 to an OD at 560 nm, of about 0.8 per mg dry weight OM; 0.1 mg lysozyme (egg white, Sigma) was added, and during incubation at 37°C the OD was measured at regular intervals [19,49]. As a control, OM suspensions were incubated at 37°C without the addition of lysozyme.

Muramic acid and rahmnose contents were determined [50,51].

The OM aqueous suspension was used for intraperitoneal injection of rats.

**Induction of arthritis in rats:**

**Animal models:**

I- **Control groups:**

- Negative control group: Consists of 10 rats, injected with phosphate buffered saline.
- Positive control group: Consists of 10 rats, injected with an aqueous suspension of OM from the strains selected for induction of arthritis, and non-treated.

II- **OM-induced arthritis 30-days groups:** Consists of 20 rats, and subclassified into:

- OM-induced arthritic Echinacea Extract Group: Consists of 10 rats, blood samples were thrown before and after treatment with 18 mg Echinacea Extract/daily for 30 days.
- OM-induced arthritic Glucosamine Group: Consists of 10 rats, blood samples were thrown before and after treatment with 54 mg Glucosamine/daily for 30 days.

III- **OM-induced arthritis 60-days groups:** Consists of 20 rats, and subclassified into:

- OM-induced arthritic Echinacea Extract Group: Consists of 10 rats, blood samples were thrown before and after treatment with 18 mg Echinacea Extract/daily for 60 days.
- OM-induced arthritic Glucosamine Group: Consists of 10 rats, blood samples were thrown before and after treatment with 54 mg Glucosamine/daily for 60 days.

All groups of rats were weighing 132-197 g. For induction of arthritis, rats were injected intraperitoneally with 0.2 ml aqueous suspension of OM (containing 24-27 gg of muramic acid) from the strains selected [19,47]. The negative control rats were injected with an equal volume of phosphate buffered saline. The animals were observed for the development of paw inflammation at regular intervals during 60 days. Paw volume of the hind limbs was measured with a plethysmometer and was expressed as an indicator of swelling. The paw volume was expressed as the absolute volume. Diameters of wrists and ankles at the height of the distal end of the radius and at the malleoli, respectively were measured with a varnier calliper several times at a week intervals, for 8 weeks. The blood sample was taken from each rat from its eye plexus, stand for clott formation, serum samples are separated, Anti-CCP2, CRP, RF and ANA were determined in each sample for laboratory investigations of rheumatoid arthritis in rats. Also the mean increase of sum paw diameter from day 1-30 and day 31-60 after cell wall inoculation, was taken as a parameter for the severity of the acute and chronic arthritis, respectively.

**Biochemical markers for rheumatoid arthritis:**

- Anti-Cyclic Citrullinated peptide (Anti-CCP2): Using Bindazyme Anti-CCP2 (code MK 090, the binding site Ltd, Birmingham, UK); a new highly sensitive and specific second generation anti-CCP2 Enzyme Immunoassay (ELA) Kit. It is an enzyme immunoassay for the quantitative deter-
mination of IgG autoantibodies to cyclic citrullinated peptides in serum. The positive results were ≥20 U/ml, while the negative results were, <20 U/ml. Anti-CCP2 autoantibodies appear extremely specific for RA (>95%) and demonstrate sensitivity in the region of 60-77% [32].

- C-reactive Protein (CRP): Using a rapid latex slide test of serum for detection of CRP [52]. Its increase occurs within 6h after insult of inflammation, and peaks at 48h, and its half-life is constant [23]. CRP is normally present in plasma at a mean concentration of less than 80 µg/dl, by using sensitive immunochemical methods are available for CRP determination, such as ELISA, immunoturbidimetry, rapid immunodiffusion and visual agglutination [25].

- Rheumatoid Factor (RF): Using a rapid latex slide test of serum for detection of rheumatoid factor [53-55]. High levels RF (20 IU/ml or 1:40) are indicative of rheumatoid arthritis (present in 80% of cases). The higher levels of RF, the higher the possibility of a more destructive articular disease [21].

- Anti-nuclear antibodies (ANA) (Antin-DNA): Using a rapid latex slide test of serum for detection of deoxyribonucleic acid antibodies [56]. The incidence of positive ANA (in percent) per condition is; Systemic lupus erythematosus (SLE) over 90%, rheumatoid arthritis 30-40%, Sjogren’s syndrome 40-70%, Felty’s syndrome 100% and Juvenile arthritis 15-30% [21].

