

## **Biochemical Studies on Synovial Fluid and Serum from Rheumatoid Arthritis Patients**

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### **ABSTRACT**

The research included biochemical studies on synovial fluid (SF) samples collected from (48) RA patients. Moreover, blood samples from (158) RA patients and (196) healthy as control were collected.

These patients were divided according to disease activity score-28 (DAS-28) into two groups which comprise severe and moderate.

Physical and chemical analysis of SF revealed of the presence of more than 1 ml, yellow green to gray color, cloudy to opaque, low viscosity, with large fat droplets, bloody fluid, low pH degree, low glucose level, high total protein and albumin levels.

The results revealed that all RA patients had a positive rheumatoid factor (RF+) and the concentration of C- reactive protein (CRP) was found to be  $(36.73 \pm 14.397$  and  $54.27 \pm 17.856)$  mg/l in serum and SF respectively. Also, the level of Erythrocyte Sedimentation Rate (ESR) was found to be  $(54.85 \pm 22.903)$  mm/hr.

Additionally, there were low significant levels of hyaluronidase (Hylase) activity in SF when compared with the serum of RA patients. Also, there was a highly significant increase in levels of Hylase, alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) in the serum of RA patients when compared with the serum of healthy control.

Moreover, there were highly significant levels of CRP, ESR, Hylase, ALP and LDH in the serum of RA patients with severe when compared with a moderate. Also there were highly significant levels of CRP, ESR and ALP in the SF of RA patients with severe when compared with a moderate.

**Keywords:** Rheumatoid arthritis, disease activity score-28, hyaluronidase.

(196)

(158)

(48)

- -

(RF+)

100/  $(17.856 \pm 54.27$   $14.397 \pm 36.73)$

(C-RP)

$$. / (22.903 \pm 54.85)$$

28-

## INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease, in which various joints in the body are inflamed, leading to swelling, pain, stiffness, articular destruction and a possible loss of function (Lee *et al.*, 2012).

Despite intensive research over many decades, the exact cause of RA remains unknown, but some agents such as smoking, alcohol intake, vitamin D deficiency, oral contraceptive use, and low socioeconomic status might increase the cause of the disease (Persson, 2012; Toms *et al.*, 2012). The smoking is the dominant environmental risk factor of developing rheumatoid arthritis (Persson, 2012). It is four times more common in smokers than non-smokers (Maxwell *et al.*, 2010; Toms *et al.*, 2012). This may be due to the effect of nicotine, which induces abnormal expression of many enzymes such as collagenase-1 (matrix metalloproteinase-1; MMP-1). This enzyme can contribute to the pathogenesis of RA (Scherer *et al.*, 2010; Hamoudatt and Mustafa, 2013). Also, female gender is a risk factor. Women are three to five times more affected by the disease than men (Pretzel *et al.*, 2009; Hamoudatt *et al.*, 2013).

Synovial fluid (SF) is a biological fluid (dialyzat of plasma), which is in contact with articular cartilage and synovial membrane (Toulouse, 2009). The major difference between synovial fluid and other body fluids derived from plasma is its high content of hyaluronic acid (also called hyaluronan; HA) (Kokebie *et al.*, 2011). Hyaluronidase (EC 3.2.1.35; Hylase) causes a decrease in the viscosity of hyaluronic acid, and therefore increases tissue permeability (Toth *et al.*, 2011). Reduction in HA levels occur in synovial fluid with mild and severe rheumatoid arthritis, osteoarthritis, and other joint diseases as reported by different studies (Altintas *et al.*, 2010; Toth *et al.*, 2011). Also, patients with rheumatoid arthritis show increased lactate dehydrogenase (EC 1.1.1.27; LDH) activity in serum, and synovial fluid of patients with rheumatoid arthritis (Walwadkar *et al.*, 2006).

Rheumatoid arthritis causing bone damage. This process is based on imbalance between bone-resorbing osteoclasts and bone-forming osteoblasts (Finzel *et al.*, 2011). Several studies showed increase alkaline phosphatase (EC 3.1.3.1; ALP) activity in RA patients (Aschenberg *et al.*, 2013).

