Possible role of leptin, and tumor necrosis factor-alpha in hypoandrogenicity in patients with early rheumatoid arthritis

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Introduction: Hypoandrogenicity is common in men with rheumatoid arthritis who have lower levels of sex hormones such as testosterone and dehydroepiandrosterone sulphate. The fat tissue hormone leptin is stimulated by tumor necrosis factor alpha (TNF-α), and was found to be associated with hypoandrogenicity.

Aim of the work: To study the inter-relation between serum levels of TNF-α, leptin and androgens in early diagnosed RA.

Patients and methods: Serum levels of TNF-α, leptin, testosterone, and (DHEAS) hormones were measured by ELISA and compared in 40 men with early RA and 30 healthy volunteers.

Results: The mean serum leptin and TNF-α were significantly elevated in patients with RA compared to control group, and both of them were positively correlated with the disease activity score (DAS28). Sex hormones (testosterone and DHEAS) were significantly decreased in male patients with RA compared to control group, and they were negatively correlated with serum TNF-α, leptin.

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1. Introduction

Rheumatoid arthritis (RA) is more common in females than males and the sex steroid hormones may in part explain this difference. The female sex predominance in RA may be related to low androgen levels prior to disease onset since adrenal and gonadal androgen deficiency can trigger inflammatory cytokines such as TNF-α and IL-6, that are responsible for the inflammatory response in RA. Alternatively, androgens may influence RA risk indirectly through conversion to estradiol by aromatase or directly by binding to the androgen receptor and affecting cell proliferation. It was hypothesized that low total and free testosterone levels and low DHEAS levels measured before the onset of disease would be associated with an increased risk of RA in women [1].

RA may be accompanied with sexual growth retardation with different degrees and it is also accompanied with an increased secretion of TNF-α and leptin. Tumor necrosis factor alpha (TNF-α) is among the cytokines that play a major role in the inflammatory process of rheumatic diseases. Its inhibition leads to substantial improvement in clinical signs and symptoms in a majority of patients [2]. TNF-α was found to be particularly increased in RA synovitis, and was able to markedly stimulate the aromatase activity in peripheral tissues and, therefore, induce the peripheral metabolism from androgens to estrogens. The effects of TNF blockers (and generally of anticytokine agents) on peripheral sex hormone levels seem to be exerted in a faster way at the level of the RA synovial tissue (before any influence on serum levels) where they seem to block the conversion from androgens (anti-inflammatory) to estrogens (proinflammatory) induced by aromatase [3].

The study of leptin has shown the intricate network that links nutrition, metabolism and immune homeostasis. Leptin is mainly produced by the adipose tissue in proportion to body mass. Although an important role of leptin is to regulate body weight, recent evidence has indicated that leptin is much more than a fat-0-stat sensor, leptin exerts peripheral functions, including regulation of endocrine function, reproduction and immunity. Several studies have shown that circulating levels of leptin increase during infection and inflammation, suggesting that leptin is part of the immune response and host defense mechanisms [4].

This study aimed at investigating serum TNF-α and leptin levels in relation to serum concentrations of androgens in patients with early diagnosed RA.

2. Patients and methods

2.1. Patients

Forty male patients fulfilling American College of Rheumatology (ACR) criteria for a diagnosis of RA [5], and thirty healthy volunteers served as controls were studied. The patients were attending the Rheumatology Department at El-Minia University Hospital in the period of 13 months. The patients had early RA where disease modifying anti rheumatic drugs (DMARDs) and/or glucocorticoids had not been introduced when the examinations were carried out. General and locomotor examination were done to detect articular and extra-articular involvement, swollen and tender joint counts of the RA patients were recorded and the disease activity score (DAS28) [6] were used to assess disease activity. Patients were assessed for symptoms or signs for hypandrogenicity (reduced libido, reduced muscle mass, and strength, depressed mood, decreased energy or vitality, increased fatigue, incomplete sexual development, breast discomfort, gynaecomastia, poor concentration and memory, and menopausal – type hot flushes). Subjects with diabetes mellitus, hypertension, or any other systemic disease and malignancy were excluded from the study.

BMI is calculated as weight in kilograms divided by height in square meters. BMI < 25 kg/m² is considered normal, from 25-30 overweight, from 30 to 40 obese, and > 40 is considered morbid obesity [7,8]. The study was ethically approved by local ethical committee.

