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TNF α and IL-8 vitreous concentrations variations with two antidiabetic therapies in patients with proliferative diabetic retinopathy: an observational study

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Abstract

Background Antidiabetic therapies are effective, but could indirectly modify the inflammatory response in the ocular microenvironment; therefore, a study was developed to evaluate the inflammatory cytokine profile in the vitreous humor of diabetic patients with retinopathy under treatment with antidiabetic drugs.

Methods Observational, comparative, retrospective, cross-sectional study. Interleukins 1 β , 6, 8, 10, and tumor necrosis factor-alpha (TNF α) were evaluated in the vitreous humor obtained from patients with type 2 diabetes mellitus, proliferative diabetic retinopathy, and concomitant retinal detachment or vitreous hemorrhage, and who were already on antidiabetic treatment with insulin or metformin + glibenclamide. The quantification analysis of each cytokine was performed by the cytometric bead array (CBA) technique; medians and interquartile ranges were obtained, and the results were compared between groups using the Mann-Whitney U test, where a p -value < 0.05 was considered significant.

Results Thirty-eight samples; quantification of TNF α concentrations was higher in the group of patients administered insulin, while interleukin-8 was lower; in the metformin + glibenclamide combination therapy group, it occurred inversely. In the stratified analysis, the highest concentrations of interleukin-8 and TNF α occurred in patients with vitreous hemorrhage; however, the only statistical difference existed in patients with retinal detachment, whose TNF α concentration in the combined therapy group was the lowest value found (53.50 (33.03–86.66), $p=0.03$). Interleukins 1 β , 6, and 10 were not detected.

Conclusion Interleukin-8 and TNF α concentrations are opposite between treatment groups; this change is more accentuated in patients with proliferative diabetic retinopathy and vitreous hemorrhage, where the highest concentrations of both cytokines are found, although only TNF α have statistical difference.

Keywords Antidiabetic therapy, Diabetic retinopathy, Inflammatory response, Insulin, Interleukins, Metformin

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Background

Diabetes mellitus (DM) is one of the most important chronic-degenerative diseases globally, [1] because it reduces the quality of life of those who suffer from it. It is mainly characterized by sustained hyperglycemia, caused by the total or partial loss of endocrine glucose regulation, either due to resistance to the action of insulin or due to a decrease in its production [2, 3]. Among the types of DM, type 2 (T2DM) has the highest prevalence; [4, 5] in all cases, the normal biochemistry of the organism is altered and new oxidant molecules that are precursors of tissue damage are produced [6]. Some of these mechanisms include the formation of advanced glycation end products (AGEs) by the non-enzymatic binding between a glucose molecule and different long half-life proteins [7–10].

When oxidant species increase, vascular complications can occur, compromising the supply of nutrients and oxygen to the tissues. To compensate for capillary damage, the body activates mechanisms such as the synthesis of vascular endothelial growth factor (VEGF), which generates new blood vessels to fulfill the metabolic demand, although these had an unstable constitution than the pre-existing ones; furthermore, this factor activates an inflammatory response that can increase vascular permeability [11, 12].

In the retinal capillaries, the thickening of the endothelial basement membrane, the decrease in intercellular tight junctions, and the loss of pericytes facilitate the release of inflammatory mediators generated by oxidative stress into the retina [13], which leads to diabetic retinopathy (DR). Some of these cytokines attract antigen-presenting cells capable of activating naïve T-lymphocytes and transforming them into differentiated precursor cells of inflammation, through the synthesis of chemokines and other inflammatory mediators [14]. It has been described that retinal damage due to inflammation is related to the degranulation of glial cells and the extravasation of cytokines and growth factors, which generates a recurring cycle of damage and inflammation that wears down the retinal tissue [15, 16].

