• Clinical Research •

Plasma and aqueous humor levels of adiponutrin and pannexin 1 in patients with and without diabetic retinopathy

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Abstract

● **AIM:** To evaluate plasma and aqueous levels of adiponutrin and pannexin 1 in patients with and without diabetic retinopathy.

● **METHODS:** The study included three age and gender-matched groups of 20 cataract patients with no diabetes or additional disease (Group C), 20 cataract patients with diabetes and no retinopathy (Group DM+C), and 20 cataract patients with diabetic retinopathy (Group DR+C). All the patients were examined with respect to body mass index (BMI), fasting plasma glucose, hemoglobin A1c (HbA1c), and lipid profile. Phacoemulsification and intraocular lens (Phaco+IOL) implantation were performed to all patients in all the groups, and aqueous samples were taken during the operation. The plasma and aqueous adiponutrin and pannexin 1 levels were analyzed using enzyme-linked immunosorbent assays.

● **RESULTS:** A statistically significant difference was determined between the groups with respect to BMI, fasting plasma glucose, and HbA1c levels (P<0.05 for all parameters tested). The plasma adiponutrin levels of Group DR+C were statistically significantly lower than those of Group C and Group DM+C (P<0.001, P=0.004). No statistically significant difference was determined in the aqueous adiponutrin levels in three groups. The plasma pannexin 1 levels of Groups DM+C and DR+C were statistically significantly lower than those of Group C (both P=0.001). The aqueous pannexin 1 levels of Group DR+C were statistically significantly higher than those of Group C and Group DM+C (P=0.001, P<0.001).

● **CONCLUSION:** Adiponutrin and pannexin 1, which play an important role in the pathophysiology of diabetes and obesity, and have a regulatory role in hyperglycemia and insulin resistance. The measurement of adiponutrin and pannexin 1 levels may support clinicians in determining the risk of DR development.

● **KEYWORDS:** diabetes mellitus; retinopathy; adiponutrin; pannexin 1

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INTRODUCTION

Diabetes mellitus (DM) is a chronic, systemic disease that develops as a result of insulin deficiency or insulin resistance, and progresses with hyperglycemia. With an impairment in the physiological mechanisms responsible for the regulation of glucose metabolism, diabetes and complications develop. All the micro and macrovascular systems may be affected, primarily the eyes, kidneys heart, and peripheral nerves. Diabetic retinopathy (DR) is a classic example of a microvasculopathy triggered by hyperglycemia. DR is the most common microvascular complication in diabetic patients and one of the main causes of acquired blindness in the world[1]. Dysfunction, death and insufficient regeneration occurring in capillary endothelial cells and pericytes in endothelial and vascular smooth muscle cells induced by hyperglycemia are thought to play an important role in the pathogenesis of diabetic microvasculopathy[2]. Although DR is defined as an angiopathy seen with leakage in the retinal vessels, the pathophysiology of the diseasee has not been fully clarified. The disease starts with a non-proliferative phase, progress to a proliferative phase correlated to the severity of the disease and can result in severe vision

loss. Diabetic microvascular complications are related to the severity and duration of the hyperglycemia. Studies of experimental models of diabetic control and complications have shown that DM-related complications are decreased with strict glucose control[3-4].

In recent years there has been increasing interest in adipokines originating from adipose tissue, because of the effects on glucose and energy metabolism.

Adipose tissue plays a key role in metabolic hemostasis by storing energy, and functioning as an endocrine organ. Energy is stored as triacylglycerol in subcutaneous and visceral fat tissue in the abdominal region. Insulin in healthy adipose tissue increases lipogenesis and the intake of glucose to fat tissue, and decreases lipolysis, but when insulin resistance develops, these effects cannot be seen[5]. Fat tissue has an effect on metabolism, energy expenditure and insulin sensitivity by expressing many endocrine and paracrine factors (*e.g.,* leptin and adiponectin), which are known as adipokines[6].

