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Comparative Analysis of TNF-alpha, TNF-R1, and TNF-R2 in Patients with Low-impact Fractures Due to Osteoporosis

Análise comparativa de TNF-alfa, TNF-R1 e TNF-R2 em pacientes com fraturas de baixo impacto decorrentes de osteoporose

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Abstract

Objective To analyze the serum levels of TNF-alpha and its TNF-R1 and TNF-R2 receptors in the blood of patients with low-impact fractures due to osteoporosis, comparing between genders and with healthy patients.

Methods The present study was conducted with a blood sample of 62 patients, divided into patients with osteoporosis and healthy patients. The results were obtained using the ELISA method. Cytokine concentrations were determined based on the absorbance values obtained.

Results Serum TNF-alpha levels were undetectable in female patients, while in males they were found only in one patient, with no significant difference. Similar results were found in the analyses of TNF-R1 and TNF-R2 levels, a significant increase in levels of TNF-alpha receptors in the groups of patients with osteoporosis compared with the control group in both sexes. There was no significant difference between the sexes in the dosage of both receptors within the group with osteoporosis. There was also a positive and significant correlation in the levels of TNF-R1 and TNF-R2 only in women.

Conclusion The significant increase in TNF-R1 and TNF-R2 levels in women with osteoporosis suggest that the release and expression of these receptors may be contributing differently to the development of osteoporosis in men and women.

Keywords
- osteoporosis
- tumor necrosis factor-alpha
- receptors, tumor necrosis factor

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Resumo

Objetivo Analisar os níveis séricos de TNF-alfa e de seus receptores TNF-R1 e TNF-R2 no sangue de pacientes com fraturas de baixo impacto, decorrentes de osteoporose, comparando entre os sexos e com pacientes saudáveis.

Métodos O estudo foi realizado com amostra de sangue de 62 pacientes, divididos em pacientes com osteoporose e pacientes saudáveis. Os resultados foram obtidos através do método de ELISA. As concentrações de citocinas foram determinadas com base nos valores de absorbância obtidos.

Resultados Os níveis séricos de TNF-alfa foram indetectáveis nos pacientes do sexo feminino, enquanto no masculino encontrou-se somente em um paciente, não havendo diferença significativa. Encontrou-se resultados semelhantes nas análises dos níveis de TNF-R1 e TNF-R2, aumento significativo nos níveis dos receptores de TNF-alfa nos grupos de pacientes com osteoporose em comparação com o grupo controle, em ambos os sexos. Não houve diferença significativa entre os sexos na dosagem de ambos os receptores dentro do grupo com osteoporose. Houve ainda correlação positiva e significativa nos níveis de TNF-R1 e TNF-R2 apenas nas mulheres.

Conclusão O aumento significativo nos níveis de TNF-R1 e TNF-R2 em mulheres com osteoporose sugerem que a liberação e expressão destes receptores pode estar contribuindo de maneira distinta no desenvolvimento da osteoporose em homens e mulheres.

Palavras-chave

- osteoporose
- fator de necrose tumoral alfa
- receptores do fator de necrose tumoral

Introduction

Osteoporosis is a systemic disease characterized by decreased bone mass with deterioration of the skeletal microarchitecture, leading to bone fragility and increasing the propensity to fractures.[1]

Bone metabolism is active throughout life, and renewal is a constant process in which osteoclast pulls out the mineral component and osteoblast resets it.[2] The normal skeletal maturation process involves the accumulation of bone mass up to about 30 years of age. From then on, there is a physiological loss of about 0.3% each year, initiating the osteopenia process, with decreased bone mineral density, weakening, and increased bone trabeculations. Osteoporosis can affect both men and women, and may occur in any region, with the main bones being the ones in the hip joint, the wrist, the spine, and the ribs. The weakening caused by low bone density is related to most low-impact fractures in the elderly.[3]

Several components of the innate and adaptive immune response have been related to the modulation of osteoclast and osteoblast activity, thus leading to direct changes in the bone matrix.[4] Several signaling pathways were identified as contributors to the interaction between osteoblasts and osteoclasts, including RANK, NF-Kb receptor activator, and its ligand (RANK-L).[5–7]

The connection of RANK-L to its receptor, RANK, provides the signal to conduct the development of osteoclasts from hematopoietic progenitor cells, in addition to activating mature osteoclasts. Osteoprotegerin, also known as an osteoclastogenesis inhibitor, is the TNF-alpha receptor-related protein (alpha tumor necrosis factor) that controls the development and function of osteoclasts. It is responsible for the negative regulation between RANK-L and its receptor, therefore inhibits bone resorption by osteoclasts.[8,9]