**Histopathological Examinations:**

At the end of the experiment the hind feet of rats were excised and fixed in 10% buffered formalin solution, fixed specimens were decalcified using 5% formic acid, and embedded in paraffin wax. Serial sections of 5 µm thickness were obtained, dewaxed, and stained with haematoxylin and eosin. The stained sections were scored microscopically for changes to joint architecture.

The effect of treatments on parameters under investigation were done.

**Results**

Two isolates namely Citrobacter spp. from the intestinal flora of RA patients and four isolates namely Escherichia coli, P. aeruginosa, Staphylococcus aureus and Enterobacter spp, are found in the normal flora of the intestines from healthy individuals were used for preparation of bacterial outer membranes (OM) and injected intraperitoneally for induction of arthritis in rats.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Control (healthy subjects) (N=10)</th>
<th>Arthritic patients (N=23)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>3</td>
<td>4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Citrobacter spp.</td>
<td>1</td>
<td>3</td>
<td>N.S.</td>
</tr>
<tr>
<td>Streptococci</td>
<td>3</td>
<td>5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>2</td>
<td>4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Enterobacter spp.</td>
<td>3</td>
<td>3</td>
<td>N.S.</td>
</tr>
<tr>
<td>Salmonella spp.</td>
<td>0</td>
<td>2</td>
<td>N.S.</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>2</td>
<td>2</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Items</th>
<th>Control (healthy subjects) (N=10)</th>
<th>Arthritic patients (N=23)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CCP2 (U/ml)</td>
<td>9.90±2.025</td>
<td>42.391±7.590*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>7.40±2.10</td>
<td>33.391±15.189*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Rheumatoid Factor (IU/ml)</td>
<td>14.60±6.30</td>
<td>49.565±15.805*</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Anti-nuclear Antibodies</td>
<td>7.60±1.50</td>
<td>30.417±18.528*</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

*: Statistical significance difference between the arthritic patients group and control (healthy subjects) group.

There were significant difference between the arthritic patients group and control group in Anti-CCP2 (42.391±7.590 Vs 9.90±2.025), CRP (33.391±15.189 Vs 7.40±2.10), RF (49.565±15.805 Vs 14.60±6.30) and ANA (30.417±18.528 Vs 7.60±1.50), p<0.05, respectively.

CRP, RF, ANA and anti-CCP2 were highly significance increase in positive control of OM-induced arthritic rats for 30-days (32.0±8.76 mg/dl, 50.0±10.95 IU/ml, 50.0±24.49 IU/ml and 33.833±5.382 U/ml, p<0.01 respectively), and for 60-days (37.33±9.24 mg/dl, 53.33±11.56 IU/ml, 53.34±23.10 IU/ml and 37.833±5.345 U/ml, p<0.01 respectively) when compared to the negative control group. Also, there were significant increase in CRP, RF, ANA and Anti-CCP2 in OM-induced arthritic rats before treatment with Echinacea extract (Immunvita) (24.0±6.753 mg/dl, 45.0±10.00 IU/ml,
revealed slight to moderate inflammatory cellular infiltrate, edema and dilated blood vessels in synovial membrane with mild proliferation of synovial cells, Figs. (2,3).

Histopathology results:

1- In OM-induced Arthritic 30-days group:

A- Before treatment:

In OM-induced arthritic non treated groups (positive control), the histological examinations revealed slight to moderate inflammatory cellular

b) After treatment:

- In OM-induced Arthritic Glucosamine-treated group:

Table (3): Biochemical markers for rheumatoid arthritis in negative control, positive control, non-treated and treated OM-induced Arthritic rats (30 days).