Adiponutrin gene (*ADPN*) expression, an adipokine thought to have an effect on glucose metabolism, was first detected from differentiated 3T3L-1 adipocytes and adipose tissues of mice during feeding[7]. Adiponutrin is a member of the patatine-like phospholipase family and is strongly associated with membranes and lipid droplets[8-9]. Defined as an intracellular molecule, the plasma level of adponutrin was first measured by Winberg *et al*[10] in 2014. Genetic studies have reported that adiponutrin on human 22q13 chromosome is a marker related to type 2 diabetes and insulin levels, and that adiponutrin gene (7q34) is associated with obesity in rats[11-12]. Adiponutrin is a key regulator of lipid droplets in hepatocytes and regulated by insulin and/or glucose in healty humans[13]. In patients with type 2 diabetes, adiponutrin levels have been determined to be low correlated with leptin and adiponectin levels associated with insulin sensitivity and resistance[13-14].

ADPN has been reported to be expressed in the retina in humans[15]. A high hyperglycemic level has been reported to reduce *ADPN* levels when there is reduced insulin sensitivity or insulin resistance, and in those with high insulin sensitivity, *ADPN* levels are high[16].

Another molecule thought to have an effect on glucose metabolism is pannexin 1, which is a member of the pannexin family and is a molecule formed from cysteine and containing 3 transmembrane protein[17]. Pannexin 1 channels are located in the cell membrane, and when activated, transfer ATP, nucleotides, and molecules up to 1 kd, to the intracellular area. Mechanostretching provides irreversible opening of the channel through A1 adrenergic/histamine stimulation, oxygen glucose deprivation, and caspase-mediated separation of the C terminal section[18].

Pannexin 1 channels have a role in the expression of nucleotides from adipocytes to the extracellular area, and are known to be very important in the insulin-mediated glucose uptake of ATP expression linked to pannexin in adipocytes, and insulin has been determined to be an important activator of pannexin 1 channels[19]. Pannexin 1 regulates insulin-stimulated glucose uptake in adipocytes and thus contributes to control of metabolic homeostasis. Mice with genetic deletion of *Panx1* in adipocytes experience exacerbated insulin resistance[19].

Pannexin 1 has a role in processes such as cell proliferation, differentiation, migration, wound healing, inflammation, cytokine expression, ischaemia, and blood flow regulation[20].

In an extensive literature scan, no study could be found showing a relationship between adiponutrin and pannexin, and DR. Adiponutrin and pannexin 1 are involved in the regulation of carbohydrate metabolism. And then again, as remembered disorder of carbohydrate metabolism is generally considered the primary culprit in the development and progression of DR. So, these proteins are a logical culprit to consider in DR. Therefore, the aim of this study was to compare the plasma and aqueous levels of adiponutrin and pannexin 1, which were thought to have an effect on DR, in patients with DR, patients with diabetes but no retinopathy, and those with neither diabetes nor retinopathy, and to thereby be able to determine the potential role of these molecules in the etiopathogenesis of DR.

SUBJECTS AND METHODS

Ethical Approval The study was performed in adherence with the tenets of the Declaration of Helsinki and was approved as a prospective study by the Ethics Committee of Firat University Faculty of Medicine (approval No.2020/10-16). Informed consent was obtained from all patients.

The study included patients who presented at the Ophthalmology Polyclinic of Elazig Health Sciences University because of reduced vision, were diagnosed with cataract (grade 2-3 nuclear cataract) after a detailed ophthalmological examination. All of the participants underwent a screening process with a complete ophthalmological examination (including evaluation of refraction and visual acuity, slit-lamp biomicroscopy, intraocular pressure measurement using Goldmann applanation tonometry, and a fundus examination. Patients who had glaucoma, corneal disease, uveitis, previous laser treatments, ocular trauma, or surgery before and during the follow-up period were excluded from the study) and underwent cataract surgery. Three groups were formed of age and gender-matched patients as a control group of 20 cataract patients without diabetes (Group C), 20 cataract patients with diabetes and without diabetic retinopathy (Group DM+C), and 20 cataract patients with diabetes and DR (proliferative retinopathy; Group DR+C).

