EFFECTS OF METHOTREXATE AND LEFLUNOMIDE THERAPY ON KNEE SYNOVUM OF ADJUVANT INDUCED ARTHRITIS AS A MODEL FOR RHEUMATOID ARTHRITIS

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KEY WORDS: ADJUVANT ARTHRITIS, RHEUMATOID ARTHRITIS MODEL (RA MODEL), LIGHT MICROSCOPY, OXIDATIVE STRESS MARKERS, NITRIC OXIDE, MALONDIALDHYDE, GLUTATHIONE, SUPEROXIDE DISMUTASE, CERULOPLASMIN

ABSTRACT

Objective: Leflunomide and methotrexate have proven to be efficacious in reducing joint inflammation and joint destruction in clinical models of arthritis and in rheumatoid arthritis. The objective of this study was to evaluate the effects of both drugs as well as their combination therapy on the synovium and cartilage of adjuvant arthritis as a model of rheumatoid arthritis (RA) in humans.

Methodology: This study was carried out on forty animals stratified into 5 groups: normal, adjuvant arthritis (AA) control, AA who received leflunomide in a dose of 20 mg/kg orally, AA who received intraperitoneal methotrexate in a dose of 0.3 mg/kg twice weekly and AA who received both leflunomide and methotrexate of the same dose given in groups 3 and 4. All animals were sacrificed after 3 weeks; the right knee was dissected and examined with light microscopy. Oxidants markers [nitric oxide (NO) and malondialdhyde (MAD)] and antioxidants markers [glutathione (GSH), erythrocyte superoxide dismutase (SOD) and ceruloplasmin (CP)] were all measured.

Results: All the treatment modalities showed variable degrees of improvement of synovial and cartilage scoring in comparison to AA (the non-treated group). The leflunomide treated group (group 3) showed the best improvement of
synovial pathology, while the combined therapy group (group 5) showed the best improvement of cartilage pathology.

The oxidative stress markers showed some changes with different modalities of treatment where, nitric oxide did not change significantly between all groups. Malondialdehyde (MAD) was significantly lower in the methotrexate (MTX) treated group as compared to AA controls. Also, superoxide dismutase (SOD) was significantly lower in the leflunomide treated group, MTX treated group as well as in the group who received combined therapy as compared to AA the controls. Glutathione (GSH) level was significantly decreased with combination therapy as compared to the leflunomide treated group. Serum ceruloplasmin (CP) showed a significant decrease in its level in the MTX treated group as compared to the AA controls. MTX treatment (group 4) was the best in controlling oxidative stress markers.

**Conclusion:** Further study is needed to evaluate the duration and dose effect of each drug on synovium, cartilage and oxidative markers.

**INTRODUCTION**

RA is a chronic inflammatory arthropathy characterized by joint destruction with invasive proliferation of the synovial cells in the articular cartilage (Weyand, 2000 and Hoshi & Hiida, 2001). In RA, lymphocytes undergo very little apoptosis (Matsumoto et al., 1996 and Sugiyama et al., 1996). The induction of apoptosis, among autoimmune lymphocytes by p53 dependent mechanism may be important in RA (Fox, 1998).

Lymphocytes that infiltrate the RA synovium are more resistant to apoptosis than normal lymphocytes due to their upregulation of bcl2 and bclx (Salmon et al., 1997). This upregulation appears to be mediated by cytokines and cell matrix interactions between the lymphocytes and interstitial cells in the synovium (Akbar & Salmon, 1997). Even though p53 is upregulated in the synovial fibroblast lining cells, it was not activated in the infiltrating lymphocytes (Firestein et al., 1996). Therefore, it might help to overcome this resistance to lymphocyte apoptosis by promoting p53 activation and lowering uridine monophosphate (UMP) which leads to cell cycle arrest in lymphocytes (Fox, 1998).

Leflunomide (LFM, HWA486) is an isoxazol derivative with antiphagocytic and novel immunomodulating properties. It has been shown to be very effective in preventing and curing arthritis (Ju et al., 1994),
improvement of patient quality of life (Scott, 1999) and retardation of radiographically assessed disease progression (Smolen et al., 1999).

A number of anti-inflammatory effects exerted by MTX seen to be related to the extracellular adenosine increase and its interaction with specific cell surface receptors, with subsequent inhibition such as IL-8 production by peripheral blood mononuclear cells (PBMC), IL-6 secretion by human monocytes, leucotriene B4 synthesis in neutrophils and decreased synovial collagenase gene expression (Bouma et al., 1994).

