Comparing the Impact of Serum GPER-1 and Oxidant/Antioxidant Levels on Retinopathy in Diabetic Patients and Healthy Individuals: A Pilot Study

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Abstract

Aims

This research sought to determine the impact of serum G receptor-mediated protein-1 (GPER-1) levels on the development of retinopathy in diabetic patients, comparing them to healthy individuals.

Methods

Forty patients with diabetic retinopathy (DR) (Group 1), 40 patients without DR (NDR) (Group 2) and 40 healthy individuals (Group 3) were included in this study. Serum progesterone, GPER-1, oestradiol, oxidant/antioxidant and thyroid-releasing hormone (TSH) levels were analysed and compared among the groups. Post hoc analysis was performed to compare the sub-groups in which statistically significant differences were found.

Results

A significant difference was found among all groups in terms of GPER-1, oxidant/antioxidant and oestradiol levels (p < 0.01), but no significant difference was found in terms of TSH or progesterone (p = 0.496, p = 0.220, respectively). In the post hoc analysis of the groups with statistically significant differences, another significant difference was found among all groups for GPER-1 and oxidant/antioxidant levels (p < 0.05). GPER-1 and oxidant levels were positively correlated, while GPER-1 and antioxidant levels were negatively correlated (r = 0.622/p < 0.01, r = 0.453/p < 0.01, r = 0.460/p < 0.01, respectively). The multiple regression analysis showed that increased GPER-1 may help prevent DR.

Conclusions

GPER-1 levels, which were highest in the DR group, increased as the oxidant/antioxidant balance changed in favour of oxidative stress. This seems to be a defence mechanism for preventing neuronal damage.

Introduction

Diabetic retinopathy (DR) is among the most common complications of diabetes; it can present with symptoms such as exudate, haemorrhage, macular oedema, microangiopathy, and neovascularisation.[1] Worldwide, it is also the most common preventable cause of blindness.[2] DR is diagnosed by clinical signs of vascular pathologies in the retina,[1] and it is divided into two main clinical stages: proliferative DR (PDR) and non-proliferative DR (NPDR).[3] Though pathologies, such as microaneurysms, haemorrhages, and exudate, are common during the NPDR stage, patients may be asymptomatic. In the PDR stage, however, serious outcomes, such as intravitreal haemorrhages or tractional retinal
detachment due to neovascularisation, may occur.[1, 3–4] DR aetiology involves many complex mechanisms. These include retinal ischemia (formed due to vascular pericytes and endothelial damage), glial cell dysfunction (caused by increased inflammatory mediators) and homeostasis disruption (due to higher levels of free reactive oxygen species (ROS), which increase as the oxidant/antioxidant balance changes).[5–7]

GPER-1 is a transmembrane oestrogen receptor, localised in the endoplasmic reticulum (ER), which binds with high affinity to 17β-oestradiol.[8–9] It is present in various areas of the body, including the reproductive, nervous, endocrine, immune and cardiovascular systems.[10] GPER-1 expression has also been found in the retina.[11]

Oxidative stress deteriorates the homeostatic balance, causing protein damage and, thus, stressing the ER. This damages cells and causes them to enter apoptosis.[12] However, prior research has shown that GPER-1 activation causes ROS to decrease, which rapidly reduces the stress being placed on the ER.[10, 12]

Currently, various treatment methods (e.g., anti-vascular endothelial growth factor [anti-VEGF], steroid and laser therapies) are used to reduce the oxidation and inflammatory mediators of DR pathogenesis.[6, 13] However, retinal neurodegeneration could be an independent pathophysiological component of DR, and new molecular mechanisms should be investigated to detect early damage and initiate treatment.[1, 14] Upon reviewing the extant literature, the present researchers found no studies examining oxidative stress and GPER-1 levels in diabetic patients as compared to healthy participants.

Therefore, this research compared the serum GPER-1 levels and oxidative stress markers (malondialdehyde [MDA], catalase [CAT] and superoxide dismutase [SOD]) in diabetic patients and healthy individuals.

Materials And Methods

Study design and participants

This prospective study included 80 patients with type 2 diabetes (40 with DR [Group 1] and 40 without DR [NDR; Group 2]), as well as a control group of 40 healthy individuals (Group 3). All participants signed written informed consent forms. The study was performed in compliance with the ethical principles of the Declaration of Helsinki, and approval for the research was obtained from the Local Ethics Committee (Protocol number: 03-2018/20 Date: 7 November 2018).

