The mechanism of leptin and IGF-1 in the diabetic rheumatoid arthritis Iraqi patients

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Abstract. We aimed here to study the impact of leptin on insulin like growth factor-1 (IGF-1) level to imply its antidiabetic effect on Iraqi rheumatoid arthritis’s, who was with and without diabetes mellitus. Rheumatoid arthritis is a chronic inflammatory disease, primarily targets the synovium and articular cartilage, which causes joint damage. Although the role of adipocytokines in mediating damages of joint has recently suggested, it is still a matter of considerable debate.

METHODS: Patients diagnosed as diabetic rheumatoid arthritis aged from 33-60 years and others diagnosed as non-diabetic rheumatoid arthritis were compared with healthy control (aged 33-46 years). Some biochemical parameters have determined, such as fasting serum glucose, glycated hemoglobin, lipid profile, serum fasting insulin, IGF-1, and leptin, using ELISA and immune radiometric assay. Results indicated an elevation in some biochemical parameters in diabetic rheumatoid arthritis’s when compared with patients without diabetes. There was a significant increase in fasting serum glucose and some of the lipid components in diabetic rheumatoid arthritis patients compared to that non-diabetic. A significant elevation of leptin in diabetic rheumatoid arthritis patients compared to control (p<0.003). However, a non-significant difference of leptin was detected between diabetic rheumatoid arthritis and non-diabetic patients. In addition, some other parameters were significantly reduced in diabetic rheumatoid arthritis patients when compared to non-diabetic patients, such as high-density lipoprotein and IGF-1. This study highlights that leptin could act as pro-inflammatory mediator in rheumatoid arthritis. The negative correlation between leptin level and IGF-1 plays an important role in understanding the metabolic pathogenesis of rheumatoid arthritis.

Keywords: Rheumatoid arthritis, diabetes mellitus, insulin like growth factor-1, Leptin, lipid profile, insulin.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic systemic autoimmune inflammatory disease affects mainly the peripheral joints, frequently foremost to damage of these joints [1]. RA has many symptoms such as symmetrical synovitis, advanced joint destruction, pain, fatigue, and walk disability. The occurrence of RA ranges from gentle remitting appearances to rapidly advanced forms with increased mortality [2]. Chronic inflammation in RA activates some disorders, such as fat mobilization, enhanced gluconeogenesis, alter protein catabolism, and negative nitrogen balance [3]. RA causes a damage of cartilage and bone, as well as, a damage of cardiovascular, pulmonary and endocrine system [4].

The insulin like growth factor-1 (IGF-1) is a protein of 70mer stimulated by the liver as a response to the stimulus of growth hormone. Therefore, IGF-1 is frequently used to measure the body’s natural biochemical foundation for proper bone, muscle and tissue [5]. It has been reported that insulin resistance (IR) is associated with some metabolic disorders, such as diabetes mellitus-type 2 (DM-T2), obesity and hypertension [6]. IR usually refers to the disability of insulin to adequately control the metabolism of glucose in the peripheral tissues. IR has been associated with 1.7-fold increase in the risk of cardiovascular diseases (CVD) and contributed to the progress of
DM-T2. On the other hand, obesity found to have an effect on the IR of RA patients [7]. Leptin is the major recognized adipokytokine. It composed of 167mer, and it is mainly expressed in adipose tissue. Leptin controls energy homeostasis and restricts by numerous neuroendocrine and immune functions. Leptin and its receptors (Ob-R) segment acts as structural and functional resemblances with cytokines family and their receptors [8]. Although arthritis does not lead to DM, diabetes does sometimes correlate with joint symptoms, where lifestyle and obesity contribute to or deteriorate both. As well as, corticosteroids have shown to restrict with glucose metabolism [9]. The pro inflammatory influences, DM and hypertension have been hypothesized as mean causes of arthritis [10]. Arthritis was previously described as a deteriorating disease, but recently is considered as a metabolically dynamic process amenable to treatment [11]. This study designed to deeply investigate the contribution of leptin hormone on the pathogenesis and mechanism of RA patients suffered DM. The interference of leptin with some biochemical parameters was studied using ELISA and immune radiometric assay.