<table>
<thead>
<tr>
<th>Items</th>
<th>Negative control</th>
<th>Positive control</th>
<th>Om-induced arthritic-echinacea exact group</th>
<th>Om-induced arthritic-glucosamine group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CCP2 (U/ml)</td>
<td>8.00±1.58</td>
<td>33.83±5.38*</td>
<td>35.5±4.20**</td>
<td>16.67±2.52</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>7.2±1.09</td>
<td>32.0±8.76*</td>
<td>24.0±6.53**</td>
<td>10.67±4.62</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RF (IU/ml)</td>
<td>16.0±5.47</td>
<td>50.0±10.95*</td>
<td>45.0±10.0**</td>
<td>33.34±11.56</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ANA (IU/ml)</td>
<td>8.8±1.10</td>
<td>50.0±24.49*</td>
<td>30.0±11.55**</td>
<td>16.67±5.78</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

p : Statistical significance difference between different groups.
* : Statistical significance difference between the positive control group and negative control group.
** : Statistical significance difference between the OM-induced arthritic before treatment rats and both OM-induced arthritic after treatment and negative control rats.

Table (4): Biochemical markers for rheumatoid arthritis in negative control, positive control, non-treated and treated OM-induced Arthritic rats (60 days).

<table>
<thead>
<tr>
<th>Items</th>
<th>Negative control</th>
<th>Positive control</th>
<th>Om-induced arthritic-echinacea exact group</th>
<th>Om-induced arthritic-glucosamine group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CCP2 (U/ml)</td>
<td>8.00±1.58</td>
<td>37.83±5.35*</td>
<td>37.2±4.43**</td>
<td>19.80±2.17</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CRP (mg/dl)</td>
<td>7.2±1.09</td>
<td>37.33±9.24*</td>
<td>26.0±4.53**</td>
<td>12.00±4.62</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RF (IU/ml)</td>
<td>16.0±5.47</td>
<td>53.33±11.56*</td>
<td>45.0±10.0**</td>
<td>35.00±10.00</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ANA (IU/ml)</td>
<td>8.8±1.10</td>
<td>53.34±23.10*</td>
<td>23.0±12.55**</td>
<td>15.02±5.78</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

p : Statistical significance difference between different groups.
* : Statistical significance difference between the positive control group and negative control group.
** : Statistical significance difference between the OM-induced arthritic before treatment rats and both OM-induced arthritic after treatment and negative control rats.
When compared to the non-treated group, the paws joint revealed no inflammation, mild cartilage damage, with slight rough edge and still edema could be observed, Fig. (4).

- **In OM-induced Arthritic Echinacea Extract-treated group:**
  
  When compared to the non-treated group, there were no inflammatory reactions, however there were edema with proliferated synovial membranes, partial damage in cartilage and bone were seen, Figs. (5,6).

2- **In OM-induced Arthritic 60-days group:**

A- **Before treatment:**
  
  Paws joints, inspected after 60 days, of initial induction, showed more damage to cartilage and bone, beginning of pannus formation, together with sever inflammation, Fig. (7).

B- **After treatment:**

- **In OM-induced Arthritic Glucosamine-treated group:**
  
  In 60% of this group, there were edema, new blood vessels formation and mild synovial cells proliferation, while in the 30% of this group, showed partial cartilage damage and irregular cartilage edge Fig. (8).

- **In OM-induced Arthritic Echinacea Extract-treated group:**
  
  There are rough edge with destructed cartilage and bone, however edema with fibroblast and blood vessels could be observed in synovial membrane of 50% of animals Figs. (9,10).

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**Fig. (1):** Paw joint of control rat, normal cartilage (C) and bone (B) structure.

**Fig. (2):** Paw joint of OM-induced arthritic 30 days non-treated group, demonstrating edema (Arrow) in synovial layer (S), damaged cartilaginous layer (C).

**Fig. (3):** Higher magnification photomicrograph of the OM-induced arthritic 30 days non-treated group, showing edema (O), blood vessel (BV) and synovial membrane (S).

**Fig. (4):** Paw joint in OM-induced arthritic glucosamine-treated 30 days group, showing cartilaginous damage (c) and irregular articular edge (arrow).
Fig. (5): Paw joint in OM-induced arthritic echinacea extract-treated 30 days group, showing inflammatory reaction in synovial membrane (Arrow) and cartilaginous damage (C).

Fig. (6): Demonstrating articular joint surface in paw of OM-induced arthritic Echinacea extract 30 days treated group, focusing on irregular edge of the articular joint (Arrow).

Fig. (7): Demonstrating paw joint after 60 days of OM-induction of arthritis, non-treated group, showing edema (Arrow).