TNF-alpha is a potent cytokine, which exerts a variety of biological effects, and can perform a pleiotrophic role in the immune response, inflammation, in addition to controlling cell proliferation, differentiation and apoptosis.[10,11] Tumor necrosis factor alpha acts on osteoclastogenesis through a mechanism involving the activation of NF-kB.[12,13] In addition, TNF-alpha acts by directly stimulating macrophages to differentiate into osteoclasts by an independent mechanism of RANK-L.[14,15]

Currently, it is known that TNF-alpha binds to 2 cell surface receptors, type 1 receptor (TNF-R1), also known as p55, and type 2 receptor (TNF-R2) or p75 sections, each receptor being responsible for mediating distinct intracellular signals.[16]

Both TNF-R1 and TNF-R2 are highly expressed in osteoclast precursors.[17] When activated by TNF-alpha, the type 1 receptor stimulates osteoclastogenesis by activating NF-Kb and inhibits the osteoblasts differentiation.[18,19] On the other hand, type 2 receptor activation showed osteoclastogenesis suppression in *in vitro* experiments.[17]

The present study aims to evaluate the levels of TNF-alpha and its receptors, TNF-R1 and TNF-R2, in elderly patients with low-impact fractures due to osteoporosis, comparing and analyzing the values between men and women and with patients in the control group.

Materials and Methods

All procedures of the present study were approved by the Research Ethics Committee of the University under protocol (CAAE) number: 51827515.4.0000.5145 and opinion number

Table 1 Distribution of study groups by gender

Group	Number	Average age (years old)	Standard error	*p-value*
Control woman	11	40.9	3.1	0.83
Control man	17	42.6	3.2	
Osteoporosis woman	21	80.1	2.1	0.14
Osteoporosis man	13	78.9	2.6	

1,375,317, and all participants signed a Free and Informed Consent Form, after clarification.

Peripheral blood samples were collected from 62 patients, 21 women and 13 men, with low-impact fractures due to osteoporosis, and 11 women and 17 men who presented high-impact trauma fractures, forming patients in the control group (►**Table 1**).

The collection was conducted in the orthopedic center of our institution. Patients with bone diseases, fractures not related to osteoporosis, immunosuppressed patients, patients with malignant neoplasms and those who did not agree to participate in the study were excluded.

ELISA For Measuring Sly Cytokines

Tumor necrosis factor alpha (TNF-alpha) and tumor necrosis factor receptor types 1 and 2 (TNFR1 and TNFR2) were evaluated by enzyme immunoabsorption assay (ELISA). Plates of 96 high-affinity bonding wells (Nunc, Roskilde, Denmark) were sensitized with monoclonal antibodies specific to each cytokine investigated (BD Biosciences, San Jose, CA, USA) under the conditions recommended by the carbonate-bicarbonate buffer manufacturer (pH 9.5) and were incubated overnight at 4°C. After incubation, the contents of the wells were discarded and the plates were blocked with 200 µL per phosphate buffer well containing 2% buffered saline solution with bovine serum phosphate/albumin (PBS-BSA) (Sigma, St. Louis, MO, USA) for 4 hours at room temperature. Bovine serum phosphate/albumin was then discarded and lines 1 to 10 were filled with diluted serum samples 1:2 in 1% of PBS-BSA for a total volume of 100 µL per well. Serial dilutions of recombinant cytokines were used to elaborate the standard curve. The plates were incubated overnight at 4°C and then rinsed 4 times with PBS solution containing Tween at 0.05% (Sigma, St. Louis, MO, USA). Next, biotin-conjugated antibodies (BD Biosciences, San Jose, CA, USA) specific to each cytokine investigated were added to wells diluted with PBS-BSA at 1% at the concentration indicated by the manufacturer for a total of 100 µL per well. The plates were incubated for 4 hours at 37°C, rinsed with 0.05% PBS Tween, treated with 100 µL per strepavidin well conjugated with peroxidase and then incubated for 3 hours at 37°C. Finally, the plates were rinsed with 0.05% PBS-Tween. A total of 100 µL of tetramethylbenzidine (TMB) development buffer was added per well under light and at room temperature; the reaction was interrupted by the addition of 50 µL of sulfuric acid. The results were obtained by measuring absorbance at 450 nm using an automatic ELISA reader (Enspire, Perkin Elmer, USA). Cytokine concentrations were determined based on linear regression using absorbance values obtained from recombinant cytokine curves and are expressed in pg/ml. The sensitivity of the method ranged from 10 to 18 pg/ml.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 7.0 and Statview (Abaccus, USA) software. The normality of quantitative variables was investigated using the Kolmogorov-Smirnov test and the Shapiro-Wilk test. The Student t-test was used to analyze clinical parameters such as age. Cytokine levels were analyzed by the Mann-Whitney test. The significance level was established at 5% ($p < 0.05$) in all quantitative tests.