Methotrexate (MTX) treatment in RA seems to reduce the production of proinflammatory monocytic/macrophagic cytokines (IL-1, IL-6, TNF-α) to increase at least, gene expression of anti-inflammatory cytokines (IL-4 and IL-10) and to decrease gene expression of pro-inflammatory cytokines (IL-2 and IFN-γ), with resulting anti-inflammatory effects (Cutoto et al., 2001).

**Aim Of The Work:**

To study the effect of leflunomide, methotrexate and their combination therapy on the histopathologic picture and oxidative stress markers in RA model

**MATERIAL AND METHODS**

Forty adult male albino rats weighing 110-140 grams each were obtained from the Faculty of Pharmacy, Cairo University.

The animals were allowed to acclimatize one week before the induction of arthritis (AIA), which was done by subcutaneous injection of complete Freund's adjuvant (CFA).

The animals were stratified into five equal groups:

- **Group-1:** normal rats representing the control group.
- **Group-2:** (AA control): CFA was injected in the right paw. Animals did not receive any treatment and served as adjuvant arthritis control.
- **Group-3:** (AA+leflunomide): CFA was injected in the right paw. Animals received leflunomide in a dose of 20 mg/kg/orally starting from day 8 of the experiment.
- **Group-4:** (AA+methotrexate): CFA was injected in the right paw. Animals received intraperitoneal methotrexate of a dose 0.3 mg/kg twice weekly, starting from day 8 of the experiment.
Group-5: (AA+combination therapy): CFA was injected in the right paw. Animals received leflunomide and methotrexate of the same dose given in groups 3 and 4 starting on day 8 of the experiment.

All animals were sacrificed after 3 weeks and the right knee was dissected and examined with light microscopy.

Method of Histopathological Study:

The knee joints were fixed in formalin and embedded in paraffin blocks. Then 5μ thick sections were cut longitudinally and stained with H&E. Both cartilage and synovium were examined with light microscopy.

The histopathological pictures of all specimens were assessed by a double blind study, where the examiner did not know the mother group of each specimen.

Histological changes of the synovium were graded according to Amano et al. (1994) into five grades from (0-4), while articular cartilage was assessed according to Colombo et al. (1983).

Measurements in Laboratory Studies:

Oxidative stress markers were measured:

- Nitric oxide (NO):

  It was assessed according to Tracy et al. (1995) where the total content of nitrate and nitrite were evaluated after reduction with nitrate reductase. The end product was measured at 540 nm

- Malondialdehyde (MAD):

  It is lipid peroxide product, its level were assessed by thiobarbituric acid test according to Uchiyama and Mihara (1987).

- Blood glutathione (GSH):

  It is measured by the supernatant after protein precipitation which reacts with Ellman's reagent and gives yellow substance measured at 412nm (Beutler et al., 1963).

- Erythrocyte superoxide dismutase (SOD):

  It is measured according to Marklund and Marklund (1974) where the erythrocytes lysed at 4°C for 2 hours and SOD was measured from the supernatant fluid.

- Ceruloplasmin (CP):

  It is measured by acidification of O-dianisidine dihydrochloride, which absorbs at 540nm as described by Schosinsky et al. (1974).
RESULTS

I. Histopathological Results:

The normal rat knee synovium in group 1 showed synovial intima formed of 1-3 layers of flat synovial cells. The subintimal stroma was loose with thin walled blood vessels with no inflammatory cellular infiltrate (Fig. 1).

![Image](image1.jpg)

Fig. (1): Normal rat knee synovium showing flat synovial cells (S), loose stroma (T).

The normal rat knee cartilage showed a superficial zone formed of flat chondrocytes arranged parallel to the surface, middle zone formed of homogenous connective tissue matrix and deep zone that consists of chondrocytes existing in relative isolation or in small clusters as lacunae perpendicular to the superficial layer and parallel to each other. There were no ulcers, fibrillation fissures, cysts or chondrocyte disorganization (Fig. 2).

Group 2 (Adjuvant arthritis group):

The synovium of AA rats showed multi-layering of synovial cells more than 3 layers with prominent villous formation (Fig. 3). The stroma ranged from loose to compact with an increase in vascularity and inflammatory infiltration (Fig. 4).
Fig (2): Normal rat knee cartilage showing superficial zone with flat chondrocytes (arrow), middle zone with homogenous matrix (M) and deep zone with chondrocytes arranged perpendicular to superficial layer (C).