Collection of Biological Samples After an 8-12h fasting period, a 10 mL blood sample was taken from each patient in the morning into a tube containing aprotinin (BD Vacutainer SST II Advance, BD, Plymouth, UK).

In all patients, body mass index (BMI, kg/m^2), fasting plasma glucose (FPG), HbA1c, and lipid profile (LDL, HDL, triglycerides) were examined. The collection, storage, and preservation of these samples were previously described[21]. The obtained blood samples were centrifuged at 4000 rpm for 10min, and the plasma obtained was placed in small volume tubes and stored at -80℃ until assay of adiponutrin and pannexin 1. All the patients in the study group were performed phacoemulsification+intraocular lens (Phaco+IOL) implantation. During the cataract operation, aqueous samples were taken and stored at -80℃ until assay.

Surgical Method Phacoemulsification was used throughout this study as described previously[22]. Thirty minutes before the operation, alprazolam (0.5 mg) was orally administered for sedation of the patient. Topical cyclopentolate (1%), tropicamide (0.5%), and phenylephrine (2.5%) were used for pupil dilation. For local anesthesia, topical 0.5% propakain HCl dropped on corneal and conjunctival surface. The cornea was incised at the 9 o'clock position with a 20-G MVR knife and aqueous samples taken from this incision from the anterior chamber. In addition another corneal incision was made at 1 o'clock. Viscoelastic material was inserted. At the 11 o'clock position, a corneal incision was made using a number 3 knife. The lens was emulsified with a balanced salt solution (BSS), followed by the horizontal chop method with hydrodissection and hydrodelineation. The remaining lens material was removed by manual irrigation and aspiration (I/A) of the cannula. A foldable IOL was installed using a cartridge system. The viscoelastic material inserted into the anterior chamber was removed using the manual I/A method. The incision site was closed with stromal hydration, and any wound leakage was controlled.

Biochemical Analyses of Biological Samples Plasma adiponutrin and pannexin 1 levels were examined using the human adiponutrin, pannexin 1 ELISA kit (Sunred Biological Technology, Shanghai, China) in a plate-washing-incubation CombiWash device (Human Diagnostics, Wiesbaden, Germany) in accordance with the study procedures determined in the kit catalogue, and the absorbance measurement was taken with a Chromate 4300 Microplate Reader (Awareness Technology, Palm City, USA).

Aqueous analyzes were performed according to a previously published methods[21]. Two aqueous liquids and blood samples were enriched with increasing amounts of adiponutrin or pannexin 1. The percentage recovery was calculated as follows: recovered value/expected value ×100.

The measurement range of the human adiponutrin kit was 0.8 to 20 ng/mL and the sensitivity was determined by the manufacturer at 0.072 ng/mL. The intra-assay and inter-assay coefficients of variation for adiponutrin were <10% and <12%, respectively. The measurement range of the human pannexin 1 kit 0.05 to 15 ng/mL and the sensitivity was the determined by the manufacturer at 0.045 ng/mL. The intra-assay and inter-assay coefficients of variation for pannexin 1 were <10% and <12%, respectively.

Assay Validation of Kits for Aqueous Fluids Aqueous assay validation was performed according to a previously published method by Aydin[21], as was briefly described below. Linearity: Two aqueous liquids and blood samples were diluted (1/2, 1/4, 1/8) with distilled water in order to find the adiponutrin and pannexin 1 linearity. Recovery: Two aqueous liquids and blood samples were enriched with pure amounts of adiponutrin and pannexin 1. The percentage recovery was calculated as follows: recovered value/expected value ×100. The coefficient of variation (CV): The intra-assay (within-day) and inter-assay variation (between days) were determined for two different aqueous liquids and blood samples using the means of 2-3 replicates of each. The CV is calculated as: CV=standard deviation (SD)/mean concentration.

