Evaluation of cytokines in type 1 diabetes patients with and without retinopathy

Avaliação de citocinas em pacientes diabéticos tipo 1 com e sem retinopatia

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ABSTRACT

Introduction: Diabetes *mellitus* is one of the greatest public health problems in the world; the numerous complications of this disease are the focus of great concern, as they are related to high morbidity and mortality. Among the complications of diabetes, diabetic retinopathy is the most common microvascular complication. **Objectives:** Evaluate the association of plasma levels of inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ), and anti-inflammatory cytokine interleukin-10 (IL-10) with diabetic retinopathy and the correlation of plasma levels of these cytokines together. **Material and method:** 125 patients with type 1 diabetes *mellitus* were included in this study – 55 with retinopathy and 70 without retinopathy. Cytokines IL-6, TNF-α, IFN-γ and IL-10 were determined by flow cytometry. **Results and discussion:** A significant association of the levels of cytokines IL-6, TNF-α, IFN-γ and IL-10 with retinopathy was not observed, possibly because measurements were performed on plasma samples, but not on vitreous humor. However, a positive correlation of cytokines among themselves ($p < 0.001$) was observed. **Conclusion:** Cytokines IL-6, TNF-α, IFN-γ and IL-10 presented a positive correlation among themselves, what suggests that they may act together in the development and progression of vascular complications in diabetes patients.

Key words: diabetes *mellitus* type 1; diabetic retinopathy; cytokines; inflammation.

INTRODUCTION

According to the International Diabetes Federation (IDF), the prevalence of diabetes *mellitus* (DM) in 2014 was 387 million, and an increase of around 205 million new cases is estimated to occur by 2035[1]. Its prevalence in Brazil can be compared with that in developed countries, where DM is considered one of the greatest public health problems[2]. The high prevalence is a cause of great concern because of the numerous complications resulting from this disease[3]. In our country, DM is one of the 10 main causes of mortality. Besides, its morbidity is associated with elevated health care costs, impaired working capacity, and reduced life expectancy of individuals affected by this disease, what characterizes a huge socioeconomic impact[4, 5].

DM type 1 (DM1) represents approximately 5%-10% of cases. In spite of the shorter frequency, it is the main form to affect children and adolescents. This type of DM is associated with deficiency in insulin production due to destruction of pancreatic beta cells, what can be measured by the action of autoantibodies or can be attributed to unknown causes, in which there is no evidence of autoimmune action[6].

Uncontrolled hyperglycemia is considered the major precipitating factor of severe chronic complications, with DM being one of the leading causes of cardiovascular diseases, blindness, kidney failure and lower-limb amputation. In addition, DM patients are predisposed to the development of certain infections[3]. Among the chronic complications, diabetic retinopathy (DR) is the most common microvascular complication. Approximately 90% of DM1 patients are estimated to present some degree of DR 20 years after the onset of the disease; among these, 60% will develop the most severe form of DR, called proliferative diabetic retinopathy (PDR)[7, 8]. DR is considered the main cause of blindness in individuals aged 20-74 years; around 12% of new cases of reduced visual acuity that impairs the conduction of routine activities are attributed

to DR. However, when diagnosis and treatment are carried out in a timely manner, that is, before irreversible alterations occur, the risk of blindness due to DR is reduced to nearly 5%[9].

Studies show that alterations in four distinct biochemical pathways are involved in the pathogenesis of DR. As a group, these alterations are responsible for causing oxidative stress that triggers the inflammatory process, which is intimately related to the pathophysiology of DR[10]. Some studies have shown that inflammation, and more specifically inflammatory cytokines, are important factors in the development of microvascular complications of DM, including retinopathy, neuropathy and nephropathy[11].

Cytokines are extracellular proteins of low molecular weight that serve as mediators of immune response. They act in highly complex pathways that regulate the inflammatory process, and they are necessary to conduct response to the lesion site. Low-grade chronic inflammation and activation of the innate immune system are known to be strongly related to the pathogenesis of DM[11].