2.2. Methods

Radiographic evaluation: Plain X-Ray was done for hands and wrists, and graded by (Larsen scale) [9] the presence of erosions was determined also using Ultrasonography (US).

Laboratory investigations included: Complete blood count, Erythrocyte sedimentation rate, C-reactive protein level, complete urine analysis, liver function tests, renal function tests, and presence of rheumatoid factor were measured by standard techniques.

3. Determination of serum leptin, TNF-α, and Androgens

3.1. Collection and preparation of the samples

Venous blood samples were collected from every subject by sterile venipuncture on the same day of history taking and clinical examination, 2 milliliters of blood was taken for ESR determination. Separated serum was kept frozen at -80 °C till the time of estimation of serum TNF-α, leptin, testosterone and dehydroepiandrosterone sulphate.

3.2. Determination of serum leptin

Kits were used for determination were (Bio Vender – Laboratori medicina, a.s. Czech Republic) .Leptin ELISA Clinical Range, standards, quality controls and samples are incubated in micro plate wells pre-coated with polyclonal anti-human leptin antibody. After 60 minutes incubation and washing, polyclonal anti-human leptin antibody, conjugated with horse-radish peroxidase (HRP) is added to the wells and incubated.
for 60 minutes with captured leptin. Following another washing step, the remaining HRP conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of leptin. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve [10].

3.3. Determination of serum TNF-α

Kits were used for determination were (BioVendor-Laboratorni medicina, a.s. Czech Republic). An anti-human TNF-α coating antibody is adsorbed onto microwells. Human TNF-α present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human TNF-α antibody is added and binds to human TNF-α captured by the first antibody. Following incubation unbound biotin-conjugated anti-human TNF-α antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin conjugated anti-human TNF-α antibody. Following incubation unbound streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of human TNF-α present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human TNF-α standard dilution [11].

3.4. Determination of serum Androgens

Measurements were done according to instructions illustrated in the kits (BioVendor-Laboratorni medicina, a.s. Czech Republic) for DHEAS and total testosterone and (GenWay Biotech, Inc. San Diego, USA) for free testosterone.

Statistical analyses were performed using SPSS version 10.0 (SPSS, Chicago, IL). Continuous data are expressed as mean ± standard deviation (SD). The differences among cases and controls were determined by independent sample t-test for continuous data. The correlation of leptin with TNF-α, and between leptin and TNF-α and serum androgens was calculated using Spearman correlation test. Statistical significance was set at p < 0.05.

4. Results

4.1. Characteristics of the patients with RA and controls

Forty consecutive male patients with early RA were recruited for the study by a rheumatologist from a rheumatology outpatient service, with a mean age of 32.83 ± 7.39 years. The mean duration of disease was 9.28 ± 2.32 months. Rheumatoid factor was positive in 26 patients (65%), DAS 28 was 3.87 ± 1.08. A control group of 30 healthy male subjects with a mean age of 34.17 ± 7.81 years no significant differences between age and BMI between patients and controls, no symptoms or signs of hypoandrogenicity were detected (Table 1).

4.2. Serum concentrations of leptin, TNF-α, testosterone and dehydroepiandrosterone sulphate

The mean serum leptin were significantly elevated in patients with RA compared to control group (32.40 ± 3.92 and 21.52 ± 2.0 ng/ml, respectively). Also the mean serum TNF-α were significantly elevated in patients with RA compared to control group (92.93 ± 20.82 and 14.73 ± 2.09 pg/ml, respectively) (Fig. 1). On the contrary DHEAS were significantly decreased in patients with RA compared to control group 68.23 ± 12.97 and 213.13 ± 62.98 µg/dl, respectively. Regarding the total testosterone it was 1.56 ± 0.50 and 6.89 ± 1.87 ng/ml in RA and control respectively and the free testosterone 8.73 ± 4.12 and 30.97 ± 8.50 pg/ml, respectively (Table 1).

4.3. Correlations between serum leptin, TNF-α with the DAS 28 score

Serum levels of leptin and TNF-α were positively correlated with the disease activity score (r = 0.58, p < 0.001 and r = 0.62, p < 0.001, respectively) (Figs. 2, 3).