2. Materials and methods

2.1. Patients

This study included RA patients suffered DM-type2 (diabetic RA), who were diagnosed by specialist physicians and weren’t under any treatment. In order to understand the role of leptin as antidiabetic, other RA patients without DM were included in the study (non-diabetic RA). Fifty RA patients with and without DM (18 males and 32 females) aged between (33- 60) and 40 healthy volunteers (15 males and 25 females) aged between 33- 46 were involved. All participates (ninety) were attended at the National Diabetic Center (NDC) of Mustansiriya University and Al-Yarmok Teaching Hospital in Baghdad. An informed consent was obtained from all patients and volunteers participants. The board of the National center for Diabetic and treatment research of Mustansiriyah University has approved the project.

2.2. Blood Collection

Ten mL blood of fasting patients and volunteers were collected from the median cubital. The blood was allowed to stand for 30 min for clotting. Serum samples were collected after centrifuge for 5 min at 10000 rpm and stored at ultra-freezer at -40°C, for further analysis.

2.3. Biochemical Parameters

Fasting serum glucose (FSG), glycated hemoglobin (HbA1c), lipids profile (total cholesterol TCh, triglyceride TrG, high density lipoprotein-cholesterol HDL-c, low density lipoprotein-cholesterol LDL-c, and very low-density lipoprotein VLDL), fasting insulin and leptin levels were all determined using ELISA (DRG kit). IGF-1 level was determined using immune radiometric assay (IRMA-Immune tech). Furthermore, IR (insulin resistance) parameters were calculated from homeostasis model assessment-2 insulin resistance (HOMA2-IR).

2.4. Methods

Determination of FBG. Serum TC, serum TAG and serum HDL-C: Each biochemical parameter was determined by its kit method using automated analyzer (BIOLABO Kenza 240TX).

Determination of serum LDL-C: LDL was calculated indirectly using the Friedewald’s equation [17].

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LDL-C = TC - [HDL-C + TAG/5].
\]

This equation is only accurate when: TG levels are below 400 mg/dl.

The Bio-Rad variant hemoglobin A1c (HbA1c): The ion-exchange high-performance liquid chromatography was used for an automatic and accurate separation of glycated hemoglobin (HbA1c) using Vearent kit.

Determination of serum Insulin and leptin: They were measured using the DRG insulin ELISA kit.

Determination of serum HOMA2-IR: This was calculated using HOMA2 calculator software.
Determination of serum IGF-1: Serum IGF-1 concentration was measured using the DIASORIN kit.

2.5. Statistical analysis
Microsoft office excel 2010 work sheet was assisted for the statistical analyses. Data were expressed as means ± standard deviation SD and differences between variables considered of statistical significance in the t-test at P level <0.05.

3. RESULTS
The diabetic and non-diabetic RA patients were introduced to some biochemical tests to investigate the chemical interference of leptin level as antidiabetic hormone with some biochemical parameters of arthritis’s.

Results in figure 1 showed a significant increase in FSG, TC, TAG, LDL-C, VLDL, and leptin in RA patients when compared with controls, (p<0.03). A significant decrease in fasting insulin level, HDL-C, and IGF-1 in RA patients were detected when compared with controls, (p<0.03).

Results in figure 2 showed the characteristics of diabetic and non-diabetic RA patients, where a significant increase in FSG, TC, TAG, and LDL-C was indicated in diabetic RA patients rather than those without DM. However, a non-insignificant elevation in HbA1C, HOMA2-IR, VLDL, and leptin in diabetic RA patients when compared with those without DM. In addition, HDL-C and IGF-1 have significantly decreased in diabetic RA patients when compared with non-diabetic RA patients, (p<0.05).

see also Table 1.

Figure 1. A descriptive analysis of the biochemical parameters in RA patients and controls. The black bars refer to the standard deviation and stars indicates the significance of parameter levels, where, *** p<0.0003, ** p<0.002 and * is for p< 0.01.
Figure 2. A comparison between levels of Biochemical parameters and gender distribution in diabetic and non-diabetic RA patients. The black bars refer to the standard deviation and stars indicates the significance of parameter levels, where, *** p<0.0003, ** p<0.002 and * is for p< 0.01.