Therefore, the study of the association of plasma levels of inflammatory cytokines with the presence of retinopathy in DM patients is important, as it can contribute to a better comprehension of the mechanisms responsible for the development of this microvascular complication, as well as to the discovery of novel therapeutic targets, what can be useful to find new treatments to avoid the development of retinopathy and delay its progression.

OBJECTIVES

The present study was aimed at evaluating the association of plasma levels of inflammatory cytokines interferon-gamma (IFN-γ), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α), and anti-inflammatory cytokine interleukin-10 (IL-10) with the presence of DR in DM1 patients, besides correlating plasma levels of these cytokines among themselves.

MATERIAL AND METHOD

This study was given an assent by the ethics research committee of Universidade Federal de Minas Gerais (UFMG) (CAAE – 0392.0.203.000-11) and by the ethics research committee of Santa Casa de Misericórdia de Belo Horizonte (006/2012). All participants were informed about the purposes

of the project, completed the baseline questionnaire, and signed the free informed consent.

One hundred twenty-five adult individuals aged 18-60 years with clinical and laboratory diagnosis of DM1 participated in the study. Those who presented any of the following conditions were excluded: liver disease, alcohol abuse, coagulopathy, cancer, ongoing infectious process, hemodialysis, history of kidney transplant, and pregnancy.

Participants were classified in two groups according to the presence or absence of retinopathy, which was evaluated by means of funduscopy – this piece of information was obtained from the analysis of patients' records.

The clinical and biochemical parameters were obtained from the analysis of patients' clinical charts, which were completed based on information given by patients and confirmed and/or complemented by analysis of medical records. Clinical and biochemical data were compiled from a period of 1-6 months prior to collection date of blood samples for cytokine measurements. Cytokines IFN-γ, IL-6, IL-10 and TNF-α were determined in plasma samples by flow cytometry, using the Human Basic Kit Flow Cytomix (eBioscience®).

The produced results were analyzed with the statistical program SPSS 20.0. For the continuous variables, the normality test Shapiro-Wilk was used. For the variables that presented normal distribution, mean and standard deviation were calculated, and Student's t test was employed for comparison between both groups. For the variables that did not present normal distribution, median and 25%-75% percentile were calculated, and Mann-Whitney U test was used for comparison between both groups. The categorical variables were presented as absolute and relative frequency, and the chi-square test was adopted for comparison among these variables. The Pearson method was used to investigate correlation among the assessed parameters. A value of $p \leq 0.05$ was considered significant.

RESULTS

Table 1 describes the characteristics of 125 DM1 patients, grouped according to the presence or absence of DR. Patients with DR presented older age ($p = 0.001$) and longer duration of diabetes ($p < 0.001$) than those without DR. Aside from that, DR patients presented levels of urea ($p < 0.001$), creatinine ($p = 0.001$), albuminuria ($p < 0.001$) and cystatin C ($p < 0.001$) higher than those without DR, besides glomerular filtration rate ($p < 0.001$) lower than patients without DR. Higher frequency of

TABLE 1 – Characteristics of type 1 diabetes patients classified according to presence or absence of retinopathy