This pathological process also stimulates the production of tumor necrosis factor-alpha (TNF α) and interleukin 8 (IL-8), responsible for the proliferation and recruitment of neutrophils at the site of damage, by activating the innate response to molecular patterns associated with damage (DAMPs) produced chronically in T2DM; interleukins that cross the blood-retinal barrier initiate an immune privileged response mediated mainly by the activation and interaction of Müller cells, [17] which causes retinal dysfunction due to the accumulation of inflammatory mediators that interfere with the electrical signaling of the neural axis, that decrease the visual function. Also, the composition of the vitreous

humor can be altered in pathophysiological conditions such as those induced by DM; [18] the role that cytokines play as inflammatory mediators has been widely studied, although the findings reported to date are still not consistent regarding their concentrations, because the balance is difficult to achieve in subjects with T2DM, particularly if they have already developed DR. Therefore, it is necessary to limit the inflammation induced by hyperglycemia through pharmacological therapies [19].

The main drug to treat T2DM is the biguanide called metformin, which decreases the synthesis of intracellular ATP and changes the concentration of adenosine monophosphate (AMP) that is involved in anti-inflammatory cellular processes [20]. On the other hand, the use of the sulfonylurea glibenclamide, a hypoglycemic agent that used to be administered in combination with metformin, has currently been restricted because it can lead to hypoglycemia; [21] even so, it was a widely used treatment in Latin America due to its low cost compared to other drugs. The use of exogenous insulin also has anti-inflammatory effects as a result of reducing oxidation due to hyperglycemia; its administration is usually reserved for cases where other drugs fail to regulate blood glucose [22].

Additionally, hypoglycemic pharmacological therapies combined with different mechanisms of action have been shown to enhance the effect observed with those reported in monotherapies [23]. In this sense, they could indirectly limit the inflammation associated with proliferative DR, which could promote a protective effect on retinal cells, but so far this has not been described; this study evaluated the concentration of intravitreal inflammatory molecules in patients suffering from T2DM and proliferative DR treated with two different pharmacological therapies of the most used antidiabetic agents.

Methods.

Observational, comparative, retrospective, and cross-sectional study. Vitreous humor samples were collected between March 2019 and February 2020. The protocol was approved by the Institutional Review Board of the Hospital where it was performed (No. 2019R18B1) and adhered to the tenets of the Declaration of Helsinki; all participants agreed to be included in the study and signed informed consent.

Mexican subjects of any sex, of legal age, with at least 10 years of having T2DM under treatment with insulin or hypoglycemic agents (metformin+glibenclamide), with or without systemic arterial hypertension (SAH), who had a previous diagnosis of proliferative DR as the following criteria: the presence of retinal neovascularization (early stage), neovascularization plus vitreous hemorrhage (high-risk), or retinal detachment (severe stage), scheduled for vitrectomy via pars plana, and who previously agreed to participate in the study and signed

written informed consent, were included by non-probabilistic sampling. Subjects with other systemic inflammatory diseases, patients who had an ocular history of proliferative vitreoretinopathy or mixed retinal detachments, or those who had undergone intraocular surgery within the 90 days before the vitrectomy, were excluded. According to their pharmacological therapy, subjects were classified into one of the following groups: (1) insulin (5U/12 h), and (2) oral hypoglycemic drugs (metformin (850 mg/12 h)+glibenclamide (5 mg/12 h)).

The vitreous samples used were in sterile polypropylene microtubes at -80 °C until the time of analysis; at the time of collection, a volume between 500 and 1000 µL (undiluted) was considered, which was obtained at the beginning of the vitrectomy before opening the infusion line, by aspiration with a 1 mL syringe. Quantification of total proteins was performed by the Bradford method with Coomassie brilliant blue (Thermo Scientific, IL, USA); the microplates were read with the ELx800 equipment (BioTek, VT, USA) at 310 nm, and the concentrations were obtained based on an albumin standard curve to assess the integrity of the samples.

For analysis, the samples were diluted 1:10 in PBS; subsequently, the quantification of interleukins was performed using the two-color CBA (Cytometric Bead Array) technique for the cytokines IL-1β, IL-6, IL-8, IL-10, TNFα (CBA Human Inflammatory Cytokines Kit, CA, USA). The preparation of standards, samples, and capture beads was based on the manufacturer's procedure, and they were analyzed on the FACSARIA Fusion flow cytometry equipment (Becton Dickinson, CA, USA). Cytokine concentrations were reported in units of picograms/milliliter (pg/mL).