Table 2 showed a correlation between leptin with other biochemical parameters in RA patients with and without DM. Results indicated that there was a significant high positive correlation between leptin level with FSG (p<0.05), TC, TAG, and LDL-C in diabetic RA patients (p<0.03). Whilst, a significant negative correlation was detected between serum leptin with HDL-C and IGF-1 in diabetic and non-diabetic RA.

| Variables    | Diabetic RA  | Non-Diabetic RA | Normal values (control) |
|--------------|--------------|----------------|-------------------------|
| FSG (mg/dl)  | 147.78±20.5  | 120.78±8.5     | 91.8±2.52               |
| HbA1c%       | 8.95±0.41    | 5.63±0.4       | 5.08±0.11               |
| Insulin (µU/ml) | 11.51±4.1   | 14.5±1.8      | 22.22±1.20              |
| HOMA2-IR     | 13.3±1.46    | 9.43±1.6       | 8.39±1.20               |
| TC (mg/dl)   | 249.22±18.9  | 183.33±24.8    | 162.46±6.131            |
| TG (mg/dl)   | 236.28±22.3  | 198.46±29.2    | 93.81±8.85              |
| HDL-C (mg/dl)| 37.49±6.1    | 47.41±4.5      | 65.01±5.20              |
| LDL-C (mg/dl)| 129±11.5     | 113.92±10.6    | 81.09±4.63              |
| VLDL (mg/dl) | 40±9.2       | 36.3±5.3      | 26.51±7.31              |
| IGF-1 (ng/ml)| 122.8±11.2   | 137.4±10.5     | 222.22±15.2             |
| Leptin (ng/ml)| 27.3±3.51  | 22.7±2.8      | 14.68±1.32              |
Table 2. A Correlation coefficient (r) between leptin and biochemical parameters on RA patients with and without DM.

| Biochemical Tests                          | Leptin (ng/ml) Diabetic RA | Leptin (ng/ml) Non-diabetic RA |
|-------------------------------------------|----------------------------|-------------------------------|
| Fasting blood sugar (mg/dL)               | 0.293*                     | 0.286*                        |
| Glycated hemoglobin c%                    | 0.236                      | 0.76                          |
| Insulin (µU/mL)                           | 0.244                      | 0.062                         |
| Homeostasis model assessment-2 insulin resistance | 0.136                      | 0.122                         |
| Total cholesterol (mg/dL)                 | 0.564**                    | 0.485**                       |
| triglyceride (mg/dL)                      | 0.623**                    | 0.323**                       |
| High-density lipoprotein -C (mg/dL)       | -0.568**                   | -0.559**                      |
| Low-density lipoprotein -C (mg/dL)        | 0.535**                    | 0.382**                       |
| Very low-density lipoprotein (mg/dL)      | 0.113                      | 0.311                         |
| Insulin like growth factor-1 (ng/mL)      | -0.269*                    | -0.263*                       |
| Leptin (ng/mL)                            |                            |                               |

* = (p<0.05), ** = (p<0.002).

4. DISCUSSION

RA is a prolonged inflammatory disease attacked the articular, as well as, the extra-articular structures. It is usually characterized by synovial hyperplasia, inflammatory cell recruitment and the advanced stage of RA in cartilage and bone destruction. Chronic inflammation in RA patients is associated with metabolic syndrome and atherosclerosis [12]. It also contributes to insulin resistance [13], which leads to some other disorders such as hyperglycemia and dyslipidemia that indirectly associated to atherosclerosis and CVD. Thus, sequenced mechanisms starting from inflammation, IR, or dyslipidemia could increase the burden of CVD risk in RA patients (14).

Herein, we indicated higher levels of FSG, TC, and TAG, but lower HDL-C levels in RA patients when compared to controls. Interestingly, these levels were high in diabetic RA patients more than that of non-diabetic patients. High levels of lipids in RA’s was previously reported [15, 16, 17 and 18]. Studies in the field showed discrepancies in the lipid values, which could be attributed to the difference in the studied population, as well as, the level of the disease activity [19].