	Patients without retinopathy	Patients with retinopathy	p
Number of individuals (n)	70	55	
Males (n, %)	25 (35.7)	20 (36.4)	NS
Age (years)	29 (23-36)	36 (30-43)*	*$p = 0.001$
BMI (kg/m²)	23.6 ± 3.2	23.3 ± 3.4	NS
Diabetes duration (years)	15 ± 6	23 ± 5*	*$p < 0.001$
Glucose (mg/dl)	158 (112-246)	134 (80-243)	NS
HbA1c (%)	8.2 ± 1.1	8.5 ± 1.4	NS
AST (U/l)	23 (19-30)	23 (18-27)	NS
ALT (U/l)	29 ± 10	30 ± 9	NS
Albumin (g/dl)	4 ± 0.4	4 ± 0.5	NS
Urea (mg/dl)	29 (26-34)	39 (30-57)*	*$p < 0.001$
Creatinine (mg/dl)	0.8 (0.68-0.92)	1 (0.73-1.51)*	*$p = 0.001$
Glomerular filtration rate (ml/min/1.73 m²)	104 (82-116)	69 (42-106)*	*$p < 0.001$
Albuminuria (mg/g)	4 (3-5)	17 (7-57)*	*$p < 0.001$
Cystatin C (mg/l)	0.76 (0.64-0.87)	1.08 (0.78-2.04)*	*$p < 0.001$
Family history of diabetes (n, %)	41 (58.6)	39 (70.9)	NS
Tobacco smoking (n, %)	6 (8.6)	5 (9.1)	NS
Physical activity (n, %)	29 (41.4)	22 (40)	NS
Neuropathy (n, %)	6 (8.6)	14 (25.5)*	*$p = 0.010$
Use of statins (n, %)	14 (20)	26 (47.3)*	*$p = 0.001$
Use of anti-hypertensive drug (n, %)	34 (48.6)	46 (83.6)*	*$p < 0.001$
Use of ASA (n, %)	5 (7.1)	16 (29.1)*	*$p = 0.001$
Use of thyroxine (n, %)	9 (12.9)	9 (16.4)	NS

Variables presenting normal distribution were expressed as mean ± standard deviation, and compared using Student's t test. Variables not presenting normal distribution were expressed as median (25%-75% percentile), and compared using Mann-Whitney U test. Categorical variables were expressed as absolute and relative frequencies n (%), and compared using the chi-square test.

$p < 0.05$ for patients with retinopathy compared with patients without retinopathy. NS: not significant; BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine transaminase; ASA: acetylsalicylic acid.

neuropathy ($p = 0.01$) was observed in the group of DR patients. Regarding medication, there was higher frequency in the use of statins ($p = 0.001$), anti-hypertensive agents ($p < 0.001$), and acetylsalicylic acid (ASA) ($p = 0.001$) by DR patients. Statistically significant differences were not verified for the variables sex, body mass index (BMI), glucose, glycated hemoglobin (HbA1c), aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, family history of diabetes, tobacco smoking, physical activity and use of thyroxine.

The correlations of plasma levels of inflammatory cytokines among themselves in DM1 patients without retinopathy, with retinopathy, with neuropathy and with nephropathy are presented in **Tables 2**, **3**, **4**, and **5**, respectively. In the patients without retinopathy, all cytokines presented significant correlation among themselves ($p < 0.01$). A similar result was observed in patients with nephropathy ($p < 0.05$ for the

correlation between IFN-γ and IL-6, and $p < 0.01$ for the other correlations). In the patients with retinopathy and those with neuropathy, all cytokines presented significant correlation among themselves ($p < 0.01$), except the correlation between IFN-γ and IL-6, which was not significant.

Table 6 presents the plasma levels of cytokines IFN-γ, IL-6, IL-10 and TNF-α of patients with and without DR, but statistically significant differences were not found among these assessed parameters.

TABLE 2 – Correlation among inflammatory cytokines in type 1 diabetes patients without retinopathy

	IFN-γ (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
IFN-γ (pg/ml)	-	-	-	-
IL-6 (pg/ml)	0.757**	-	-	-
IL-10 (pg/ml)	0.826**	0.602**	-	-
TNF-α (pg/ml)	0.54**	0.461**	0.686**	-

Correlations (R²) were evaluated using the Pearson method.

*IFN-γ: interferon-gamma; IL: interleukin; TNF-α: tumor necrosis factor-alpha; **$p < 0.01$.*

TABLE 3 – Correlation among inflammatory cytokines in type 1 diabetes patients with retinopathy

	IFN-γ (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
IFN-γ (pg/ml)	-	-	-	-
IL-6 (pg/ml)	0.256	-	-	-
IL-10 (pg/ml)	0.956**	0.373**	-	-
TNF-α (pg/ml)	0.393**	0.477**	0.51**	-

Correlations (R²) were evaluated using the Pearson method.