Statistical analysis

The proportions of the qualitative variables were obtained and compared, when needed, with Fischer's exact test; additionally, the means, standard deviation (SD), median, and interquartile ranges (IR) were calculated for the quantitative variables; after the analysis

of normal distribution with the Shapiro-Wilk test, the concentration of each cytokine was compared between the groups with the Mann-Whitney U test, and variables such as age, last fasting blood glucose and duration of DM by Student's t-test. Statistical analysis was performed using IBM SPSS software version 25 for Windows, and a *p*-value < 0.05 was considered significant.

Results

Thirty-eight eyes of 38 subjects were evaluated, with a mean age of 58.18 (8.36) years; 52.6% were women, and 47.4% were men. The mean duration of diabetes was 17.26 (7.37) years, and the fasting blood glucose concentration was 143.68 (54.48) mg/dL; 60.5% had presented retinal detachment and were classified as severe proliferative DR, 39.5% presented high-risk proliferative DR with vitreous hemorrhage, and 65.79% stated that they had SAH convergent with T2DM. Patients were divided according to their type of treatment: 20 to the insulin group, and 18 to the oral hypoglycemic drugs group (metformin+glibenclamide). When comparing the variables age, sex, last fasting blood glucose, and duration of DM between the groups, there was no statistical difference (*p*>0.05, Table 1). The mean total protein was 1.48 (0.87) µg/mL, which allowed the viability of the protein content of the samples to be validated.

Regarding the analysis of inflammatory mediators in the vitreous humor, the group treated with insulin alone had a TNFα concentration slightly higher than the group with metformin+glibenclamide (*n*=20, 76.50 pg/mL (64.46–104.72) vs. *n*=18, 61.26 pg/mL (34.34–89.20), *p*=0.03, Fig. 1); however, IL-8 had a concentration 37.15 pg/mL higher in the oral hypoglycemic drugs group compared to the insulin group (*n*=18, 324.68 (264.97–420.21) vs. *n*=20, 287.53 (233.14–369.79)), which reflected an inverse effect between both molecules, although this difference was not significant (*p*=0.44).

The groups were stratified according to the complications associated with T2DM-induced inflammation and concomitant to proliferative DR; these were (a) retinal detachment (*n*=23), and (b) vitreous hemorrhage (*n*=15). In the comparison between treatment groups, a higher concentration of IL-8 and TNFα was found in cases of vitreous hemorrhage compared to those with retinal detachment. In this last subgroup, TNFα was significantly higher in the insulin group than in the oral hypoglycemic drugs group (Table 2, *p*=0.03), meanwhile, IL-8 was higher in the second treatment group without a statistical difference (Table 3). Concentrations of both molecules were not statistically different in patients with and without SAH, in each treatment group (*p*>0.05); the rest of the cytokines were found below the detection threshold of the assay, so they could not be quantified.

Table 1 Clinical characteristics of each treatment group

	Insulin (<i>n</i> =20)	Metformin+glibenclamide (<i>n</i> =18)	<i>p</i> *
Age	57.70 (7.91)	58.72 (9.02)	0.71
Sex (% , <i>n</i>)	50% (<i>n</i> =10)	55.6% (<i>n</i> =10)	0.76**
Man	50% (<i>n</i> =10)	44.4% (<i>n</i> =8)	
Woman			
Last fasting blood glucose	140.37 (64.09)	147.17 (43.72)	0.71
Duration of DM	19.05 (6.55)	15.27 (7.89)	0.11

*Student's t-test

** Fischer's exact test

Mean (standard deviation)