Fig (3): Synovium of AA showing prominent villous proliferation (arrow) (x140).
Fig (4): Synovium of AA showing multilayers of synovial lining, loose stroma, increase vascularity (V) and inflammatory infiltrate (I).

Fig (5): Cartilage of AA showing V shaped fissures (arrow) and erosion (E) of the superficial layer of the articular cartilage with disorganization of the chondrocytes (C).
The articular cartilage showed mild loss of the superficial layer and ulceration, fibrillation, fragmentation, few fissures and cysts with minimal disorganization of chondrocytes, without chondrocyte loss or subchondral bone exposure (Fig. 5).

In comparison of pathological score of synovium of group 1 and group 2 there was a highly significant difference as regards epithelial cells, villous formation, blood vessels and total synovial score, there was a significant difference between both groups as regards inflammatory cells, while there was no significant difference of stroma between both groups (Table 1).

The cartilage showed a highly significant difference in loss of superficial layer and ulceration or erosion and there was no significant difference as regards the rest of pathological scoring of cartilage as shown in table 1.

Table (1): Comparison between group 1 and group 2 as regard both synovium and cartilage changes:

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
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<th>p</th>
<th>Sig.</th>
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<tbody>
<tr>
<td>I. Synovial changes:</td>
<td></td>
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</tr>
<tr>
<td>- Epithelial cells</td>
<td>0.31±0.49</td>
<td>2.44±0.70</td>
<td>5.4</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Villous formation</td>
<td>0.60±0.50</td>
<td>2.4±0.69</td>
<td>4.3</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Blood vessels</td>
<td>0.46±0.79</td>
<td>2.0±0.87</td>
<td>2.8</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>- Stroma</td>
<td>0.46±1.19</td>
<td>0.75±0.68</td>
<td>0.4</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Inflammatory cells</td>
<td>0.0±0.0</td>
<td>0.72±0.01</td>
<td>0.6</td>
<td>&lt;0.05</td>
<td>S</td>
</tr>
<tr>
<td>- Total score</td>
<td>1.0±1.2</td>
<td>9.44±0.25</td>
<td>12</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>II. Cartilage changes:</td>
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<td></td>
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<tr>
<td>- Loss of superficial layer</td>
<td>0.29±0.5</td>
<td>1.8±0.9</td>
<td>3.6</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Ulceration or erosion</td>
<td>0±0</td>
<td>0.76±0.66</td>
<td>3.1</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>- Fibrillation and fragmentation</td>
<td>0±0</td>
<td>1.29±0.49</td>
<td>5.7</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Fissure V shaped</td>
<td>0±0</td>
<td>0.25±0.7</td>
<td>0.8</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Cysts</td>
<td>0±0</td>
<td>0.20±0.5</td>
<td>1.2</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Osteo or chondrocytes</td>
<td>0±0</td>
<td>0.12±0.33</td>
<td>0.8</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Chondrocyte loss</td>
<td>0±0</td>
<td>0.28±0.74</td>
<td>0.8</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
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</table>

Leflunomide treated adjuvant arthritis group (group 3):

The synovium showed marked improvement of the total score which reflects the sum of all changes in the synovium. There was moderate improvement of epithelial lining where it appeared flat synoviocytes, stroma varied from loose to compact. Decrease in villous proliferation was noted and the inflammatory infiltrate and vascularity were persisted in the sections (Figs. 6, 7).
Fig. (6): Leflunomide treated adjuvant arthritis group showing multilayered synovial membrane with loose stroma and inflammatory infiltrate.

Fig. (7): Leflunomide treated adjuvant arthritis group showing flattening of synoviocytes, decrease in villous formation, compact stroma and persistent
The cartilage of adjuvant arthritis showed significant improvement in the loss of superficial layer with flat chondrocytes arranged parallel to the surface. The other pathological features of cartilage did not show any changes (Fig. 8).

Comparing group 2 (AA) to group 3 (leflunomide treated AA); there was a highly significant improvement in the stroma (which became more compact), in inflammatory infiltration and total score. Significant improvement appeared in the epithelial lining with no significant difference between both groups as regards villous formation and vascularity.

On the other hand, there was only significant improvement in the loss of the superficial layer of hyaline cartilage but still no significant difference between both groups and the rest of pathological changes of articular cartilage (Table 2).

**Methotrexate treated adjuvant arthritis group (group 4):**

The histopathological features of the synovium showed moderate improvement in the form of improvement of total score and decrease in villous formation. There was an increase in vascularity, inflammatory infiltration and mild flattening of epithelial lining. The hyaline cartilage was
mildly improved (Fig. 9).