*IFN-γ: interferon-gamma; IL: interleukin; TNF-α: tumor necrosis factor-alpha; **$p < 0.01$.*

TABLE 4 – Correlation among inflammatory cytokines in type 1 diabetes patients with neuropathy

	IFN-γ (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
IFN-γ (pg/ml)	-	-	-	-
IL-6 (pg/ml)	0.41	-	-	-
IL-10 (pg/ml)	0.811**	0.732**	-	-
TNF-α (pg/ml)	0.744**	0.713**	0.885**	-

Correlations (R²) were evaluated using the Pearson method.

*IFN-γ: interferon-gamma; IL: interleukin; TNF-α: tumor necrosis factor-alpha; **$p < 0.01$.*

TABLE 5 – Correlation among inflammatory cytokines in type 1 diabetes patients with nephropathy

	IFN-γ (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	TNF-α (pg/ml)
IFN-γ (pg/ml)	-	-	-	-
IL-6 (pg/ml)	0.312*	-	-	-
IL-10 (pg/ml)	0.939**	0.406**	-	-
TNF-α (pg/ml)	0.412**	0.492**	0.535**	-

Nephropathy was defined by the presence of albuminuria ≥ 30 mg/g and/or glomerular filtration rate < 60 ml/min/1.73 m². Correlations (R²) were evaluated using the Pearson method.

*IFN-γ: interferon-gamma; IL: interleukin; TNF-α: tumor necrosis factor-alpha; *$p < 0.05$; **$p < 0.01$.*

TABLE 6 – Cytokines of type 1 diabetes patients classified according to the presence or absence of retinopathy

	Patients without retinopathy	Patients with retinopathy	p
IFN-γ (pg/ml)	98.4 ± 23.2	104.8 ± 29.8	NS
IL-6 (pg/ml)	16.4 ± 2.6	16.3 ± 2	NS
IL-10 (pg/ml)	$1,166 \pm 215.6$	$1,154.5 \pm 214.5$	NS
TNF-α (pg/ml)	169.1 ± 101.9	175.8 ± 94.9	NS

Variables were expressed as mean ± standard deviation, and compared by means of Student's t *test.*

*p < 0.05 for patients with retinopathy compared with patients without retinopathy.

IFN-γ: interferon-gamma; IL: interleukin; TNF-α: tumor necrosis factor-alpha; NS: not significant.

DISCUSSION

DR patients present older age ($p = 0.001$) and longer duration of DM ($p < 0.001$) than those without DR. According to Esteves *et al*. (2008), duration of DM is an important factor associated with DR. In DM1, whose onset may be easily identified, it is estimated that after around 20 years of disease, almost all DM1 patients manifest some degree of DR[12, 13].

Moreover, DR patients present levels of urea ($p < 0.001$), creatinine ($p = 0.001$), albuminuria ($p < 0.001$), and cystatin C ($p < 0.001$) higher than patients without DR, and a glomerular filtration rate ($p < 0.001$) lower than patients without DR, what indicates association of DR with diabetic nephropathy, described in the literature. Jawa *et al*. (2004) observed that DM1 patients that presented nephropathy in most cases display other signs of microvascular complications (neuropathy and retinopathy), with the presence of advanced retinopathy almost always associated with histological alterations in renal glomeruli, and also with increased urinary protein excretion; increased albuminuria, urea, and plasma creatinine are strong indicators of the presence of retinopathy[14, 15].

The group of patients with DR presents higher frequency of neuropathy ($p = 0.01$), one of the microvascular complications of DM, intimately related to patient's age, duration of diabetes, and glycemic control. Commonly, the presence of diabetic neuropathy is associated with other microvascular complications, such as retinopathy and nephropathy[16, 17].