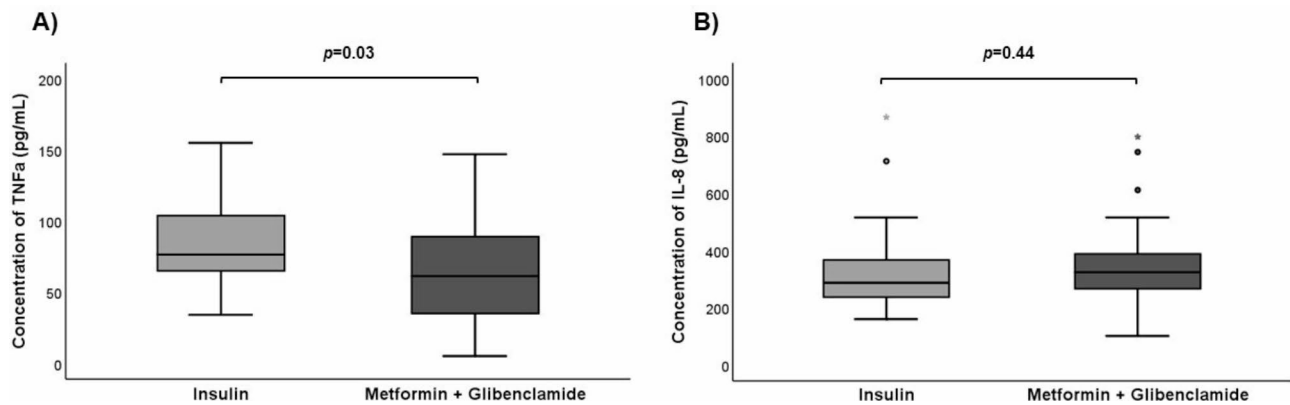


Fig. 1 Cytokine concentrations in the vitreous humor of patients with proliferative DR. All reported concentrations are in pg/mL, and the comparison was performed with Mann-Whitney’s U test. **A:** TNF α concentration was significantly higher in the insulin treatment group. **B:** there was a higher concentration of IL-8 in the oral hypoglycemic drugs group, but the difference was not statistical. Mild outliers are marked with a circle (o), and extreme outliers as an asterisk (*)

Table 2 Comparisons of vitreous TNF concentrations between treatments and concomitant diseases to diabetic retinopathy

	Insulin	n	Metformine + Glibenclamide	n	p*
Retinal detachment	73.02 (63.93–101.91)	11	53.50 (33.03–86.66)	12	0.03
Vitreous hemorrhage	82.12 (55.64–115.28)	9	70.62 (51.23–89.20)	6	0.46

*Mann-Whitney’s U test
Median (interquartile range)

Table 3 Comparisons of vitreous IL-8 concentrations between treatments and concomitant diseases to diabetic retinopathy

	Insulin	n	Metformine + Glibenclamide	n	p*
Retinal detachment	287.53 (255.69–356.52)	11	316.72 (262.32–555.54)	12	0.38
Vitreous hemorrhage	319.37 (223.85–613.91)	9	332.64 (286.21–420.21)	6	0.95

*Mann-Whitney’s U test
Median (interquartile range)

Discussion

In this study, we found that TNF α was significantly superior in the group treated with insulin, and IL-8 was slightly higher in the group treated with hypoglycemic agents, but without statistical difference. Furthermore, the concentrations of both cytokines were higher in cases where vitreous hemorrhage occurred concomitantly with DR, regardless of the treatment used, although the inverse effect of IL-8 and TNF α was notable, possibly related to the mechanisms of action of each drug; nonetheless, these differences were no significant either. By the immunological technique employed, interleukins 1 β , 6, and 10 could not be detected.

Some studies have described that the increase in IL-1 β can cause neurodegeneration, apoptosis of retinal capillary cells, and dysfunction of the blood-retinal barrier, [24–27] which would partly explain the visual dysfunction present in patients with retinal damage. However, IL-1 β is not the only one that increases its concentration when DR occurs; Capitão et al. mention that IL-1 β , IL-8, and TNF α increase as the disease progresses [25]. In contrast, Lee et al [28]. reported that although there

may be alterations in the cytokine profile in cases where proliferative DR coexists with vitreous hemorrhage, the role of IL-1 β could be weak in cases of retinal capillary nonperfusion, given that the concentration of this interleukin is low (0.39 ± 0.08 pg/mL), which is similar to the result reported in the study by Koskela et al. (0.3 ± 0.9 pg/mL) [29].