Non-diabetic RA patients showed an increased in HOMA-IR index when compared with controls, which was in agreement with others [20]. Results here suggest that the early secretion of insulin could damage the β cell after glucose has stimulated. On the contrary, the HOMA-β levels were higher in RA patients, which could be due to overproduction of insulin and HOMA-β to maintained normal FSG levels. Therefore, the increase in the HOMA-β index is not to improve the function of β-cell, but merely to compensate the decrease of insulin sensitivity [21].

Results in Figure 1 showed non-significant low levels of serum insulin in RA patients, significant low levels of IGF-1 in diabetic RA patients compared with that of control or of non-diabetic RA. The mitogenic effects of insulin regulate the utilization of carbohydrates. IGF-1 as an important growth factor involve in cell growth, differentiation, and survival of cells [22]. Insulin along with IGF-1 contribute to the pathology of some inflammatory diseases including RA [23]. Therefore, the insulin resistance/IGF-1R may play a significant role in the development of the disease. IGF-1 contributes to the synthesis of cartilage and bone extracellular matrix proteins within RA synovium [24, 25]. Previous studies showed that high IGF-1 is related with low-grade inflammation, and that production of IGF-1 is repressed in patients with RA [26, 27].

Serum leptin level of RA patients was significantly more than that of controls (p<0.02), in agreement with others [28]. However, no significant differences between leptin of diabetic RA patients and that of non-diabetic RA patients. Leptin increases the production of the pro-inflammatory cytokines through activation of monocyte/macrophage cells [29]. Accordingly, we were able to suggest a mechanism of
leptin and IGF-1 in diabetic RA based on the statistical results of this study, see figure 3. Diabetic RA patients had a significant high TC, TG and LDL, as well as hyperglycemia. However, insulin and IR were within normal (see figure 1), which could indicate high gluconeogenesis and lipogenesis in the liver and therefore, higher level in the blood and adipose tissue. This will stimulate more secretion of leptin, which will send a positive feedback to the hypothalamus to store the excess of glucose and lipids in the adipose tissue and muscle and leads to obesity RA patients. Diabetic RA patients had a significant low IGF-1 in the serum (figure 2), which may result from unsteady state of the cartilage due to the chronic inflammatory caused by RA and immune dysfunction. Low IGF-1 in the serum was negatively correlated with leptin (Table 2), thus high leptin level in the serum (figure 2). Another study on RA patients revealed a decrease in serum leptin concentration and a shift towards Th2 cytokine production. These alterations observed in RA patients and the experimental models suggest that leptin may play a role in inflammatory mechanisms of arthritis [29, 30].

On the other hand, female appeared to suffer RA more than male of around 3:1 (figure 1b). Genetic factors and hormonal features are probably involved [31], however, sex variance in functional capacity for RA patients has been previously distinguished [32, 33].

![Diagram of leptin and IGF-1 in diabetic RA](image)

**Figure 3.** A suggested mechanism of leptin and IGF-1 in diabetic RA based on the statistical results of this study. Fasting blood sugar (FBS), total cholesterol (TC), triglyceride (TG) and low-density lipoprotein (LDL). Red lines with negative signs refer to negative effect and low level and green lines with positive signs refer to positive effect and high level.

5. CONCLUSION

The present study found that leptin act as pro-inflammatory mediator in RA. Results also showed that females had higher chance to have RA than male. The negative correlation between leptin and IGF-1 level in diabetic and non-diabetic RA patients could play an important role in the metabolic disease or the disease activity of RA.

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**Authors contributions**
Authors have contributed equally in designing the experiments, interpreting the results and preparing of the manuscript.

**Conflict of interest**
The authors declare no conflict of interest, financial or otherwise.

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No animals were used in this research. All human research procedures followed were in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national) authorized by The National Diabetes Center for Treatment and Research. Authors have gain a permission from all patients and volunteers to do clinical research.

**Consent for publication**
Not applicable.

**Availability of data and materials**
Authors can declare that data generated or analyzed during this study are all included in this article.

**Authorship**
All authors have read and approved the manuscript.

**References**

[1] A. Hinojosa-Azaola, J. Alcocer- varela (2014) Art and Rheumatology: The artist and the rheumatologist’s perspective, Rheumatology. 53(10) 1725-1731.