The aim of this work is to study some physical and biochemical properties of synovial fluid from rheumatoid arthritis patients.

## MATERIALS AND METHODS

### Subjects

#### 1- Patients Group

Samples were collected over a period of twenty two months, (1<sup>st</sup> February-2011 to 1<sup>st</sup> November-2012).

The study included 158 rheumatoid arthritis patients (26 males, 132 females), their ages ranged between (19-83) years, admitted to rheumatology unit at Ibn- Sina Teaching Hospital in Nineveh governorate, and diagnosed by a rheumatologist. These patients met the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) criteria for the diagnosis of RA (Arnett *et al.*, 1988; Aletaha *et al.*, 2010). All patients were diagnosed as having RA for at least one year. Moreover, they were all under treatment with non-steroidal-anti-inflammatory drugs (NSAIDs) and disease modifying anti-rheumatic drugs (DMARDs) with or without combination. The cases were not affected by other diseases.

#### 2- Control Group

Control subjects (196) were randomly selected from volunteers and companions of other patients. Members of this group were with negative history of any major illness including arthritis, arthralgia and other diseases (apparently healthy). Their ages between (17–70) years from both sexes which comprised (65) males and (131) females.

### Samples

#### 1- Synovial Fluid

Synovial fluid from (48) patients (16 males, 32 females) ranging in their ages between (30- 83) years was collected.

The SF (10) ml was collected from knee joint by sterile syringe, then immediately transferred into a clean dry plain tube (Matsumoto *et al.*, 2006). Synovial fluid's appearance, color, volume, pH, viscosity, sugar, total protein and albumin were measured.

The sample was then centrifuged at (3000 xg) for 20 minutes to remove cellular debris (Peak *et al.*, 2005). The supernatant was refrigerated at (-20°C) (Matsumoto *et al.*, 2006).

#### 2- Blood

Venous blood sample (10) ml was drawn from both control and patients groups, then transferred immediately into a clean dry plain tube, incubated for ten minutes in water bath at (37°C), and centrifuged at (3000 xg) for 10 minutes to separate the serum. The separated serum was then kept in deep freeze at (-20°C) (Matsumoto *et al.*, 2006).

For RA patients, (2) ml of blood sample were placed in a tube containing anticoagulant, and gently inverted for several times for determination of Erythrocyte Sedimentation Rate (ESR) (Saadeh, 1998).

#### Estimation of Various Parameters

1- Synovial fluid viscosity was estimated, using sterile syringe with needle 23 Gx1 (Lipowitz, 2012).

2- The pH of SF was measured by a pH meter (Tokushima *et al.*, 1998).

3- Glucose concentration (Glu) was estimated in synovial fluid by enzymatic method using a kit manufactured by Cypress Diagnostics (Burtis and Ashwood, 1999).

4- Hyaluronidase activity (HAase) was estimated in serum and SF using the enzymatic method from sigma protocol with slight modification (Mathews and Dorfman, 1955).

5- Alkaline phosphates activity (ALP) was estimated in serum and SF using a kit manufactured by Bio-Merieux (France) (Kind and King, 1954).

6- Lactate dehydrogenase activity (LDH) was estimated in serum and SF using a kit manufactured by Bio Labo SA (France) (Burtis and Ashwood, 1999).

**7-** Total lipids concentration (T. lipid) was estimated in serum and SF using colorimetric method. The reaction included heating a small amount of serum or SF with concentrated sulfuric acid. The mixture was then treated with phosphor vanillin reagent to give a red to violet colored complex (Charbrol and Chardonnet, 1937).

**8-** Total protein concentration (T.P) was estimated in serum and SF by Biuret method (Burtis and Ashwood, 1999) using a kit manufactured by Bio Labo (Farance).

**9-** Albumin concentration (Alb.) was estimated in serum and SF calorimetrically using a kit manufactured by Bio Labo (France) (Burtis and Ashwood, 1999).