Statistical Analysis Data obtained in the study were analysed statistically using the Statistical Package for the Social Sciences (SPSS) version 22.0 software (SPSS Inc., Chicago, IL, USA). To determine the signifiance of the difference between the groups in respect of age, gender, FPG, HbA1c, lipid profile, plasma and aqueous adiponutrin and pannexin 1 levels, the Mann-Whitney *U* test was applied. A value of $P<0.05$ was accepted as statistically significant.

RESULTS

The validation of the kits we used has been made in our laboratory. Results of the linearity of used kits in biological samples were summarized in Table 1. Table 2 indicated recovery assay results of kits used through this study. Intra assay values were calculated as 9.4% and 12.2% for adiponutrin and pannexin 1 in our laboratory, while inter assay values were recorded as 12.8% and 15.2% for adiponutrin and pannexin 1, respectively, in our laboratory (Tables 1 and 2).

The Group C was formed of 20 cataract patients with no diabetes and no retinopathy, comprising 11 females (55%) and 9 (45%) males with a mean age of 65.6±3.26y. The Group DM+C was formed of 20 cataract patients with diabetes and no retinopathy, comprising 10 females (50%) and 10 (50%) males with a mean age of 64.75±2.42y. The Group DR+C was formed of 20 cataract patients with DR, comprising 8 females (40%) and 12 (60%) males with a mean age of 64.35±2.73y. No statistically significant difference was determined between the groups in respect of age and gender (all $P>0.05$; Table 3).

Table 1 Linearity of kits in biological samples used in this study

ng/mL (%)

Samples	Undiluted	1/2	1/4	1/8
Adiponutrin				
Aqueous-1	5.55	4.82 (86.8)	4.88 (87.9)	5.94 (107)
Aqueous-2	6.78	5.43 (80)	6.66 (98.2)	6.68 (98.5)
Blood-1	2.80	3.2 (114)	2.76 (98.5)	2.76 (98.5)
Blood-2	2.96	2.78 (93.9)	3.2 (108)	2.84 (95.9)
Pannexin 1				
Aqueous-1	1.51	1.42 (94)	1.62 (107)	1.48 (98)
Aqueous-2	1.61	1.56 (96.8)	1.46 (90.6)	1.56 (96.8)
Blood-1	0.76	0.82 (107)	0.66 (86.8)	0.74 (97.3)
Blood-2	0.80	0.88 (110)	0.82 (102)	0.92 (115)

Table 2 Recovery assay of kits in biological samples used in this study

ng/mL

Samples	Initial concentration	Added	Recovered	Expected	Recovery (%)
Adiponutrin					
Aqueous-1	6.76	12	21.2	18.76	113.0
Aqueous-2	8.80	12	18.9	20.80	90.8
Blood-1	0.99	12	14.2	12.99	109.3
Blood-2	1.11	12	12.94	13.11	98.7
Pannexin 1					
Aqueous-1	0.93	4	5.4	4.93	109.5
Aqueous-2	0.79	4	4.92	4.79	102.7
Blood-1	0.40	4	4.48	4.40	101.8
Blood-2	0.51	4	4.62	4.51	102.4

Table 3 Demographic characteristics of the patients

Characteristics	Group C	Group DM+C	Group DR+C
Age (y)	65.6±3.26	64.75±2.42	64.35±2.73
FPG (mg/dL)	92.7±7.14	157.1±21.24[a]	172.35±14.58[b]
HbA1c (%)	5.54±0.26	7.03±0.23[a]	8.32±0.39[b]
BMI (kg/m^2)	25.07±1.79	32.11±1.39[a]	34.84±2.40[b]
HDL (mg/dL)	47.64±2.43	40.38±1.33[a]	39.38±1.83[b]
LDL (mg/dL)	128.19±5.93	156.84±7.21[a]	165.33±7.51[b]
Triglyceride (mg/dL)	169.79±5.66	177.08±5.36[a]	190.06±5.38[b]

C: Cataract; DM: Diabetes mellitus; DR: Diabetic retinopathy; BMI: Body mass index; FPG: Fasting plasma glucose; HbA1c: Hemoglobin A1c; HDL: High density lipoprotein; LDL: Low density lipoprotein. [a]P<0.05 compared with Group C (Mann-Whitney U), [b]P<0.05 compared with Group C and Group DM+C (Mann-Whitney U).