[2] P. Ruscitti, F. Ursini, P. Cipriani, V. Liakouli, F. Carubbi, O. Berardicurti, R. Giacomelli (2017) Poor clinical response in rheumatoid arthritis is the main risk factor for diabetes development in the short-term: A 1-year, single-centre, longitudinal study, PLOS One. 12(7): e0181203.

[3] K. Amer, W. M. Fathy (2012) Serum visfatin is specific significant predictor of rheumatoid arthritis severity: A comparative study versus interleukin-6 and clinical severity scores, life science Journal. 9(1):35-38.

[4] A. A. Wagan, T.E. Mahmud, A. Rasheed, Z. A. Zafar, Rehman, A. Ali (2016) Cardiovascular risk score in rheumatoid arthritis, Pakistan Journal of Medical Sciences. 32(3):534–538.

[5] A. Philippou, M. Maridaki, S. Pneumaticos, M. Koutsilieris (2014) The complexity of the IGF1 gene splicing, post-translational modification and bioactivity, Mol Med Camb Mass. 20(1):202-214.

[6] J. Nicolau, T. Lequerr´e, H. Bacquet, O. Vittecoq (2017) Rheumatoid arthritis, insulin resistance, and diabetes, Joint Bone Spine. 84(4):411–416.

[7] S. Manrique-Arija, I. Ureña, P. Valdivieso (2016) Insulin resistance and levels of adipokines in patients with untreated early rheumatoid arthritis, Clinical Rheumatology. 35(1):43–53.

[8] M. Rosenbaum, L. Leible (2014) 20 years of leptin: role of leptin in energy homeostasis in humans, J Endocrinology. 223(1): T (83-96).
[9] P. Ruscitti, F. Ursini, P. Cipriani, F. Ciccia, V. Liakouli, F. Carubbi, R. Giacomelli (2017) Prevalence of type 2 diabetes and impaired fasting glucose in patients affected by rheumatoid arthritis, Medicine (Baltimore). 96(34):e7896.

[10] K. Powers, D. Day, J. Fantarella (2009) Type II diabetes, osteoarthritis, and inflammatory diet correlate with poor periodontal, Health nature reviews rheumatology. 217(18):1-3.

[11] C.Y. Wen, Y. Chen, H. J (2013) Tang. Bone loss at subchondral plate in knee osteoarthritis patients with hypertension and type 2 diabetes mellitus, Osteoarthritis and Cartilage. 21(11): 1716–1723.

[12] N. Busso, A. So, V. Chobaz-Peclat, C. Morard, E. Martinez-Soria, D. Talabot-Ayer (2002) Leptin signaling deficiency impairs humoral and cellular immune responses and attenuates experimental arthritis, J Immunol. 68:875–882.

[13] M. Cojocaru, I. M. Cojocaru, I. Silosi, C. D. Vrabie (2012) Metabolic syndrome in rheumatoid arthritis, Maedica (Buchar). 7:148–152.

[14] B. S. Kumar, G. Sivaram Naik, D. T. Katyarmal, D. Prabarath Kumar, V. Suresh, P. Srinivasa Rao (2013) Metabolic syndrome. Patients with rheumatoid arthritis: clinical implications, J Clin Sci Res. 2:94–100.

[15] P. H. Dessein, M. Tobias, M. G. Veller (2006) Metabolic syndrome and subclinical atherosclerosis in rheumatoid arthritis, J Rheumatol. 33:2425-32.

[16] A. N. Georgiadis, E. C. Papavasiliou, E. S. Lourida, Y. Alamanos, C. Kostara, A. D. Tselepis (2006) Atherogenic lipid profile is a feature characteristic of patients with early rheumatoid arthritis: effect of early treatment a prospective, controlled study, Arthritis Res Ther. 8(3): R82.

[17] Z. S. Hassen, N. F. Hassine, N. Sakly, M. Jguirim, W. Korbaa, M. Younes (2011) Lipid profile in Tunisian patients with rheumatoid arthritis, Clin Rheumatol. 30:1325-1331.