### Table 1: Demographic, clinical, and laboratory data in RA patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>RA (n = 40) (mean ± SD)</th>
<th>Control (n = 30) (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>32.83 ± 7.39</td>
<td>34.17 ± 7.81</td>
</tr>
<tr>
<td>Disease Duration (ms.)</td>
<td>9.28 ± 2.32</td>
<td>3.87 ± 1.08</td>
</tr>
<tr>
<td>Rheumatoid nodules No. (%)</td>
<td>4 (10%)</td>
<td>23.30 ± 1.06</td>
</tr>
<tr>
<td>No. of swollen Joints</td>
<td>3.9 ± 2.8</td>
<td>23.40 ± 3.92</td>
</tr>
<tr>
<td>ESR</td>
<td>30.67 ± 14.10</td>
<td>24.19 ± 1.57</td>
</tr>
<tr>
<td>RF + ve No (%)</td>
<td>26 (65%)</td>
<td>32.40 ± 3.92</td>
</tr>
<tr>
<td>DAS28</td>
<td>3.87 ± 1.08</td>
<td>14.73 ± 2.09</td>
</tr>
<tr>
<td>BMI</td>
<td>23.30 ± 1.06</td>
<td>21.52 ± 2.0</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>32.40 ± 3.92</td>
<td>14.73 ± 2.09</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>92.93 ± 20.82</td>
<td>6.89 ± 1.87</td>
</tr>
<tr>
<td>Total testosterone (ng/ml)</td>
<td>1.56 ± 0.50</td>
<td>8.73 ± 4.12</td>
</tr>
<tr>
<td>Free testosterone (pg/ml)</td>
<td>68.23 ± 12.97</td>
<td>30.97 ± 8.50</td>
</tr>
<tr>
<td>DHEAS (µg/dl)</td>
<td>213.13 ± 62.98</td>
<td>68.23 ± 12.97</td>
</tr>
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ESR (erythrocyte sedimentation rate), DAS28 (disease activity score), RF (rheumatoid factor), BMI (body mass index), TNF-α(tumor necrosis factor alpha), DHEAS (dehydroepiandrosterone sulphate).

* Significant difference from Control at p < 0.05.
Figure 1  Box plot showing the comparison of the serum leptin and TNF-α level in both patients and controls. *Significant difference from Control at $p < 0.05$.

Figure 2  Correlation studies between the serum leptin level with (A) DAS28, (B) DHEAS, (C) total and (D) free testosterone in RA patients.
4.4. Correlations between serum leptin, TNF-α, and androgens

There was a negative correlation between serum leptin and serum androgens (DHEAS, total testosterone and free testosterone) \( r = -0.42, p < 0.007; r = -0.48, p = 0.002 \) and \( r = -0.048, p = 0.771 \), respectively (Fig. 2).

There was a negative correlation between serum TNF-α and serum androgens (DHEAS, total testosterone, and free testosterone). \( r = -0.58, p < 0.001; r = -0.54, p < 0.001 \) and \( r = -0.34, p = 0.031 \), respectively (Fig. 3). Correlation results of the studied parameters are shown in Table 2.

5. Discussion

Chronic inflammatory diseases in human such as rheumatoid arthritis are characterized by a dramatic decrease of serum levels of adrenal androgens. Treatment with dehydroepiandrosterone (DHEA) as the starting point of androgen conversion has been proved to be a therapeutic alternative in systemic lupus erythematosus and possibly in patients with chronic inflammatory bowel disease. This indicates that androgens have anti-inflammatory influence, which is switched off during long term systemic inflammatory responses [12].

The fat tissue hormone leptin may be an important link between hyperandrogenicity and obesity. A large number of cell types can produce this hormone (for example white adipose tissue cells, endothelial cells, T-lymphocytes, bone marrow cells, spleen cells, platelets, etc.) [13]. The role of leptin for food intake (inhibitory effect) and metabolic and endocrine functions has been extensively described. However, leptin also regulates immunity, inflammation, haematopoesis and adrenal androgen secretion [14].

Tumor necrosis factor alpha (TNF-α) is one of the most common pro-inflammatory cytokines responsible for various inflammatory disorders. It plays an important role in the...
origin and progression of rheumatoid arthritis and also in other autoimmune disease conditions. TNF-\(\alpha\) is a multifunctional cytokine involved in inflammation, apoptosis, cell survival and immunity acting via two receptors TNF-R55 and TNF-R75 [3,4]. It causes severe damage when produced in excess amount and is also a strong inducer of other pro-inflammatory cytokines such as IL-1\(\beta\), IL-6, and IL-8 [15]. This study aimed at investigating serum TNF-\(\alpha\) and leptin levels in relation to serum concentrations of adrenal androgens in patients with RA.