The plasma adiponutrin levels were determined as 2.61±1.8 ng/mL in Group C, 2.69±2.61 ng/mL in Group DM+C, and 1.21±0.7 ng/mL in Group DR+C. The plasma adiponutrin levels of Group C and DM+C were statistically significantly higher than those of Group DR+C (P<0.001, P=0.004) and the difference between Groups C and DM+C was not statistically significant (P=0.189; Figure 1).

The aqueous adiponutrin levels were determined as 2.54±0.69 ng/mL in Group C, 2.49±0.89 ng/mL in Group DM+C, and 3.02±1.73 ng/mL in Group DR+C. No statistically significant difference was determined of the aqueous adiponutrin levels in three groups (all P>0.05; Figure 1).

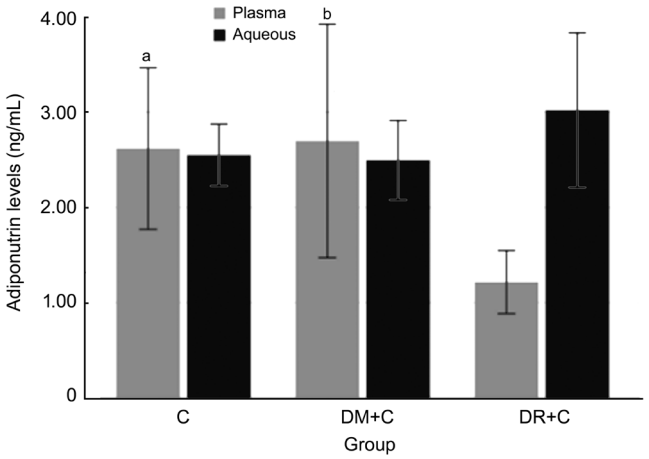

Figure 1 Adiponutrin levels in the aqueous humor and blood of patients with DR+C, DM+C and C [a]P<0.001, [b]P=0.004 *vs* Group DR+C plasma.

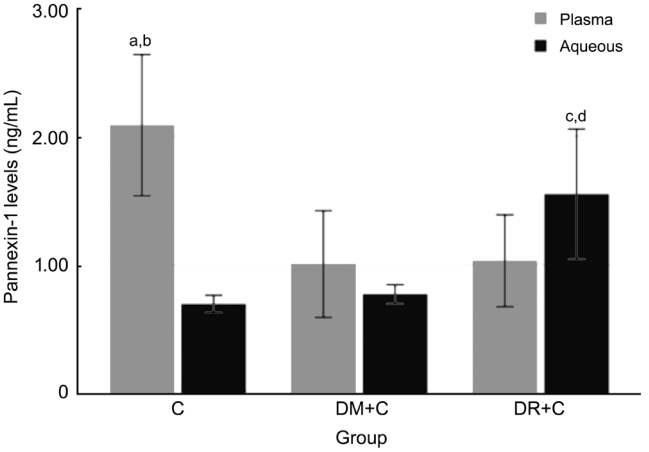

Figure 2 Pannexin 1 levels in the aqueous humor and blood of patients with DR+C, DM+C and C [a]P=0.001 *vs* Group DM+C plasma, [b]P=0.001 *vs* Group DR+C plasma, [c]P<0.001 *vs* Group C aqueous, [d]P=0.001 *vs* Group DM+C aqueous.

The plasma pannexin 1 levels were determined as 2.08±1.17 ng/mL in Group C, 0.98±0.65 ng/mL in Group DM+C, and 1.05±0.91 ng/mL in Group DR+C. The plasma pannexin 1 levels of Groups DM+C and DR+C were statistically significantly lower than those of Group C (both P=0.001) and the difference between Groups DR+C and DM+C was not statistically significant (P=0.417; Figure 2).