Higher frequency of the use of statins ($p = 0.001$), anti-hypertensive medication ($p < 0,001$), and ASA ($p = 0.001$) by patients that present DR was observed. Statin is a medicine used to treat hypercholesterolemia, considered a risk factor for DR[18]. Hypertension is also considered a risk factor for the development of DM complications. Several studies have demonstrated its strong association with the development and progression of DR[17, 19, 20]. ASA is classified as an antiplatelet drug, often used clinically at the dose of 75 mg/day by patients that present high risk for cardiovascular disease (hypertension, diabetes, dyslipidemia, tobacco use, and family history of cardiovascular diseases) to reduce the risk of cardiovascular events[21].

Statistically significant associations were not observed between retinopathy and the levels of glucose, HbA1c, and plasma albumin. A possible reason for the divergent results found in the present study is the fact that assessment of HbA1c was not done over the years in the DM1 patients of this study: it is important to consider the possibility that patients with DR can have presented high levels of HbA1c in years preceding DR diagnosis. Concerning the plasma levels of albumin, a plausible explanation is that albuminuria in patients with DR is slightly to moderately increased. This picture can have a significant association with DR, but it was not enough to cause reduced plasma levels of albumin.

DM1 is defined as an inflammatory disease of pancreatic islets, in which beta cells are destroyed by autoantibodies, cytotoxic T cells, and inflammatory mediators[22]. Histologically, DM1 is represented by a lymphocytic mononuclear inflammatory infiltrate (insulitis) and by the absence of pancreatic beta cells. When migration of inflammatory cells characteristic of the insulitis process occurs, there is the important participation of several cytokines, especially the pro-inflammatory ones, such as TNF-α, IFN-γ and interleukin-1 beta (IL-1β),which favor the process of autoimmune and inflammatory response typical of DM1[23]. As stated by Dogan *et al*. (2006)[24], it is possible to detect high levels of inflammatory markers in newly diagnosed DM1 patients, what suggests that systemic inflammation is related to the onset of the disease. However, the levels of these markers are known to remain high during DM progression, and they can possibly contribute to the development of complications.

Reis *et al*. (2012)[25] analyzed plasma samples of 42 DM1 patients and 24 healthy patients as a control group, finding higher circulating levels of IL-6 in DM1 patients than in the control group. He *et al*. (2014)[26] carried out a study with 35 children with DM1 and 30 healthy individuals, in which levels of cytokines (IL-1, IL-6, IL-10, IL-12 and TNF-α) were investigated. The authors observed levels of cytokines higher in children with DM1 than in the control group.

IL-10 is an anti-inflammatory cytokine that seems to act trying to block DM evolution[27]. Its increase in DM1 patients

can be resulting from a compensatory mechanism to the increase of pro-inflammatory cytokines. For this reason, the cytokines involved in the pathogenesis of DM1 are important for the development of the disease, acting both in the positive control (pro-inflammatory cytokines) and in the negative control (anti-inflammatory cytokines) of the inflammatory process, and they can act in a cascade or alone[25, 26, 28].

Thus, the results obtained in the present study are in agreement with the literature, which confirms the relationship of plasma levels of cytokines among themselves in DM1 patients without retinopathy, and also in those with DM complications, such as retinopathy, neuropathy, and nephropathy. Therefore, it is possible to suggest that cytokines can act in group, promoting a subclinical inflammatory state in DM1 patients, which can contribute to the development and the progression of DM complications.

There was no significant association of plasma levels of inflammatory cytokines IL-6, TNF-α, IFN-γ and IL-10 with DR in DM1 patients. Prolonged hyperglycemia is known to cause several biochemical alterations related to this disease, such as the alteration in the gene expression, non-enzymatic glycosylation of proteins, the increased oxidative stress that leads to an excess of oxidation end products, and, at last, infiltration of inflammatory cells followed by cytokine production in the diabetic retina[29]. Accordingly, some authors characterize DR as a low-level chronic inflammation, with the presence of inflammatory cytokines, and elevated numbers of circulating activated leukocytes[30, 31].