[18] D. Dursunolu, H. Evrengül, B. Polat, H. Tanriverdi, V. Cobankara, A. Kaftan (2005) Lipoprotein and lipids in patients with rheumatoid arthritis: serum levels and relationship to inflammation, Rheumatol Int. 25:241-245.

[19] C. S. Crowson, E. Myasoedova, J.M. Davis, E. L. Matteson, T. M. Therneau (2011) Increased prevalence of metabolic syndrome associated with rheumatoid arthritis in patients without clinical cardiovascular disease, J Rheumatol. 38(1): 29-35.

[20] J. N. Hoes, M. C. VanDerGoes, D. H. VanRaalte (2011) Glucose tolerance, insulin sensitivity and β-cell function in patients with rheumatoid arthritis treated with or without low-to-medium dose glucocorticoids, Annals of the Rheumatic Diseases, 70(11):1887–1894.

[21] J. Giles, S. Danielides, M. Szklo (2015) Insulin resistance in rheumatoid arthritis: disease-related indicators and associations with the presence and progression of subclinical atherosclerosis, Arthritis and Rheumatology, 67(3):626–636.

[22] P.T. Walsh, L. M. Smith, R. O’Connor (2002) Insulin-like growth factor-1 activates Akt and Jun N-terminal kinases (JNKs) in promoting the survival of T lymphocytes, Immunology. 107(4):461–671.

[23] J. D. Isaacs (2008) Therapeutic T-cell manipulation in rheumatoid arthritis: past, present and future, Rheumatology (Oxford). 47(10):1461–1468.

[24] G. M. Keysze, A. H. Heer, J. Kriegsmann, T. Geiler, C. Keysser. R. E (1995) Gay. Detection of insulin-like growth factor I and II in synovial tissue specimens of patients with rheumatoid arthritis and osteoarthritis by in situ hybridization, J Rheumatol. 22(2):275–281.

[25] C. J. Malemud (1993) The role of growth factors in cartilage metabolism, Rheum Dis Clin North Am, 19(3):569–580.

[26] A. Lemmey, P. Maddison, A. Breslin, P. Cassar, N. Hasso, R. McCann (2001) Association between insulin-like growth factor status and physical activity levels in rheumatoid arthritis, J Rheumatol. 28(1):29–34.

[27] P. V. Kasperkowitz, T. C. Timmer, T. J. Smeets, N. L. Verbeet, P. P. Tak, G. van-L. Baarsen (2005) Fibroblast-like synoviocytes derived from patients with rheumatoid arthritis show the
imprint of synovial tissue heterogeneity: evidence of a link between an increased myofibroblast-like phenotype and high-inflammation synovitis, Arthritis Rheum. 52(2):430–441.

[28] P. Sarraf, R. C. Frederich, E. M. Turner, G. Ma, N. T. Jaskowiak, D. J. Rivet (1997) Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia, J Exp Med. 185(1):171–175.

[29] M. Wislowska, M. Rok, B. Jaszczyk, K. Stdpiej, M. Cicha (2007) Serum leptin in rheumatoid arthritis, Rheumatol Int. 27:947–54.

[30] D. A. Fraser, J. Tho, J. E. Reseland, O. Forre, J. Kjeldsen-Kragh (1991) Decreased CD4+ lymphocyte activation and increased interleukin-4 production in peripheral blood of rheumatoid arthritis patients after acute starvation, Clin Rheumatol. 18(5):394–401.

[31] S. M. O’Brien, P. Fitzgerald, P. Scully, A. Landers, L. V. Scott, T. G. Dinan (2007) Impact of gender and menstrual cycle phase on plasma cytokine concentrations, Neuroimmunomodulation. 14(2):84–90.

[32] P. K. Pandey, A. Swami, T. K. Biswas, R. Thakuria (2017) Prevalence of metabolic syndrome in treatment naive rheumatoid arthritis and correlation with disease parameters, Arch Rheumatol. 32(1):46-52.

[33] Z. Al-Youzbaky, Z. Abdulla (2015) Serum leptin level in rheumatoid arthritis and its relationship with disease activity. Tikrit Journal of Pure Science. 20(2):49-53.