The aqueous pannexin 1 levels were determined as 0.69±0.14 ng/mL in Group C, 0.78±0.17 ng/mL in Group DM+C, and 1.54±1.08 ng/mL in Group DR+C. The aqueous pannexin 1 levels of Group DR+C were statistically significantly higher than those of Group C and Group DM+C (P<0.001, P=0.001) and the difference between Groups C and DM+C was not statistically significant (P=0.088; Figure 2).

DISCUSSION

The results of this study showed that the plasma adiponutrin levels of the Group DR+C were statistically significantly lower than those of Groups C and DM+C (P<0.001, P=0.004). It has been reported that adiponutrin levels decrease in cases of high glycemic level, decreased insulin sensitivity, or insulin resistance; while adiponutrin levels are high in those with high insulin sensitivity[16]. Adiponutrin is a molecule expressed from the liver and adipose tissue, which has both lipogenic and lipolytic effects[23].

Adiponutrin hepatic expression in humans is controlled by the nutritional status. In a fasting status responding to glucose-dependent insulin levels, expression is decreased, and with food intake, expression is stimulated[24]. Faraj *et al*[14] determined low adiponutrin levels in basal and insulin stimulating conditions in type 2 diabetic patients. In addition, adiponutrin levels have been determined to be low in rats with defective insulin and insulin receptors[25].

In cases with insulin resistance despite high insulin levels, adiponutrin levels have been determined to be low[24]. In patients with type 2 diabetes, adiponutrin levels have been determined to be low correlated with leptin and adiponectin levels associated with insulin sensitivity and resistance[14]. Previous studies have shown that insulin must be present effectively in the environment for the effectiveness of adiponutrin. In the current study, the adiponutrin levels were determined to be significantly low in the DR+C group. This significantly low level of adiponutrin in the DR+C group is an expected finding in patients with type 2 diabetes, characterised especially by insulin deficiency and resistance. This suggests that at advanced stages of the disease, as the insulin level decreases and insulin resistance increases, the adiponutrin efficacy and levels will gradually decrease. With the removal of the physiological effects of adiponutrin in insulin and glucose metabolism, it is thought that patients become more predisposed to the complications of diabetes.

Adiponutrin has been reported to be expressed in the human retina[15]. However, in the comparison of the three groups in the current study, no statistically significant difference was determined in the levels of adiponutrin in the aqueous humor. This finding suggests that the effect of adiponutrin on DR does not originate from local expression, but could be because of systemic effects on chronic hyperglycemia and insulin sensitivity.

The plasma pannexin 1 levels were found to be statistically significantly lower in both the DM+C and DR+C groups than in Group C in the current study. The pannexin 1 channels control the expression of ATP and other nucleotides from several cell types to the extracellular area[26]. Insulin sensitivity and glucose level is affected by pannexin-mediated ATP expression in adipose tissue. If the insulin signal is too low to manage the process, an excessive amount of ATP is released from adipocytes, and therefore the autocrine and paracrine effects of adipose tissue change[27]. In an experimental study using rats with pannexin gene deletion, insulin resistance was seen to increase following a high fat diet[19]. Purinergic signalling plays a role in several physiological and pathohysiological processes in mammalian cells. Extracellular nucleosides and nucleotides exert their effects through purigenic receptors (adenosine, P2X, P2Y). These receptors, the functions of which have been well defined, could be therapeutic targets in neurological and cardiovascular diseases, inflammation, osteoporosis, cancer and diabetes[28].