In the present study, cytokine levels did not show correlation with the presence of DR. A possible explanation for this fact is that cytokine levels can increase predominantly in retina, at a specific site, presenting a systemic small increase in blood circulation in DR patients. This hypothesis can be confirmed by the result of a study conducted by Cheung *et al*. (2012)[32], in which the authors assessed levels of different cytokines in samples of vitreous/aqueous humor, and found association between levels of IL-6 and DR. Besides, some authors demonstrated that cytokine levels are higher in the aqueous humor than in the plasma of patients with DR[33, 34], so justifying the results found in the present study.

Despite the relevant results, this work presented some limitations: its design is cross-sectional, what did not enable patients' follow-up over the years; data collection based on completion of the baseline questionnaire by patients, which were confirmed by medical records, did not prove adequate, presenting some incomplete pieces of information, what precluded the analysis between patients with non-proliferative DR (NPDR) and proliferative DR (PDR); and the fact that the used sample was plasma, not vitreous humor.

CONCLUSION

It has been suggested that plasma levels of inflammatory cytokines play a fundamental role in DR, contributing to the development of a low-grade chronic inflammatory state, which can promote the emergence and the aggravation of lesions in the retina. In the present work, association of cytokines IL-6, IL-10, IFN-γ and TNF-α with DR was not observed, possibly because the used samples were plasma, not aqueous/vitreous humor, what suggests the hypothesis that the increased levels of these inflammatory mediators occur predominantly at the local level, in the retina, during the development of this complication. However, positive correlations were observed of the assessed cytokines among themselves in patients without retinopathy and in those with DM complications, such as retinopathy, neuropathy, and nephropathy, suggesting that they can act as a group in the development and progression of microvascular and neuropathic complications of patients with diabetes. Also it was not possible to evaluate association of cytokines with the different DR stages, but it would be very interesting to verify if plasma measurement of cytokines can be useful for prognosis and monitoring of DR evolution. Therefore, it is necessary that further studies be conducted in this area, aimed at better comprehension of mechanisms involved in development and progression of DR, which can be useful for the discovery of new laboratory markers and the development of more effective treatments, to avoid that this complication develops or to retard its progression.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interest regarding the publication of this paper.

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RESUMO

Introdução: O diabetes mellitus *é considerado um dos maiores problemas de saúde pública no mundo; as numerosas complicações decorrentes dessa patologia são foco de grande preocupação, pois estão relacionadas com elevadas morbidade e mortalidade. Entre as complicações decorrentes do diabetes, a retinopatia diabética é considerada a complicação microvascular mais comum.* **Objetivos:** *Avaliar a associação dos níveis plasmáticos das citocinas inflamatórias interleucina 6 (IL-6), fator de necrose tumoral alfa (TNF-α) e interferon gama (IFN-γ) e da citocina anti-inflamatória interleucina 10 (IL-10) com a retinopatia diabética, bem como a correlação dos níveis plasmáticos dessas citocinas entre si.* **Material e método:** *Foram incluídos no estudo 125 pacientes diabéticos tipo 1, sendo 55 portadores de retinopatia e 70 sem retinopatia. As citocinas IL-6, TNF-α, IFN-γ e IL-10 foram determinadas pelo método de citometria de fluxo.* **Resultados e discussão:** *Não foi observada associação significativa dos níveis das citocinas IL-6, TNF-α, IFN-γ e IL-10 com a retinopatia, possivelmente devido às dosagens terem sido realizadas em amostras de plasma e não de humor vítreo/aquoso. Porém, foi observada correlação positiva das citocinas entre si (*p < 0,001). **Conclusão:** *As citocinas IL-6, TNF-α, IFN-γ e IL-10 apresentaram correlação positiva entre si, o que sugere que elas podem atuar em conjunto no desenvolvimento e na progressão das complicações vasculares nos pacientes diabéticos.*

Unitermos: diabetes mellitus *tipo 1; retinopatia diabética; citocinas; inflamação.*

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