Table (2): Comparison between group 2 (adjuvant arthritis) and group 3 (leflunomide treated arthritis).

<table>
<thead>
<tr>
<th></th>
<th>Group 2</th>
<th>Group 3</th>
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<th>Sig.</th>
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<tr>
<td>I. Synovial changes:</td>
<td></td>
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</tr>
<tr>
<td>- Epithelial cells</td>
<td>2.44±0.70</td>
<td>1.88±0.23</td>
<td>1.8</td>
<td>&lt;0.05</td>
<td>S</td>
</tr>
<tr>
<td>- Villous formation</td>
<td>2.40±0.69</td>
<td>2.00±0.0</td>
<td>1.2</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Blood vessels</td>
<td>2.00±0.87</td>
<td>2.63±0.23</td>
<td>1.2</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Stroma</td>
<td>0.75±0.68</td>
<td>2.13±0.23</td>
<td>4.2</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Inflammatory cells</td>
<td>0.72±0.01</td>
<td>1.25±0.27</td>
<td>5.3</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Total score</td>
<td>9.44±0.25</td>
<td>1.95±0.16</td>
<td>11</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>II. Cartilage changes:</td>
<td></td>
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<tr>
<td>- Loss of superficial layer</td>
<td>1.80±0.90</td>
<td>1.0±0.76</td>
<td>1.9</td>
<td>&lt;0.05</td>
<td>S</td>
</tr>
<tr>
<td>- Ulceration or erosion</td>
<td>0.76±0.66</td>
<td>1.0±0.76</td>
<td>0.3</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Fibrillation and fibrillation and fragmentation</td>
<td>1.29±0.49</td>
<td>1.25±0.89</td>
<td>0.2</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Fissure V shaped</td>
<td>0.20±0.50</td>
<td>0.25±0.46</td>
<td>0.0</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Cysts</td>
<td>0.12±0.33</td>
<td>0.00±0.00</td>
<td>0.8</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Osteo or chondrocytes</td>
<td>0.28±0.74</td>
<td>0.50±0.53</td>
<td>0.7</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Chondrocyte loss</td>
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</table>
Comparing group 2 (AA group) and group 4 (MTX treated group), there was a significant improvement in group 4 in the form of decrease in number of villous formation and there was a highly significant improvement in stroma (which became more compact), and in total pathological score.

There was no significant difference between both groups as regards epithelial cells (Table 3). There was a highly significant improvement in the superficial layer of cartilage where the loss of superficial layer was decreased. Also, there was a decrease in the number of the ulcers and erosions, but it was statistically insignificant (Table 3) chondrocyte disorganization was noted with highly significant difference from group 2 (p<0.01) (Table 6).

Table (3): Comparison between group 2 and group 4:

<table>
<thead>
<tr>
<th></th>
<th>Group 2</th>
<th>Group 4</th>
<th>t</th>
<th>p</th>
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<tr>
<td>I. Synovial changes:</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>- Epithelial cells</td>
<td>2.44±0.70</td>
<td>2.33±0.34</td>
<td>0.5</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Leflunomide and methotrexate treated group (group 5) showed moderate improvement of synovium in the form of decrease in villous formation, flattening of epithelial cells, compact stroma, decrease in inflammatory infiltrate. As regards cartilage; it showed marked improvement in this group where there was a decrease in the loss of superficial layer, decrease in the number of ulcers, erosions, fragmentation, fissures, with no chondrocyte loss or disorganization (Fig. 10).</td>
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</table>
Fig (10): synovium and cartilage of rat knee joint of group 5 showing flattening of epithelial cells, compact stroma, decrease in inflammatory infiltration, vascularity with improvement of hyaline cartilage.

Table (4): Comparison between group 2 and group 5:

<table>
<thead>
<tr>
<th></th>
<th>Group 2</th>
<th>Group 5</th>
<th>t</th>
<th>p</th>
<th>Sig.</th>
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<tbody>
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<td>I. Synovial changes:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>- Epithelial cells</td>
<td>2.44±0.70</td>
<td>2.42±0.13</td>
<td>0.3</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Villous formation</td>
<td>2.40±0.69</td>
<td>1.33±1.03</td>
<td>1.9</td>
<td>&lt;0.05</td>
<td>S</td>
</tr>
<tr>
<td>- Blood vessels</td>
<td>2.00±0.87</td>
<td>3.50±0.00</td>
<td>3.2</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>- Stroma</td>
<td>0.75±0.68</td>
<td>2.00±0.00</td>
<td>3.9</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Inflammatory cells</td>
<td>0.72±0.01</td>
<td>0.50±0.02</td>
<td>31</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Total score</td>
<td>9.44±0.25</td>
<td>2.09±0.08</td>
<td>77</td>
<td>&lt;0.001</td>
<td>HS</td>
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<tr>
<td>II. Cartilage changes:</td>
<td></td>
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</tr>
<tr>
<td>- Loss of superficial layer</td>
<td>1.80±0.90</td>
<td>0.33±0.52</td>
<td>3.6</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>- Ulceration or erosion</td>
<td>0.76±0.66</td>
<td>0.00±0.00</td>
<td>3.1</td>
<td>&lt;0.01</td>
<td>HS</td>
</tr>
<tr>
<td>- Fibrillation and</td>
<td>1.29±0.49</td>
<td>0.00±0.00</td>
<td>5.7</td>
<td>&lt;0.001</td>
<td>HS</td>
</tr>
<tr>
<td>fragmentation</td>
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<td></td>
</tr>
<tr>
<td>- Fissure V shaped</td>
<td>0.25±0.70</td>
<td>0.00±0.00</td>
<td>0.8</td>
<td>&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Cysts</td>
<td>0.20±0.50</td>
<td>0.00±0.00</td>
<td>1.2</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Osteo or chondrocytes</td>
<td>0.12±0.33</td>
<td>0.00±0.00</td>
<td>0.8</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>- Chondrocyte loss</td>
<td>0.28±0.74</td>
<td>0.00±0.00</td>
<td>0.8</td>
<td>&gt;0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

In comparison with adjuvant arthritis group (2), there was a highly
significant difference between both groups as regards stroma, blood vessels, inflammatory infiltration, total score, loss of superficial layer and ulcers erosions of articular cartilage. There was a significant change as regards villous formation (Table 4).

The leflunomide treated group showed the best improvement of the total synovial score while combination therapy group showed the best improvement of the cartilage (Tables 5 and 6).

II. Laboratory results:

Oxidant and antioxidants results:

1. Serum nitric oxide NO (μM): There was no significant difference between all groups where normal group (mean 41.82±4.09), adjuvant arthritis group (42.01±2.49), leflunomide treated group (43.3±2.8), methotrexate treated group (44.5±2.46) and leflunomide and methotrexate treated group was (41.3±1.7) (Fig. 11).

2. Malondialdehyde (lipid peroxide): There was significant increase in its value in adjuvant arthritis (3.18±0.62) in comparison to normal (2.05±0.42).

There was no significant difference between normal and other groups (leflunomide treated group (3.05±0.633), MTX treated group (2.16±0.192) and combination therapy group (2.9±1.17).
There was significant decrease in MDA level in MTX treated group (2.16±0.192) and adjuvant arthritis group (3.18±0.62) (fig. 12).

Fig. (12): Lipid peroxide MAD (malondialdehyde) in (nmol/ml) in all groups* = significant difference from normal control at p<0.05, @ = significant difference from control arthritis at p<0.05.

3. Erythrocytic SOD (μ/mL): There was a significant increase of SOD in adjuvant arthritis (266.07±23.82), leflunomide treated group (194.39±12.6) and MTX treated group (194.66±15.76) with normal (147.20±30.01).

There was a significant decrease of SOD in leflunomide treated group, MTX treated group, combination therapy treated group (188.22±29.0) in comparison to adjuvant arthritis (Fig. 13).
Fig (13): Erythrocyte superoxide dismutase in (U/ml) in all groups. *= significant difference from normal control at p<0.05, @ = significant from control arthritis at p<0.05.

4. Blood glutathione (μMol/mL): There was a significant increase of blood glutathione level in adjuvant arthritis (1.40±0.18), leflunomide treated group (1.58±0.125) and MTX treated group (1.21±0.26) in comparison to normals (0.90±0.087). There was a significant increase of blood glutathione in leflunomide and MTX treated groups (1.23±0.1) in comparison to the leflunomide treated group (Fig. 14).

Fig (14): Blood glutathione in (μmol/ml) in all groups. *= Significant difference from normal group at p<0.05. b= Significant difference from leflunomide group at p<0.05.