To be included in this study, 40 individuals were required to have DR; 40 were required to have NDR; and 40 were required to be healthy, without any systemic disease. People were excluded from the study if they had glaucoma, ocular trauma sequelae, pathological myopia, non-diabetic retinopathy, a history of previous ocular surgery, endocrine disorders (e.g., thyroidopathy, adrenal gland disorders, pituitary pathologies), and opacities interfering with fundus examination (e.g., corneal opacity, lens opacity,
vitreous cloudiness other than diabetic haemorrhage). Those who had received hormone replacement therapy or were addicted to alcohol and/or controlled substances were also excluded, as were premenopausal women (to avoid gender-related complexities). Fasting venous blood samples were taken from the sampled participants, following a complete ophthalmological examination. In the serum obtained, GPER-1, MDA, CAT, SOD, oestradiol, TSH, and progesterone levels were examined, and comparisons were drawn among the groups.

**Preparation of blood samples**

Fasting blood samples (5ml) were taken from the participants’ median cubital veins. Circadian variations were avoided by always drawing samples between 8:00 am and 9:00 am.

**Serum**

The blood samples were immediately centrifuged at 2,000rpm for 10min at 4°C. Supematant serum was separately stored at 80°C before enzyme-linked immunosorbent assay (ELISA) was used to measure serum oestrogen, progesterone, and GPER-1 levels.

**Biochemical analysis**

Serum TSH, progesterone, GPER-1 and oestradiol levels were measured using a quantitative sandwich ELISA method via a commercial kit (SEG 045 Hu, Cloud-Clone Corp., Houston, TX, USA) according to the manufacturer’s instructions.

SOD activity was measured in the samples according to the method described by Fridovich.[15] This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye, which is measured at 505nm. The assay medium contained 0.01M phosphate buffer; a 3-cyclohexilamino-1-propanesulfonic acid (CAPS) buffer solution (50mM CAPS, 0.94mM ethylenediaminetetraacetic acid [EDTA], and saturated sodium hydroxide [NaOH]) with a pH of 10.2; a substrate solution (0.05mM xanthine, 0.025mM INT); and 80UL xanthine oxidase. SOD activity was expressed as U/mg protein.

CAT activity was determined by measuring the decrease in hydrogen peroxide concentration at 230nm via Beutler's method.[16] The assay medium contained 1M Tris hydrochloride (HCl), 5mM disodium (Na₂) EDTA buffer solution (pH 8.0), 10mM hydrogen peroxide (H₂O₂), and a blood sample, creating a final volume of 1.0ml.

MDA levels in the samples were measured with the thiobarbituric acid (TBA) test.[17] Each reaction mixture contained 0.1ml of a blood sample, 0.2ml of 8.1% sodium dodecyl sulphate (SDS), 1.5ml of 20% acetic acid and 1.5ml of 0.8% TBA aqueous solution. The pH of the mixture was adjusted to 3.5, and the volume was increased to 4.0ml with distilled water. Then, 5.0ml of an n-butanol and pyridine (15:1, v/v) mixture was added. The final reaction mixture was shaken vigorously. After centrifugation at 4,000rpm for 10min, the absorbance of the organic layer was measured at 532nm.

**Statistical analysis**
The data obtained were statistically analysed using the Statistical Package for the Social Sciences (SPSS) for Windows, version 22.0. The data's conformity to normal distribution was assessed via the Shapiro–Wilk test. The categorial data were analysed using the chi-squared test. An analysis of variance (ANOVA) was performed to compare the groups. The data in these group comparisons were normally distributed. Post hoc analysis was performed to determine the difference between the significant variables. Continuous data were expressed as mean ± standard deviation (SD) values, and the categorial data were presented as numbers (n) and percentages (%). The results were considered statistically significant at p < 0.05.

Results

Table 1 presents the demographic data for the groups, as well as information concerning the participants’ duration of diabetes. No significant differences were found between the groups in terms of age or sex (p=0.527, p=0.699, respectively). However, a significant difference was found for diabetes duration (p<0.01), and the DR group had the longest diabetes duration.

A significant difference was also found among all groups in terms of serum GPER-1, MDA, CAT, SOD, and oestradiol (p <0.01), though there was no significant difference among the groups in terms of progesterone and TSH (p=0.496, p=0.220, respectively). In the post hoc analysis of parameters with statistical differences, a significant difference was found between GPER-1, MDA, CAT, and SOD in all groups, and a significant difference for oestradiol was determined between Group 1 and Group 3 (p<0.01) (Table 2).

When correlation analysis was performed between GPER-1 and oxidant/antioxidant levels, they correlated positively with MDA and negatively with SOD and CAT (r=0.622/p<0.01, r=0.453/p<0.01, r=0.460/p<0.01, respectively) (Figure 1).

Multivariate regression analysis was performed to assess the researchers’ model, which considers GPER-1 (a possible retinopathy preventative) alongside age, sex and diabetes duration (factors known to influence DR development). This analysis determined that GPER-1 and diabetes duration were statistically significant factors in DR pathogenesis (Table 3).