Fig. (8): A photomicrograph of paw joint of OM-induced arthritic glucosamine treated rat (60 days gp), demonstrating edema(arrow), blood vessel (BV), in synovial layer.

Fig. (9): A photomicrograph of paw joint of OM-induced arthritic Echinacea treated (60 days) group, demonstrating articular surface with irregularity of the edge (Arrow).

Fig. (10): A photomicrograph of paw joint of the OM-induced arthritic Echinacea extract treated (60 days) treated group, showing edema (arrow) and fibroblast (double arrow) in synovial layer.
**Microbiological results:**

The protein content and lysozyme sensitivity for OM of isolated strains:

<table>
<thead>
<tr>
<th>Numbers of isolated strains</th>
<th>Protein content of their OM*</th>
<th>Lysozyme sensitivity for each**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40.5</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>37</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>137.5</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>73.5</td>
<td>39</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>21</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td>63.08±37.81</td>
<td>40.0±14.81</td>
</tr>
</tbody>
</table>

* μg/g of body weight.

** Decrease of the optical density at 560 nm after 8 h of incubation with Lysozyme compared with the optical density at 560 nm at time 0.

Isolated strains (1 - 4) from Non-RA individuals.

Isolated strains (5 - 6) from RA patients.

**SDS polyacrylamide gel electrophoresis for OM of 6 isolates:**

Protein content 40.5, 37, 137.5, 45, 73.5 and 45 μg/g of body weight for the isolates respectively.

**Discussion**

Autoimmune diseases require that the affected individual have a defect in the ability to distinguish foreign molecules from the body’s own. The genetic association with HLA-DR4 is believed to play a major role in the factors that allow the inflammation in rheumatoid arthritis, as well as the newly discovered associations with the gene PTPN22 and with two additional genes, all involved in regulating immune responses [3,57]. Thus in theory, rheumatoid arthritis requires susceptibility to the disease through genetic endowment with specific markers and an infectious event that triggers an autoimmune response. Once triggered, B-lymphocytes produce immunoglobulins and rheumatoid factors of the IgG and IgM classes that are deposited in the tissue. This subsequently leads to the activation of the serum complement cascade and the recruitment of the phagocytic arm of the immune response, which further exacerbates the inflammation of the synovium, leading to edema, vasodilation and infiltration by activated T-cells, mainly CD4 in nodular aggregates and CD8 in diffuse infiltrates. Early and intermediate molecular mediators of inflammation include tumor necrosis factor alpha (TNF-α), interleukins (IL-1, IL-6, IL-8 and IL-15, transforming growth factor-β, fibroblast growth factor and platelet-derived growth factor. Synovial macrophages and dendritic cells further function as antigen peresenting cells by expressing MHC class II molecules, leading to an established local immune reaction in the tissue. The disease progresses in contact with formation of granulation tissue at the edges of the synovial lining (pannus) with extensive angiogenesis and production of enzymes that cause tissue damage. Once the inflammatory reaction is established, the synovium thickness, the cartilage and the underlying bone begins to disintegrate and evidence of joint destruction accues [3,21].

The patients and controls investigated in this study, were all outpatients, they found no significant differences in total numbers of microorganisms between these groups, Table (1). While there were significant differences in biochemical markers between the arthritic patients and healthy subjects, as, there were significant increase in Anti-CCP2, CRP, RF and ANA in arthritic patients when comparing to control group, Table (2). In this study, showed that the OM prepared from the selected strains from human intestinal flora induced acute and chronic arthritis in rat models Tables (3,4) and Figs. (2,3,7), in which anti-CCP2, CRP, RF and ANA were significantly increased in positive control and in OM-induced arthritic non-treated groups when compared to the negative control group Tables (3,4).