5. Serum ceruloplasmin (μ/mL): There was a significant increase in its level in the adjuvant arthritis group (0.203±0.06) and combination therapy group (0.229±0.065) in comparison to normal group (0.128±0.022). There was a significant decrease in MTX treated group (0.114±0.024) in comparison to adjuvant arthritis group. Also, there was a significant
increase in its value in combination therapy group in comparison to MTX treated group (Fig. 15). There was no significant change of leflunomide treated group (0.196±0.058) with any of the other groups.

Fig (15): Serum ceruloplasmin (U/ml) in all groups. a=Significant difference from MTX treated group at p<0.05. * = Significant difference from normal control at P<0.05. @ = Significant difference from control arthritis at p<0.05.
DISCUSSION

The RA joint is replete with cytokines and growth factors that exert a synergistic mitogenic effect on synovial tissue fibroblasts to exhibit deviated gene expression of proto-oncogenes such as c-Myc, c-Ras and c-Jun and apoptosis inhibitors such as BCL-2. At the same time, RA synovial tissue fibroblasts contain mutations in tumor suppressor or genes such as P53. The altered rates of proliferation and apoptosis of RA synovial cells result in hyperplasia of synovial tissue and in concert with the chronic inflammatory environment ultimately lead to destruction of the RA joint (Michael & Alisa, 2000).

In this study, leflunomide, methotrexate and their combination showed variable degrees of improvement in both synovial and cartilaginous changes of adjuvant arthritis.

Leflunomide improved significantly the total score of the synovium. The stroma became more compact and villous proliferation was decreased, but it didn't reach statistical significance. There was an improvement in epithelial lining, and a decrease in the loss of superficial layer of cartilage however, ulceration and erosion persisted. These findings were in agreement with Ju and his colleagues (1994) who reported that leflunomide and its metabolite A771726 elicited an inhibitory effect on cytokines (IL-1β, IL-6, TNF-α and GM-CSF) induced DNA synthesis of synovial cells. This inhibition of cytokines induced proliferation of synovial cells by leflunomide, might partially explain its anti-rheumatic activity.

Fox et al. (1999) explained that the effects of leflunomide are through inhibiting the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which plays a key role in de-novo synthesis of pyrimidine ribonucleotide uridine monophosphate (ruMP). Cells such as activated T lymphocytes that predominantly synthesize pyrimidine via the de-novo pathway appear to be especially sensitive to the effects of leflunomide (Somlen & Emery, 2000).

In vitro experiments on the cellular level revealed that leflunomide inhibits the proliferation of activated T cells that are important in inflammation and degradation of synovial tissues (Hughes & Moreland, 2001) and prevents the expansion of activated and autoimmune lymphocytes by interfering with the cell cycle progression due to inadequate production of ruMP and by utilizing mechanisms involving P53 (Fox et al., 1999).

Our findings were in agreement with Reece et al. (2002) who found that the improvement of synovial inflammation in active rheumatoid arthritis as measured by dynamic gadolinium-enhanced magnetic resonance

95
imaging- was significantly better with leflunomide than with methotrexate over 4 months of therapy. Similarly; Smolen et al. (1999) reported that leflunomide retards radiographically assessed disease progression.

The administration of leflunomide for the treatment of RA was statistically superior to placebo and equivalent to MTX treatment. It improved signs and symptoms of active RA, delayed disease progression as demonstrated by X-ray films and improved the function and health related quality of life (Vibeke Strand et al., 1999).

This study showed that MTX improved the synovial pathology in the form of a decrease in villous proliferation, the stroma became compact and improved total score but to a degree less than leflunomide. It improved the cartilage pathology in the form of a decrease in the loss of the superficial layer of cartilage, improved the fibrillation and fragmentation to a degree better than leflunomide. These findings were in accordance to those reported by Viewiowsk & Graczyk (2000) who found that MTX holds up proliferation of monocytes, macrophages and synoviocytes. Moreover, MTX decreases the synthesis of leukotriene in the neutrophils, decreases the level of neutral proteases, holds up cellular immunity and has an anti-proliferative influence on endothelial cells. Its anti-inflammatory effects are achieved by reduction of expression of endothelial cells adhesive proteins and synthesis of chemotactic factors, stopping migration of leucocytes to tissues.

In this study there was a chondrocyte disorganization noted with MTX treatment. There were no recorded data for this finding but it could be an adverse effect of MTX on chondrocytes in the animal model.