**10-** Rheumatoid factor (RF) was tested in serum by a RF-latex method agglutination test using Rheumatic RF kit from Plasmatic Laboratory products (United Kingdom) (Hansen *et al.*, 1980).

**11-** Erythrocyte Sedimentation Rate was estimated by westergren method (Saadeh, 1998).

**12-** High sensitive C-reactive protein concentration (hs-CRP) was estimated in serum and SF using a kit manufactured by Bio ditch Med Inc/ United Kingdom.

The *i*-CHROMA<sup>TM</sup> hs-CRP test is based on fluorescence immunoassay technology ( Brooks *et al.*, 1999 ).

### **13- Calculation of Disease Activity Score-28 (DAS-28)**

Disease activity score-28 (DAS-28) is based on the calculated number of tender and swollen joints (28-joint count). The 28 joints count includes the shoulders, elbows, wrists, first to fifth metacarpophalangeal (MCP) joints, first to fifth proximal interphalangeal (PIP) joints, and knees on both sides of the body, and erythrocyte sedimentation rate (ESR) or serum C-reactive protein (CRP) level (Fransen *et al.*, 2003; Aletaha *et al.*, 2010).

Disease activity score-28-ESR (DAS28-ESR) was calculated using an online calculator by entering the data of tender joint count, swollen joint count, and ESR for each patient (Fransen *et al.*, 2003).

### **Statistical Analysis**

All values were expressed as mean  $\pm$  SD. The analysis of data was performed using the statistical program (SPSS) (Version 20, 2012) (Kirkwood, 1988).

## **RESULTS AND DISCUSSION**

The results revealed that all RA patients had a positive RF (RF+) and elevated concentration of CRP ( $36.73 \pm 14.397$  and  $54.27 \pm 17.856$ ) mg/l in serum and SF respectively. In addition, the value of ESR was found to be ( $54.85 \pm 22.903$ ) mm/hr.

Normal SF is slightly yellow, clear, viscous in consistency, no clotting, viscid no fat droplets and blood. Also, SF is secreted in small quantities (Echevaria *et al.*, 2004). In contrast, the result in Table (1) showed that the physical properties of SF from RA patients appeared (volume of SF was more than (1) ml), yellow green to gray color, cloudy to opaque, low viscosity, with large fat droplets, and bloody fluid. This result agrees with other investigated finding (Echevaria *et al.*, 2004; Altintas *et al.*, 2010).

The results obtained in Table (1) showed differences in chemical parameters in SF of RA patients compared with published chemical analysis. The degree of pH was ( $6.92 \pm 0.74$ ) lower than normal SF (7.39) (Echevaria *et al.*, 2004; Lipowitz, 2012). Glucose concentration in this study ( $4.8 \pm 2.4$  mmol/l) was decreased in SF of RA patients. Similar findings were reported by other authors (Echevaria *et al.*, 2004; Altintas *et al.*, 2010). This is probably due to increased numbers of leukocytes in SF, and decreased SF glucose owing to the glycolytic activities of white cells (Echevaria *et al.*, 2004; Altintas *et al.*, 2010; Lipowitz, 2012). While the concentrations of total protein and albumin ( $6.4 \pm 1.17$  g/100 ml) and ( $3.69 \pm 1.5$  g/100 ml) respectively were increased in RA patients fluid as compared with the results of (Echevaria *et al.*, 2004), which were (1.72 g/100ml and 1.02 g/100ml) respectively. The

variation of protein concentration in SF reflect the degree of joint inflammation (Frisbie *et al.*, 2008; Altintas *et al.*, 2010).