Discussion

The existing treatment strategies for DR, including intravitreal pharmacological agents, laser photocoagulation and vitreous surgery, aim to manage microvascular complications. However, inadequate response to these treatments indicates the presence of other underlying mechanisms.[5–7] A growing body of laboratory and clinical evidence suggests that inflammation and retinal neurodegeneration may play a role as independent pathogenesis pathways in DR.[1, 6]

Retinal neurodegeneration can occur even without DR development.[1] In diabetic animals, an increase in proapoptotic molecules triggers apoptosis in neurons, which leads to retinal thinning before DR
development. It has also been suggested that neuronal degeneration significantly increases with the formation of mitochondrial damage and oxidative stress due to high glucose exposure. Thus, neuronal damage may be reduced by suppressing oxidative stress.

GPER-1 is a rapidly acting, membrane-bound oestrogen receptor, independent of gene regulation. Though GPER-1 has been reported to mediate more than one type of oestrogen activity in vivo, growing evidence indicates that GPER-1 also has gender-independent effects. Its presence has been demonstrated in various areas of the body (e.g., the reproductive, nervous, endocrine, immune and cardiovascular systems) and in the retina. Evidence has been reported that GPER-1 plays a crucial role in metabolic regulation (lipids and glucose homeostasis, insulin production and action, etc.), blood pressure, and immune functions. Previous studies indicate that GPER-1 activation increases the efficiency of glucose transporters, reduces oxidative stress products, and regulates the inflammatory response; thus, it plays a neuroprotective role.

In diabetic patients, the oxidant/antioxidant balance changes in favour of oxidation due to increased oxidative stress in the body. Tawk et al. reveal that increased oxidative stress impairs blood–retinal barrier function and induces apoptosis in the retinal neurons and glial cells. As a vicious circle, ROS increase as diabetes duration increases and as diabetes complications develop. In the current study, a significant difference was found between Groups 1, 2 and 3 regarding GPER-1 and ROS. The presence of diabetes and the risk of developing complications increase as ROS levels increase. At the same time, serum GPER-1 levels also increase. Therefore, the present researchers assert that this rise in GPER-1 occurs in response to higher ROS levels.

Mancino et al. show that mitochondrial ROS production increases in hyperglycaemia and suggest that mitochondrial abnormalities lead to irreversible consequences. They demonstrate that such mitochondrial ROS production continues even if a patient shifts to normoglycemia. This phenomenon has been termed the ‘metabolic memory’ or ‘inheritance effect’. Via in vitro experiments, Wu et al. show that retinal pericytes, which have been exposed to hyperglycaemia, continue to overproduce ROS, even in a normoglycemic environment. This situation supports the abovementioned results. In the current study, the highest ROS levels were found in individuals with DR, evidencing an increased risk of complications. GPER-1 levels also increased to protect against ROS formation and consequences. This finding is supported by the positive correlation between SOD and CAT and GPER-1 and the negative correlation between MDA and GPER-1 demonstrated in this research.

The mean oestradiol level in the diabetic group differed significantly from that in the control group. The current authors believe that the GPER-1 levels were higher in diabetic patients than in the healthy controls (being highest in individuals with DR) indicates that it is produced to prevent cellular damage and apoptosis. They are also of the opinion that GPER-1 is upregulated as oestrogen levels decrease in individuals with diabetes.

Retinal neurodegeneration begins before clinical retinopathy develops, and it is crucial to take earlier measures to prevent this progression. In this study, serum GPER-1 levels began rising before DR
development and reached their highest amount in patients with DR, indicating that GPER-1 is produced in response to ROS increase. This could be an important finding for preventing neurodegeneration. Han et al. [23] demonstrate that G-1, which is a GPER-1 agonist, increases the viability of microglia in neuronal damage. It has also been revealed that this beneficial effect on microglia is reduced with G-15, which is a GPER-1 antagonist. Rong et al. [10] suggest that activating GPER-1 reduces ROS production by decreasing mitochondrial damage and, thus, increasing neuronal survival.

This study does have several limitations. For instance, to minimise hormonal differences between the studied male and female individuals, the researchers did not include postmenopausal women in the sample. They also did not measure the amounts of serum oxidative stress molecules in the participants’ blood samples by interfering with GPER-1 agonist agents, such as G-1. Nevertheless, this is the first research to reveal the correlation between serum GPER-1 levels and oxidative stress molecules in DR patients. It will, therefore, light the way for further studies and novel treatments.