In the current study concentrations of leptin were significantly higher in patients with RA compared with control subjects and the increase in leptin concentration was positively correlated with the disease activity. Concentrations of leptin did not differ significantly among patients with seropositive and seronegative RA. These results are in consistent with other studies [14,16] and are contradictory with others [17,18]. The reasons for this discrepancy between our study and these studies may be related to the effects of medications on their patients and the higher body mass index in their study patients compared to ours.

This study showed a significant increase in TNF-\(\alpha\) in patients with RA which was positively correlated with the disease activity. Also, there was a positive correlation between levels of leptin and levels of TNF-\(\alpha\) in patient with RA. These results are in consistent with another study [19] that reported that the chronic inflammation of the joints in case of RA leads to diffuse thickening and hyperplasia of the synovium. It is infiltrated with numerous inflammatory cells that produce several proinflammatory cytokines including interleukin (IL)-6 and TNF-\(\alpha\). These proinflammatory cytokines in turn increase circulating leptin concentrations [20]. It has been reported that TNF-\(\alpha\) can act directly on adipocytes to stimulate leptin secretion, it was explained that this hyperleptinemia is likely mediated via transcriptional activation of the leptin gene which leads to subsequent increase in leptin gene expression [20,21].

The results of this study showed that RA patients had a lower androgen level than controls and this result is in agreement with another study [22] that concluded that during a chronic inflammatory process like active RA, levels of both serum testosterone and, in particular, serum DHEAS become lower. Since testosterone and its precursors DHEAS and DHEA have anti-inflammatory properties, the decline in levels of these hormones further supports the pro-inflammatory process. Furthermore, others [23] found that in early RA, current inflammation seemed to affect the hypophyseal gonadal axis (HPG axis), mainly at the gonadal rather than the hypothalamic-pituitary level, and a prospective studies are indicated to determine if low HPG activity may be a cause rather than a consequence of a chronic inflammatory state.

In the current study serum testosterone and DHEAS levels are inversely correlated with RA activity and these results are in agreement with the results of Cutolo [24] who found that serum testosterone levels are inversely correlated with RA activity and dehydroepiandrosterone sulfate (DHEAS) plasma levels are inversely correlated with both disease duration and clinical severity in patients already affected by active RA.

The results of this work showed that there were negative correlation between serum leptin levels and the levels of secreted androgens in the patients of RA. These results are in consistent with other results in bovine adrenocortical cells demonstrated that leptin induced inhibition of 17-\(\alpha\)-hydroxy-lase P450 mRNA expression which is the key enzyme for androgen production [25]. The same study demonstrated that leptin induced inhibition of P450c21 and P450scc mRNA expression. Moreover, it has been reported that a similar inhibitory influence of leptin exists on human adrenocortical cells [26]. The concept that leptin induced inhibition of an important enzyme step of the 17/20-lyase (2\textsuperscript{nd} reaction of the P450c17) in patients with RA has been supported [12].

We found a negative correlation between testosterone, and DHEAS levels and TNF-\(\alpha\) and this result go in hand with the results of Emnestam and coworkers [22] who concluded that in the adrenal and gonadal glands, the loss of DHEA and DHEAS is attributed to a synthetic blockade of the second step of the enzyme P450c17, again induced by inflammatory cytokines such as IL1\(\beta\) and TNF.

From the previous data we found that in patients with RA there were significant increases in serum leptin levels compared to controls which may be related to the increase in the level of TNF-\(\alpha\). Increase in both serum leptin and TNF-\(\alpha\) are associated with a significant decrease in serum androgens. All these data constitute a strong ring which if can be broken at any point may improve the progress of the rheumatic disease.

Furthermore, understanding the complex relation between inflammatory cytokines and hormones may contribute to the development of new therapeutic targets for clinical intervention in RA. Hypoandrogenism is present in male patients with RA and may be relevant in disease pathogenesis. However, whether these hormonal abnormalities are intrinsic to RA or the consequence of any non-specific disorders cannot be distinguished from the current data. Further studies involving a larger number of subjects and inclusion of other disease controls are needed.

References

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