**Table 1: Physical properties and chemical compositions of synovial fluid obtained from Rheumatoid Arthritis patients compared with normal synovial fluid**

| Physical properties of SF | RA patients (No.=48) | *Normal             |
|---------------------------|----------------------|---------------------|
| Volume                    | >1 m l               | < 1m l              |
| Color                     | Yellow green to gray | Slightly yellow     |
| Appearance                | Cloudy to opaque     | Clear               |
| Viscosity                 | Simi weak            | Viscous consistency |
| Clotting                  | +                    | -                   |
| Large fat droplets        | +                    | -                   |
| Bloody fluid              | +                    | -                   |
| Chemical composition      |                      |                     |
| Parameters                | value                | *value              |
| Mean $\pm$ SD (No.=48)    |                      |                     |
| Degree of pH              | 6.92 $\pm$ 0.74      | 7.39                |
| Protein (g/100m l)        | 6.4 $\pm$ 1.17       | 1.72                |
| Albumin (g/100m l)        | 3.69 $\pm$ 1.53      | 1.02                |
| Glucose (m mol/l)         | 4.8 $\pm$ 2.4        | 5.24                |

\*From (Echevaria *et al.*, 2004).

Moreover, the present study revealed that serum hyaluronidase activity was significantly elevated ( $p < 0.0001$ ) in RA patients ( $18.67 \pm 5.01$  U/L) compared with control ( $8.04 \pm 4.83$  U/L) as shown in Table (2). This observation is consistent with the previous reports on Hylase activity elevation in patients with RA and osteoarthritis (OA) when compared with control (Nagaya *et al.*, 1999). In addition, a significant difference was observed in hyaluronidase activities between serum and SF of RA patients ( $18.67 \pm 5.01$  U/L, vs.  $9.23 \pm 3.78$  U/L,  $P < 0.0001$ ) respectively, as shown in Table (2). This might be due to the loss of HA from SF of RA patents due to the presence of many inflammatory agents such as cytokines which induce degradation of HA in synovial joint and leads to loss of HA from SF and decrease the hyaluronidase activity (Altintas *et al.*, 2010; Tetik *et al.*, 2010).

Additionally, the results in Table (2) showed a significant difference in serum ALP activity between RA patients and control ( $146.79 \pm 40.67$  U/L, vs.  $50.48 \pm 29.51$  U/L,  $P < 0.0001$ ) respectively. A similar finding was reported by other investigators (Caglayan and Aydog, 1997; Finzel *et al.*, 2011; Aschenberg *et al.*, 2013). These data suggested that bone markers might be useful in reflecting bone damage in RA (Garner *et al.*, 2010). The alteration in activity of ALP is caused by enhanced expression of inflammatory cytokines, which foster the differentiation of osteoclasts and hamper formation of osteoblasts (Finzel *et al.*, 2011). On the other hand no significant difference was observed between ALP activity in serum and SF of RA patients ( $146.79 \pm 40.67$  U/L, vs.  $147.53 \pm 48.79$  U/L,  $P = 0.916$ ) respectively, as shown in Table (2).

The results in Table (2) showed a significant increase in the LDH activity in the serum of patients with RA when compared with control ( $326.42 \pm 98.53$  IU/L, vs.  $132.6 \pm 13.64$  IU/L;  $p < 0.01$ ) respectively. These results were found to be compatible with the results obtained by other investigators (Lindy *et al.*, 1971; Walwadkar, 2006). Enzyme activity in synovium was increased about two fold in RA patients when compared to control. This may be due to the increased anaerobic metabolism in the synovial tissue in RA patients (Lindy *et al.*, 1971). No significant difference was observed in LDH activity between the serum and SF of RA patients ( $326.42 \pm 98.53$  IU/L, vs.  $355.75 \pm 128.35$  IU/L) respectively.

Also, the results in Table (2) showed that in the total lipids level, no significant difference was found between neither synovial fluid and serum of RA patients ( $320.82 \pm 78$ . mg/100ml and  $308.8 \pm 78.8$  mg/100ml;  $p = 0.337$ ) respectively.