In conclusion, GPER-1 appears to be a remarkable receptor in following up diabetic patients’ disease progression, detecting DR at an early stage and preventing neuronal damage. Unlike natural oestrogen or conjugated oestrogens, applying GPER-1-specific medications in G-1-like retinal cells devoid of other endocrinological effects could be a potential therapy for early DR intervention and for unresponsive DR in combination with existing treatments. To explore this possibility, further studies, with larger samples, are needed.

**Declarations**

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**Data availability statement:** Data are available upon request.

**Code Availability:** Not applicable

**Authors’ contributions:** AB is the lead author and was responsible for the conception of the research idea. EBK conducted biochemical analysis and recorded results. AB, YK, AÇ and AM have contributed to the literature review, data collection, analysis of results and formulation of the manuscript. All authors read and approved the final manuscript.

**Compliance with ethical standards:** Local Ethics Committee (Kahramanmaras Sutcu Imam University) (decision no: 285, dated: 24-2018 / 12)

**Ethical Standards:** All the procedures applied to human participants in this study were compatible with the ethical standards of the national research committee and the 1964 Helsinki Declaration and its later amendments.
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Tables

Table 1: Demographic and clinical characteristic of groups

| Characteristic | Group 1 (n₁:40) | Group 2 (n₂:40) | Group 3 (n₃:40) | p value* |
|---------------|----------------|----------------|----------------|----------|
| Age (years)   | 57.47±6.40     | 56.02±6.79     | 55.75±8.52     | 0.527*   |
| Gender        | 19/21          | 21/19          | 20/20          |          |
| (Male/Female) | 47.5% / 52.5%  | 52.5% / 47.5%  | 50.0% / 50.0%  | 0.699*   |
| DM (years)    | 11.82±5.95     | 6.1±2.47       | N              | <0.01*   |

n₁: Number of Diabetic Retinopathy subject.
$n_2$: Number of Non-Diabetic Retinopathy subject.
$n_3$: Number of Control subject.
DM; Diabetus Mellitus, SD; Standard deviation.
*Chi-square test.
$P$ value of $<0.05$ was considered as significant, shown in bold.

**Table 2:** Comparison of groups in terms of GPER-1, hormones and oxidative markers.

|                      | Group 1\(^a\) | Group 2\(^b\) | Group 3\(^c\) | $P$ value |
|----------------------|---------------|---------------|---------------|-----------|
| GPER-1 (ng/ml)       | 0.501±0.09\(^b,c\) | 0.455±0.06\(^a,c\) | 0.379±0.03\(^a,b\) | $<0.01$   |
| Estradiol (ng/ml)    | 56.27±5.85\(^c\) | 58.27±5.14       | 62.65±11.84\(^a\)  | 0.02      |
| Progesterone (ng/ml) | 11.56±1.12     | 11.66±0.77       | 11.92±0.83       | 0.220     |
| TSH (ng/ml)          | 1.29±0.10      | 1.28±0.06        | 1.27±0.09        | 0.496     |
| MDA (nmol/mgprt)     | 4.97±1.38\(^b,c\) | 2.97±0.56\(^a,c\) | 1.92±0.53\(^a,b\) | $<0.01$   |
| CAT (u/mgprt)        | 93.09±5.98\(^b,c\) | 122.25±9.91\(^a,c\) | 174.82±11.33\(^a,b\) | $<0.01$   |
| SOD (u/mgprt)        | 0.79±0.11\(^b,c\) | 1.16±0.19\(^a,c\) | 1.55±0.28\(^a,b\)  | $<0.01$   |

$n_1/n_2/n_3$: Number of groups subject.
GPER-1; G-protein coupled estrogen receptor-1, TSH; Thyroid stimulating hormone, MDA; Malondialdehyde, CAT; Catalase, SOD; Superoxide dismutase, SD; Standard deviation.
Groups were coded with the letters a, b, c. One-way ANOVA test was used to compare the means. Tamhane's T2 post-hoc analysis was performed in paired comparison. Statistical significance was $p<0.05$, shown in bold. In paired comparisons, the letters shown as superscript indicate the group in which there is a significant difference between them.

**Table 3:** Multivariate logistic regression analysis in the model created with GPER-1, age, gender and duration of diabetes.
|                          | P value | OR  | 95% Cl          |
|--------------------------|---------|-----|-----------------|
| **Age (years)**          | 0.418   | 0.962 | 0.875-1.057    |
| **Gender** (Male/Female) | 0.423   | 0.633 | 0.207-1.937    |
| **DM (years)**           | <0.01*  | 1.531 | 1.284-1.825    |
| **GPER-1 (ng/ml)**       | 0.045*  | 1.023 | 1.012-1.943    |

DM; Diabetus mellitus GPER-1; G-protein coupled estrogen receptor-1

*P value of <0.05 was considered as significant.

*Shown in bold

**Figures**
Figure 1

Showing the correlation of GPER-1 with MDA, CAT and SOD.