Anti-CCP2 autoantibodies are specific-IgG autoantibodies against second generation cyclic citrullinated peptides (CCP2) in sera. Anti-CCP2 autoantibodies occur early in RA, this is particularly valuable in aiding diagnosis when clinical symptoms may not be easily distinguished from other conditions [29,32-34]. In this study anti-CCP2 was significantly increased in arthritic patients (42.391 ± 7.590 Vs 9.90±2.025) U/ml, and in OM-induced...
arthritic rats (positive control) either 30 days (33.83 ± 5.38 Vs 8.00 ± 1.58 U/ml) or 60 days (37.83 ± 5.35 Vs 8.00 ± 1.58 U/ml) Tables (2-4). CCP antibodies alone give a sensitivity of around 66% in early rheumatoid arthritis, similar to RF though they have a much higher specificity of >95% (compared with around 80% for RF), these antibodies have turned up as powerful biomarkers, which are accepted as a major diagnostic tool in diagnosing rheumatoid arthritis (RA) already in a very early stage of disease. Most of clinical utility of the anti-CCP2 test is associated with high disease specificity [58] and the presence of anti-CCP antibodies in early phases of RA [59]. Moreover it has been demonstrated that the appearance of anti-CCP antibodies in the circulation may occur several years before the RA onset and represent a marker of future disease [60].

The combined use of anti-CCP and RF tests is now considered to be the Gold standard in the early detection of the RA [27-31].

Not all people with rheumatoid arthritis have detectable rheumatoid factor (called seronegative). Also, there is positive results can occur due to causes other than arthritis, including, chronic hepatitis, leukemia, dermatomyositis, infectious mononucleosis, Scleroderma and systemic lupus erythematosus (SLE) and negative results do not rule out disease. But in combination with signs, symptoms, and other diagnostic tests, it can be diagnostic and prognostic [22]. High levels of RF are indicative of rheumatoid arthritis (present in 80% of cases). The higher levels of RF, the higher the possibility of a more destructive articular disease [21]. In this study RF was significantly increased in arthritic patients (33.39 ± 15.189 Vs 7.40 ± 2.10 mg/dl), and in OM-induced arthritic rats (positive control) either 30 days (32.0 ± 8.76 Vs 7.20 ± 1.09 mg/dl) or 60 days (37.33 ± 9.24 Vs 7.20 ± 1.09 mg/dl) Tables (2,3,4).

Also in respect to the histopathological studies, revealed that slight to moderate inflammatory cellular infiltrate, edema, dilated blood vessels in synovial membrane with mild proliferation of synovial cells occur in the positive (non-treated) groups Figs. (2,3). Also the paw joints in the OM-induced arthritic 60 days groups, showed more damage to cartilage and bone, also the pannus formation begun, together with sever inflammation Fig. (7). These results ensure the theory said that, the rheumatoid arthritis requires susceptibility to the disease through genetic endowment with specific markers and an infectious event that triggers an autoimmune response [3]. Also, this study agree with other researchers whose found that some infectious organisms suspected of triggering rheumatoid arthritis [17], and the bacterial load from the intestinal flora as a whole may well be a trigger for autoimmune synovitis [19].

After treatment of the experimental models either with Echinacea extract or glucosamine, showed significant decrease in the anti-CCP2, CRP, RF and ANA levels when compared to their levels in each group before treatment, Tables (3,4), the decrease in these titters of antibodies, predict the suppression of joint damage. While in respect to the histopathological studies, showed that no inflammatory reaction, mild cartilage damage and still edema with proliferated synovial membrane Figs. (4-10). This indicating that, there is no known cure for rheumatoid arthritis, but many different types of treatment can alleviate symptoms and/or
modify the disease process. The goal of treatment is two-folds; alleviating the current symptoms, and preventing the future destruction of the joints. Pharmacological treatment of rheumatoid arthritis can be divided into disease-modifying antirheumatic drugs, anti-inflammatory agents and analgesics [35,36].

**Conclusion:**

The OM prepared from the selected strains from human intestinal flora induced acute and chronic arthritis in rat models. Serum concentrations of antibodies against cyclic citrullinated peptide (anti-CCP) seem to be just as sensitive and more specific than rheumatoid factor for diagnosing rheumatoid arthritis and predicting its progression. Also, the appearance of anti-CCP antibodies in the circulation may occur several years before the RA onset and represent a marker of future disease.

Early diagnosis and treatment of rheumatoid arthritis are crucial to avoid irreversible damage to the joints. Because rheumatoid factor can be present in the plasma of healthy people and people with autoimmune diseases other than rheumatoid arthritis, using anti-cyclic citrullinated peptide antibodies can be of great help in making the diagnosis.

Finally, the decrease in titers of anti-CCP autoantibodies, even if high titer at baseline, may predict the suppression of joint damage.

**References**

agglutination of sheep red corpuscles. APMIS, 115 (5): 422-38, 1940.