Results

Tumor necrosis factor alpha serum levels were undetectable in all female patients. In the male group, it was detected in only one patient. The median was zero in both groups, with no significant difference (Mann-Whitney; $p = 0.38$) (data not shown).

Tumor necrosis factor-R1 serum levels ranged from 1,209 to 4,918 pg/ml (median of 2,720 pg/ml) in the group of women with osteoporosis. In men with osteoporosis, levels ranged from 1,257 to 4,273 pg/ml (median of 2,680 pg/ml). There was no significant difference between the groups of patients with osteoporosis when analyzed by gender (Mann-Whitney; $p = 0.74$). In the control group, TNF-R1 serum levels ranged from 369 to 2,000 pg/ml (median of 689 pg/ml) in the group of women. In men, levels ranged from 198 to 2,417 pg/ml (median of 608 pg/ml). Tumor necrosis factor-R1 levels showed a significant increase in the osteoporosis group compared with the control group in both females and males ($p < 0.0001$) (►**Fig. 1**).

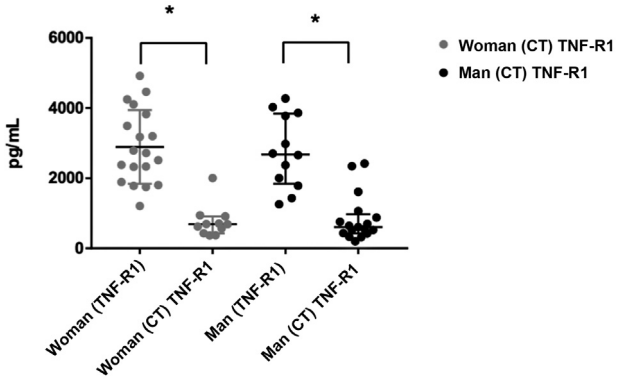

Fig. 1 Comparison of TNF-R1 serum levels between study groups in patients of both sexes.

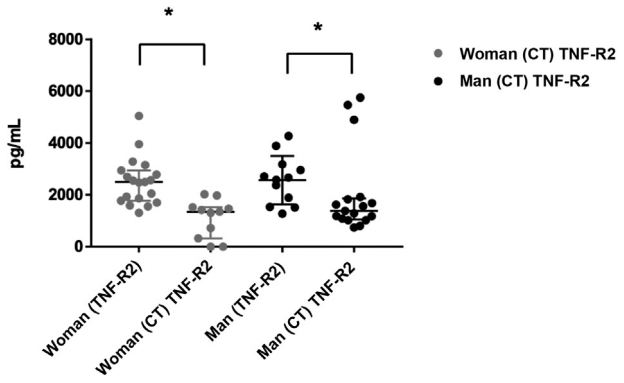

Fig. 2 Comparison of TNF-R2 serum levels between study groups in patients of both sexes.

Tumor necrosis factor-R2 serum levels had similar results, ranging from 1,313 to 5,042 pg/ml (median of 2,495 pg/ml) in the group of women with osteoporosis. In men with osteoporosis, the levels ranged from 1,276 to 4,271 pg/ml (median of 2,618 pg/ml). There was no significant difference between the groups of patients with osteoporosis when analyzed by gender (Mann-Whitney; $p = 0.85$). In the control group, TNF-R2 serum levels ranged from undetectable to 2,023 pg/ml (median of 1,350 pg/ml) in the group of women. In men, the levels ranged from 744 to 5,753 pg/ml (median of 1,380 pg/ml). Tumor necrosis factor-R2 levels showed a significant increase in the osteoporosis group compared with the control group in both females and males ($p < 0.0001$ and 0.033, respectively) (►**Fig. 2**).

As a significant increase in TNF-R1 and TNF-R2 was observed in men and women with osteoporosis, we tested whether there would be a correlation between TNF-R1 and TNF-R2 serum levels in osteoporosis, both in males and females. The results presented in ►**Figs. 3** and **4** show that there is a positive and significant correlation only in females (Sperman; $p = 0.049$).