Moreover, the results showed no significant difference between the serum of RA patients and control ( $308.8 \pm 78.8$  mg/100ml and  $303.21 \pm 90.2$  mg/100ml;  $p = 0.935$ ) respectively. These results were in agreement with other study by Van Halem *et al.*, (2007). However, they demonstrated that only a very small percentage of the difference in lipid levels between RA patients and control could be explained by changes in CRP (Van Halem *et al.*, 2007). The results of this study might differ from results of the previous study, which demonstrated changes in lipid level in RA patients (Myasoedova *et al.*, 2010).

Additionally, The results in Table (2) showed that in the total protein level no significant difference was found between synovial fluid and serum of RA patients ( $6.5 \pm 0.92$ . g/100 ml and  $6.41 \pm 1.0$  g/100 ml;  $p = 0.373$ ) respectively. These results were in accord with other (Lipowitz, 2012). Also, the results showed no significant difference between the serum of RA patients and control ( $6.41 \pm 1.0$  g/100ml and  $6.4 \pm 1.2$  g/ 100 ml;  $p = 0.171$ ) respectively. These results are in agreement with the results obtained from other investigators (Aulanni'am *et al.*, 2012).

The results in Table (2) showed that the albumin level had no significant difference between synovial fluid and serum of RA patients ( $3.86 \pm 1.1$  g/100ml and  $3.82 \pm 1.5$  g/100 ml;  $p = 0.373$ ) respectively. Also, the results showed no significant difference in albumin level between the serum of RA patients and control ( $3.82 \pm 1.5$  g/100ml and  $3.68 \pm 1.1$  g/100 ml;  $p = 0.171$ ) respectively. These results were different from the results of others who found higher albumin level in RA patients (Tetik *et al.*, 2010). This might be due to the type of dietary intake.

**Table 2: Comparison between serum of RA patients and control, and between serum and SF of RA patients for all biochemical parameters**

| Parameters               | Serum/control<br>(No.=196)<br>(A) | Serum/RA<br>(No.=158)<br>(B) | SF/RA<br>(No.=48)<br>(C) | p- value |         |
|--------------------------|-----------------------------------|------------------------------|--------------------------|----------|---------|
|                          |                                   |                              |                          | A vs. B  | B vs. C |
|                          | Mean ± SD                         |                              |                          |          |         |
| <sup>#</sup> HAase (U/L) | 8.04± 4.8                         | ***18.66± 5.0                | °°°9.23± 3.8             | 0.0001   | 0.0001  |
| <sup>##</sup> ALP (U/L)  | 50.48 ± 29.5                      | ***146.79± 40.7              | 147.53± 48.8             | 0.0001   | 0.916   |
| <sup>###</sup> LDH (U/L) | 132.6± 13.64                      | ***326.42± 98.53             | 355.75± 128.3            | 0.0001   | 0.095   |
| Total lipid (mg/100ml)   | 303.2± 90.2                       | 308.8± 78.8                  | 320.82± 78.5             | 0.596    | 0.337   |
| Total protein (g/100 ml) | 6.4± 1.2                          | 6.41± 1.0                    | 6.5± 0.92                | 0.171    | 0.373   |
| Albumin (g/100 ml)       | 3.86± 1.1                         | 3.83± 1.2                    | 3.68± 1.5                | 0.808    | 0.223   |

\*\*\* Significant difference with serum of RA patients at ( $p \leq 0.0001$ ), <sup>o</sup>Significant difference with serum of control at ( $p \leq 0.05$ ), <sup>ooo</sup> Significant difference with serum of control at ( $p \leq 0.0001$ ).

<sup>#</sup>One unit will cause a change in absorbance of (0.33) per minute at pH ( 5.7) at (37)°C for (45) minute assay.

<sup>##</sup>One kind and king unit is the amount of enzyme which, in given conditions, liberates 1 mg of phenol in 15 minutes at 37°C.

<sup>###</sup>One IU/L will cause a change in absorbance per minute at (37)°C at (340) nm.

### Disease Activity Score-28 (DAS-28)

The results in Table (3) showed the analyzed parameters when RA patients were divided according to DAS-28 activity into two groups, which comprise severe and moderate. The number of RA patients which have severe (high disease activity; DAS28 > 5.1) was 114 (72.2%) patients, and the number of RA patients which have moderate (DAS28 (3.2–5.1)) was 44 (27.8 %) patients.