Combination therapy caused a significant improvement in villous proliferation, compact stroma, total synovial score, and loss of the superficial layer of the cartilage, ulceration, erosion, fibrillation and fragmentation. These findings were similar to those reported by Kraan et al. (2000) who demonstrated that both compounds showed similar effects on synovial tissue. They added that patients treated with both leflunomide and methotrexate exhibited a decreased metalloproteinase-1/tissue inhibitor metalloproteinase-1 ratio in synovial tissue. This interferes with the mechanism involved in joint inflammation and destruction of joint integrity.

This study revealed that nitric oxide level was high in adjuvant arthritis as compared to normals. However, the difference was not statistically significant. This contradicts Darlington & Stone (2001) who found that the generation of reactive oxygen species (free radicals) is an important factor in the development and maintenance of RA in humans and
animal models. The over-production of NO was also noted by Wanchu et al. (1999) in several inflammatory disorders as in RA and SLE.

Methotrexate did not change the NO level in adjuvant arthritis and this was in agreement with DeGendt et al. (1998). On the other hand, Robbins et al. (1998) reported that MTX caused a dose and time dependent inhibition of nitrite and nitrate, which is an index of NO production.

Our finding contradicted that of Hayem et al. (2000) who found that MTX in a therapeutic concentration in vitro inhibits the production of NO by un-stimulated chondrocytes. The inhibition of NO production after administration of low dose of MTX (450 μg once a week for 4 weeks) in Balb/c mice was explained by significant inhibition of systemic release of NO synthase 2 (NOS2) which is responsible for increased expression of NO and this also found by Omata et al. (1997); Durez et al. (1998) and Perkins et al. (1998).

As regards the effect of leflunomide treatment on NO level, it did not change NO level. This can be explained as dose dependent. This finding agreed with Hamilton et al. (1999) who found that leflunomide and or A771726 (active metabolite of leflunomide) inhibit the induction of Cox-2 or inducible nitric oxide synthase (INOS) protein only at much higher drug levels. This finding contradicts with Jankovic et al. (2000) who found that an inhibitory effect of A771726 on both NO production and INSO mRNA expression was observed in IFN-γ, lipopolysaccharide (Lps) activated and rat primary fibroblasts.

Trajkovic (2001) also reported that leflunomide can directly modulate cytokine and/or Lps induced NO production in various cell types in vitro, probably by interfering with iNOS gene transcription or catalytic activity of iNOS enzyme.

In our study, malondialdehyde (MAD) was increased in adjuvant arthritis. This was in agreement with Hashem et al. (1991) but contradicts with Simonini et al. (2001) who found that MAD was not increased significantly in juvenile chronic arthritis patients’ sera. They considered that MAD is not a major marker of oxidative stress in JCA. Again in our study, MTX decreased MAD level and this was in accordance with Helmy et al. (2001).

Reduction of MAD levels after leflunomide treatment was reported and explained by suppression of TNF-induced reactive oxygen intermediate generation and lipid peroxidation (Manna et al., 2001). This contradicts to our finding where no significant changes of MAD with leflunomide. This
can be explained by the fact that our study was conducted in vivo whilst their study was done in vitro.

Antioxidants are potent scavengers of free radicals and serve as inhibitors of neoplastic process. A large number of synthetic and natural antioxidants have been demonstrated to induce beneficial effects on human health and disease prevention (Bagachi et al., 2000).

Results of our study revealed a decrease in GSH level after MTX administration. However, it did not reach a statistical value. This finding was in agreement with Koster et al. (1986) who found no change of GSH with MTX treatment. On the other hand, Rafter (1994) reported a reduction of GSH after MTX administration and explained that by inhibition of over production of leukotriene-B4 by neutrophils from rheumatoid arthritis patients. It is postulated that neutrophil intracellular reduced glutathione is decreased by increased cellular copper, which results in leukotriene-B4 over-production. The percentage increase in activity of glutathione peroxidase GPX was highest in patients taking the antioxidant combination and least in those taking standard treatment including MTX (Helmy et al., 2001). The beneficial effects of carotene and selenium, which is a component of the antioxidant enzyme glutathione peroxidase, was noted by Darlington & Stone in 2001 in amelioration of rheumatoid arthritis and related disorders.

In our study, ceruloplasmin was decreased by MTX. Rafter (1994) postulated that neutrophil intracellular reduced glutathione is decreased by increased cellular copper, which results in leukotriene-B4 over-production. In arthritic patients, there is an inappropriate amount of a copper donor form of ceruloplasmin that contains a reduced copper that was formed during ceruloplasmin oxidation of plasma cysteine, oxidation of increased amounts of plasma homocysteine, present during methotrexate administration. This restores ceruloplasmin's redox state leading to decreased copper transport into cells.