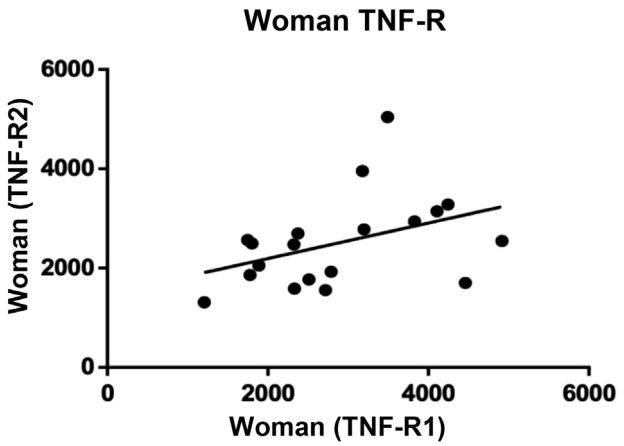

Fig. 3 Correlation between TNF-R1 and TNF-R2 serum levels in women with osteoporosis.

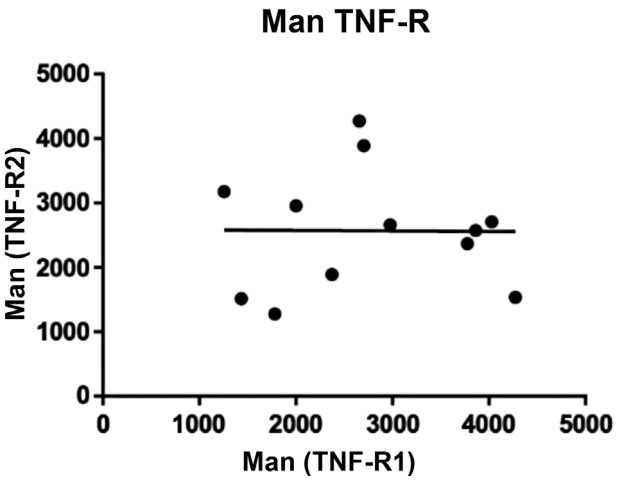

Fig. 4 Correlation between TNF-R1 and TNF-R2 serum levels in men with osteoporosis.

Discussion

Osteoporosis develops from an imbalance in bone remodeling, which results from both hormonal changes and immunological factors. In the present study, we analyzed the presence of TNF-alpha, a potent cytokine in the inflammatory role, and its receptors, TNF-R1 and TNF-R2, in patients with osteoporosis and in the control group, also comparing between genders.

The results showed significant differences in the amount of TNF-R1 and TNF-R2 receptors. Women with osteoporosis have high levels of TNF-R1 and TNF-R2 receptors when compared with women in the control group. Similarly, men with osteoporosis have high levels of TNF-R1 and TNF-R2 receptors when compared with men in the control group.

Studies show that the increase in proinflammatory cytokines, such as TNF-alpha, may be directly related to aging, and inappropriate production of this cytokine or sustained activation of its signaling pathways are factors related to the development of osteoporosis.[20,21] However, as well as the present study, other authors have demonstrated no association between changes in TNF-alpha levels between normal women and men and those with osteoporosis.[22,23]

In the present study, we also evaluated the serum levels of TNF-alpha receptors. The evaluation of soluble forms of TNF-alpha receptors should be evaluated,[24] since the dosage of circulating levels of TNF-alpha based on bone mass status is inconsistent and controversial.[22,23] Studies show that the activation of TNF-R1 leads to increased osteoclastogenesis and decreased differentiation of osteoblasts.[21]

In the present study, serum levels of TNF-alpha, TNF-R1, and TNF-R2 receptors were analyzed. We found a significant increase when comparing TNF-R1 levels in women and men with osteoporosis with those in the control group, corroborating that there is a relationship between the physiopathogenesis of osteoporosis and the effects of TNF-R1 activation. Similarly, we showed a significant increase in TNF-R2 serum levels in patients with osteoporosis, both in the male and female groups. The activation of TNF-R2 has been described

as suppressive of osteoclastogenesis.[25] Our data show that TNF-alpha was not detected in the serum of patients with osteoporosis and that serum levels of their TNF-R1 and TNF-R2 receptors are increased when compared with the control group. Signaling of these receptors is associated with modulation of osteogenesis and, if stimulated inadequately, it may result in bone homeostasis imbalance as demonstrated in studies that observed imbalance in bone deposition, in the occurrence of weakened differentiations of osteoblasts and adipocytes.[26]

An interesting finding was the fact that there is a positive and significant correlation between the levels of TNF-R1 and TNF-R2 only in women, suggesting that the two receptors are released homogeneously only in females.

Conclusion

The present study demonstrates a significant increase in TNF-alpha, TNF-R1, and TNF-R2 receptors in patients with osteoporosis in men and women. In females, serum levels of these receptors present a significant and positive correlation, while this correlation was not observed in males. These data suggest that the expression and release of these receptors may be contributing differently to the development of osteoporosis in men and women.

Conflict of Interests
The authors have no conflicts of interest to declare.

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