The results in Table (3) demonstrated that there was a significant increase in the serum and SF of severe compared with the serum and SF of moderate patients of ALP activity, CRP and ESR. These results were in agreement with (Caglayan and Aydog, 1997; Persson, 2012; Zamani *et al.*, 2012). This might reflect the increase of bone metabolism in these patients and the degree of inflammation (Garner *et al.*, 2010; Syversen *et al.*, 2010).

Also, the study found that serum hyaluronidase activity was significantly elevated ( $p < 0.0001$ ) in patients with severe compared with patients who have moderate as shown in Table (3). This observation is consistent with the previous studies on hyaluronidase elevation in patients with severe (Nagaya *et al.*, 1999; Altintas *et al.*, 2010; Tetik *et al.*, 2010). This might be due to the fact that patients with severe are more exposed to reactive oxygen species (ROS) and reactive nitrogen species (RNS), which play major roles in inducing hyaluronidase activity, than in patients with moderate (Aryaein *et al.*, 2011). While no significant difference in hyaluronidase SF was observed between severe and moderate as shown in Table (3).

**Table 3: Analyzed parameters of RA patients classified according DA-S28 (moderate (3.2-5-1) and severe (> 5.1))**

| Parameter                | Serum                                   |                                  | Synovial Fluid                         |                                  |
|--------------------------|-----------------------------------------|----------------------------------|----------------------------------------|----------------------------------|
|                          | Moderate<br>DAS28 (3.2-5.1)<br>No. = 44 | Severe<br>DAS28 >5.1<br>No. =114 | Moderate<br>DAS28 (3.2-5.1)<br>No. = 6 | Severe<br>DAS28 >5.1<br>No. = 42 |
|                          | Mean $\pm$ SD                           |                                  | Mean $\pm$ SD                          |                                  |
| CRP (mg/l)               | 33.07 $\pm$ 14.9                        | *38.1 $\pm$ 14.0                 | 30.33 $\pm$ 6                          | ***57.69 $\pm$ 16.3              |
| ESR (mm/ h)              | 53.21 $\pm$ 16.01                       | *58.32 $\pm$ 20.34               | 52.23 $\pm$ 30                         | **59.04 $\pm$ 31.09              |
| Hylase (U/L)             | 17.05 $\pm$ 4.7                         | *19.28 $\pm$ 5.0                 | 6.9 $\pm$ 1.1                          | 9.56 $\pm$ 3.91                  |
| ALP(U/L)                 | 120.7 $\pm$ 33.8                        | ***156.86 $\pm$ 38.7             | 102.0 $\pm$ 54.4                       | *154.03 $\pm$ 45.0               |
| LDH(U/L)                 | 311.75 $\pm$ 95.7                       | 332.1 $\pm$ 99.4                 | 323.5 $\pm$ 146.2                      | 360.4 $\pm$ 126.9                |
| Total lipid (mg/100ml)   | 306.21 $\pm$ 86.9                       | 308.82 $\pm$ 78.8                | 281.03 $\pm$ 52.5                      | 307.51 $\pm$ 80.34               |
| Total protein (g/100 ml) | 6.50 $\pm$ 1.05                         | 6.56 $\pm$ 0.87                  | 5.98 $\pm$ 1.66                        | 6.46 $\pm$ 1.1                   |
| Albumin (g/100m)         | 3.78 $\pm$ 0.78                         | 3.79 $\pm$ 1.2                   | 3.4 $\pm$ 1.03                         | 3.73 $\pm$ 1.6                   |

Where: \* Significant difference with DAS (3.2-5.1) at ( $p \leq 0.05$ ), \*\* Significant difference with DAS (3.2-5.1) at ( $p \leq 0.01$ ) and \*\*\* Significant difference with DAS (3.2-5.1) at ( $p \leq 0.0001$ ).

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