Moreover, Gustavsson and his group could not find detectable values of IL-1 β in vitreous samples from patients with proliferative DR by a chemiluminescence assay equivalent to ELISA tests, whose lower limit of detection was 5 pg/mL for this interleukin [30]. Likewise, we could not detect IL-1 β concentrations by the CBA technique, which uses antigen-antibody binding as a principle similar to other immunoenzymatic assays. Retinal microglia also synthesize other cytokines such as TNF α , found in early stages of DR, [31] and as we mentioned above, can increase during the disease development. A Korean group reported concentrations of 0.49 ± 0.28 pg/mL,²⁸ much lower than the values of the meta-analysis of Yao et al., [32] where the means of TNF α range from 1.08 ± 0.22 to 973.2 ± 115.4 pg/mL, while in

a Brazilian study, the concentrations of this factor were undetectable; [33] these heterogeneous values may be due to the different detection techniques used. Our result exceeded 50 pg/mL in each group evaluated and was higher in the insulin treatment group.

TNF α has also been correlated with IL-6, [34] an interleukin with pro- and anti-inflammatory responses whose concentrations in DR cases have varied from 1.08 ± 0.22 to 276.1 ± 13.9 pg/mL; [35] values within this range were also reported by Mallman et al. (69.37 pg/mL), in patients with proliferative DR who had mean glucose values of 165.1 ± 59.4 mg/dL, and who had associated complications such as vitreous hemorrhage or retinal detachment [33]. Furthermore, it is known that the regulatory capacity of IL-6 changes over time and that its concentration tends to decrease as the duration of DM increases [36, 37] During the development of T2DM there is also the suppression of some genes that encode proteins that regulate the immune response, such as IL-10, an anti-inflammatory cytokine that inhibits the translocation and infiltration of pro-inflammatory cells from damaged capillaries due to oxidative stress; the reduction of this cytokine and other dual inflammatory response proteins, as well as some transcription factors that are precursors of antioxidant enzymes, could have a repercussion in the progression of retinal damage [36].

On the other hand, previous research such as those conducted by Pessoa et al. or Yang et al. suggests that the presence of IL-8 in the retina is the result of the degranulation of immune regulatory cells in the eye, [38, 39] as a consequence of the presence of TNF α that intervenes in the mechanism of the enzyme nitric oxide synthase (NOS), increases the production of nitric oxide (NO) and promotes oxidative stress, a precursor to the activation of these cells [40]. IL-8 is a cytokine that stimulates neovascularization; [41] in patients with proliferative DR, vitreous humor concentrations of 79.6 ± 9.7 mg/dL have been found, which exceed those found in cadaveric controls (19.0 ± 3.9 pg/mL) without a history of ocular or systemic disease [42]. This increase would be related to the proliferation of vessels in DR, but the detectable concentrations of IL-8 have also been contradictory.

In a study from Slovenia that evaluated 68 patients with T2DM and proliferative DR, where more than 80% were insulin-dependent, a mean of 358 ± 698 pg/mL of IL-8 was found, [43] which was similar to our mean result (346.18 ± 174.57), although the first had a higher statistical dispersion; furthermore, the duration of diabetes was similar to the reported in our study (19.02 ± 6.86 versus 17.26 ± 7.37 years), and subjects who had vitreous hemorrhage or macular detachment were included in the analysis. However, none of these reports evaluated the cytokine profile of antidiabetic treatments or other pharmacological therapies included in their studies.