There are several studies in literature that have shown that there could be an association between adipose tissue dysfunction and impaired glucose hemostasis, and diabetes and obesity, and there is also evidence that purinergic signal pathologies have an effect in these diseases[29]. Substrates that are effective on these receptors, such as ATP, are expressed from cells to the extracellular area *via* ion channels in the plasma membrane. High levels of extracellular ATP formation at a continuously large amount cause an increase in lipolytic processes and a decrease in insulin sensitivity. The continuing lipolysis and subsequent increase in free acid levels is extremely important in the pathogenesis of insulin resistance, diabetes and cardiovascular diseases[30-31]. In addition, P2X7R, which is another purinergic signal receptor, has an active role in the expresssion of insulin from pancreas beta cells, could play a role in the micro and macrovascular complications of diabetes, and findings have been demonstrated supporting insulin resistance, such as evident glucose intolerance and increased body weight in rats lacking P2X7R[27,32-35].

Insulin resistance is the inability of insulin to increase glucose uptake into the tissue due to a defect in the intracellular insulin signalling pathway. Houstis *et al*[36] determined that reactive oxygen radicals were increased in insulin resistance accompanying type 2 diabetes and obesity. It has been reported that insulin resistance decreases with antioxidant activity, and in many studies, oxidative stress is associated with insulin resistance[37]. Pannexin channel inhibitors, such as carbenoxolone, probenecide, and pannexin 1 KO, cause an increase in diet-induced insulin resistance in adipocytes. Insulin resistance is thought to develop associated with the effects of pannexin on the regulation of oxidative stress[20].

The several mechanisms mentioned above show that pannexin 1 is extremely important in glucose metabolism. The results obtained in this study support that pannexin 1 is low in diabetic patients. That there was no statisticlly significant difference between the DR+C and DM+C groups suggests that pannexin 1 levels have a role in the pathogenesis of diabetes but there is

no significant effect on retinopathy. However, the low number of patients, especially those with DR, may be insufficient to explain the effect of low pannexin 1 on the disease.

Pannexin is known to have local effects on the eye. Cui *et al*[38] showed that as a result of changes occurring in pannexin levels in the nerve endings of corneal tissue in diabetic patients, there was an increase in ATP expression, these changes caused hypersensitivity in the corneal nerves and this was found or thought to have an effect on refraction, corneal healing and anterior chamber aqueous dynamics.

Purinergic P2X7 receptors cause cell death associated with inflammation, oxidative stress and apoptosis by triggering the formation of large, non-selective membrane pores in the cell membrane. Pannexin plays a role in the process of the formation of these pores[39-40]. In addition P2X7 receptor up-regulation has been found to be correlated with retinal cell degeneration[41-42]. Changes may occur such as neoplastic formation, inflammation and immune response mediated by P2X7 receptors. Inflammation plays an important role in the progression and exacerbation of DR.

P2X7 receptor activation triggered by hyperglycemia leads to IL-1 beta expression from pericytes and loss of pericytes, which causes impairment of the blood-retina barrier, vascular leakage and macular edema. In an experimental DR model an increase was determined in inflammatory cytokine expression mediated by P2X7 receptor up-regulation[43-44]. These receptors are an important target for DR therapy, and receptor JNJ47965567 has been found to be the most effective in DR treatment[45].

BMI, VLDL, triglyceride, HbA1c, FPG levels were significantly higher in the DR+C group compared to the C group (P<0.05). This is particularly associated with changes in glucose metabolism and lipid metabolism associated with the metabolic syndrome. The decrease in the plasma levels of pannexin and adiponutrin, which have important effects on both glucose and lipid metabolism, especially in patients with advanced diabetes, is also effective on BMI, VLDL, triglyceride, HbA1c, FPG levels.

In conclusion, the results of the current study showed that the aqueous pannexin 1 levels of Groups DR+C were statistically significantly higher than those of Group C and DM+C. That pannexin, which plays a role in ATP expression necessary for receptor activation, was determined to be high in the aqueous suggests that it could be important to lower pannexin levels in the local treatment of this disease. In addition we think that the lower detection of plasma adiponutrin levels in Group DR+C patients indicates that this patient group may be more prone to complications. Nevertheless, there is a need for further more extensive series and experimental animal studies to support these findings.

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