Erythrocyte superoxide dismutase and ceruloplasmin levels were found to be higher in adjuvant arthritis more than in controls. This finding is in agreements with Cimen et al. (2000). It contradicts with Disilvestro et al. (1992) who found that ceruloplasmin activity to protein ratio and erythrocyte superoxide dismutase values were significantly lower in rheumatoid arthritis patients than normal controls.

**Conclusion**

All the treatment modalities showed variable degrees of improvement of the histopathological picture and oxidative stress markers. The leflunomide treated group showed the best improvement of synovial
pathology, while the combined therapy group showed the best improvement of cartilage pathology. The oxidative stress was best controlled by MTX where in MTX treated group; MTX was able to decrease MAD (a lipid peroxide marker) induced by oxidative stress as it controlled the oxidative stress to a degree sufficient to decrease the protective antioxidant SOD & CP. Further study is needed to evaluate the duration and dose effect of each drug on synovium, cartilage and oxidative stress markers.

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تأثر العلاج بالميثوتريكسات واللوفولوناميد على مفصل الركبة في مرض التهاب المفاصل المساعد

الرقبة المفصلية

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الفكرة: أثبتت مركبات اللوفولوناميد والميثوتريكسات أنها ذات تأثير في تقليل التهاب المفاصل. وتم مقارنة الفعالية في المفاعلات والمحور من هذه الدراسة فوق تأثير العلاج بالميثوتريكسات واللوفولوناميد، والمركب معاً على الغشاء الزالاني والغضروف في المفاصل المعاصرة كنموذج لمرض الرقبة المفصلية للإنسان.

الطريقة: أجريت هذه الدراسة على أربعين حيواناً من حيوانات التجارب وقد تم تقسيمهم إلى خمس مجموعات هي: المجموعة الضابطة ومجموعة التهاب المفاصل المساعد الذين لم يتلقوا أي علاج، ومجموعة التهاب المفاصل المساعد الذين تلقوا العلاج باللوفولوناميد (20 مجم/كم) عن طريق الفم ومجموعة تلقوا ميثوتريكسات (0.3 مجم/كم) مرتين أسبوعياً عن طريق الحقن في التجوية البريتوني ومجموعة تلقت المركبين معاً بنفس الجرعات، ثم تم قتل الحيوانات بعد 3 أسابيع وتم تدوير الركبة اليمنى لتقصي تحت المجهر العادي وتم قياس الشفافية الحرة (نترنك أكسيد ومالونديلهايد) وأيضاً كأساسات للكشفية الحرة (جلوتاثيون – سوبر أكسيد ديميثيوتير والسريلوبولامين).

النتائج: ولقد أظهرت النتائج عن وجود تحسن بدرجات مقارنة في التمثيل الباثولوجي للغشاء الزالاني والغضروف حيث أن المجموعة التي تلقى اللوفولوناميد أو ضربت أحسن نتائج في التمثيل الباثولوجي للغشاء الزالاني أما المجموعة التي تلقى المركبين معاً أو ضربت أحسن نتائج في التمثيل الباثولوجي للغضروف. يتضح أن النتائج الحرة (نترنك أكسيد) لم يتغير في جميع المجموعات والمالونديلهايد قلت بدرجة ذات دلالة إحصائية من العلاج بالميثوتريكسات مقارنة بالتهاب المفاصل المساعد.

أما بالنسبة للأساسات للكشفية الحرة فوجد أن سوبر أكسيد ديميثيوتير قلت نسبة بدرجة ذات دلالة إحصائية في كل المجموعات التي تلقى العلاج بالمقارنة مع مجموعة التهاب المفاصل المركبين معاً وضربت نسبة بدرجة ذات دلالة إحصائية في المجموعة التي تلقى المركبين معاً بالمقارنة بالميثوتريكسات فقط. أما بالنسبة للسريولوبولامين فقد أوضحت النتائج نفس عن سيدوتوريكسات بالمقارنة بالتهاب المفاصل المساعد الذين لم يتلقوا علاج.

وقد وجد أن العلاج بالميثوتريكسات في المجموعة التي تلقى الميثوتريكسات كانت الأحسن في التحكم في مؤثرات الشفافية الحرة وكاسحات الشفافية الحرة ونحت للأداة لدراسة إضايفة لتقسيم أثار الجرعات والوقت من كل مركب على الغشاء الزالاني والغضروف والشفافية الحرة وكاسحات الشفافية الحرة.

103