In this sense, pharmacotherapy has demonstrated its effect on the progression of DR independently of glycemic control. In the review by Saw et al. (2019), [44] different antidiabetic therapies were addressed, such as metformin, whose cardioprotective effect has been widely described, so it is considered that its antiangiogenic and anti-inflammatory effects could also limit the microvascular alterations typical of DR [45]. Glibenclamide has also shown that it can prevent the progression of DR, although its effect is inferior to other sulfonylureas such as glycoside; [46] even when this drug is not considered among the first-line therapies in the recent years, it is commonly found in the therapeutic scheme of T2DM patients in our country. As part of the most common antidiabetic therapies, insulin, and its analogs are key to maintaining the normal functioning of the retinal microvasculature. Since a patient with T2DM usually has defects in the action of this hormone, the secretion of insulin-like growth factor 1 (IGF-1) decreases in the systemic blood circulation, affecting vascular homeostasis (including retinal microvasculature); therefore, it is considered that insulin and its analogs may have direct and indirect effects involved in the development of DR [47].

A 2023 study identified that both insulin and its analogs could be associated with an increased risk of developing clinically significant DR; however, these results should be taken with caution due to differences in the mechanisms of action of the drugs, and a possible “worsening effect” arising from the rapid reduction of serum glucose levels and the transient increase in inflammatory mediators and growth factors in response to changes in the environment [48].

In our study, the cytokine profile in the vitreous was analyzed and compared between the antidiabetic treatments commonly administered for T2DM, to evaluate the indirect therapeutic effect that some drug combinations can have on diseases for which they are not directed in the first place, which could serve to guide therapeutic indications in patients with T2DM who present microvascular complications in the retina. Still, as potential limitations, we did not consider body weight, body mass index (BMI), or other metabolic variables, and the specific effect of the types of insulin, or other therapies such as those administered in patients with SAH, which could modify the inflammatory response through additional signaling pathways that indirectly participate in the proliferative DR development. Even though the presence of molecules such as IL-8 and TNF α in both treatment groups suggests an ongoing pro-inflammatory response, it would be necessary to take these results with caution, because the sample sizes in the stratified groups (vitreous hemorrhage vs. retinal detachment) were small enough

to give us a full outlook of the possible indirect effects of the treatments.

Also, we did not include other immunoassays for the detection of cytokines that could have added value to this study; however, multiplex studies such as the one used, have demonstrated their reproducibility and usefulness for the simultaneous measurement of proteins even at low concentrations, with a broad cost-benefit advantage. The fact that some of the cytokines of interest, such as IL-1 β , IL-6, and IL-10, have not been detected, could be due to the duration of T2DM or the activity of each cytokine, but this will require further analysis, such as molecular biology techniques, to evaluate the gene expression that coded those proteins.

In summary, the concentration of TNF α in the vitreous is superior in patients receiving insulin treatment, and IL-8 is higher in the oral hypoglycemic drugs group of metformin + glibenclamide; however, in this interleukin, the concentrations did not reach a significant difference between the groups. Yet, this response is more pronounced in patients with vitreous hemorrhage, possibly due to the migration of cytokines from the retina. Until the last review of the literature, no similar reports were found that compared the cytokine profile in vitreous between different antidiabetic therapies, so these findings emphasize the impact that treatments can have on vascular and microvascular complications, and could expand the outlook for interdisciplinary management of preventive therapies in patients with retinal complications associated with T2DM.

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Author contributions

OML: Conceptualization, Methodology, Formal analysis, Validation, Visualization, Writing - Original draft; ORC: Visualization, Investigation, Formal analysis, Writing - Review & Editing; PLS: Validation, Investigation, Resources, Writing - Review & Editing; HJPC: Conceptualization, Visualization, Investigation, Formal analysis, Writing - Review & Editing; OGL: Validation, Investigation, Resources, Writing - Review & Editing; VLG: Methodology, Investigation, Formal analysis, Writing - Review & Editing; SASV: Conceptualization, Methodology, Formal analysis, Investigation, Validation, Visualization, Supervision, Project administration, Writing - Original draft, Writing - Review & Editing.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the institutional ethics committee of Hospital de la Luz (number 2019R18B1), and all the participants included signed informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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