Journal of Diabetes and Its Complications 33 (2019) 208–211

Contents lists available at ScienceDirect

Journal of Diabetes and Its Complications

journal homepage: WWW.JDCJOURNAL.COM

Retinal photoreceptor apoptosis is associated with impaired serum ionized calcium homeostasis in diabetic retinopathy: An *in-vivo* analysis

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ARTICLE INFO

Article history:
Received 6 October 2018
Received in revised form 3 December 2018
Accepted 3 December 2018
Available online 8 December 2018

Keywords:
Diabetic retinopathy
Ionized calcium
Spectral-domain optical coherence tomography
Retinal photoreceptor apoptosis
Autophagy
Ellipsoid zone

ABSTRACT

Purpose: The aim of this work was to study the association of serum ionized calcium with retinal photoreceptor apoptosis on spectral domain optical coherence tomography (SD-OCT) in diabetic retinopathy (DR).
Methods: Sixty consecutive cases with Type 2 diabetes mellitus were categorized into three groups: no diabetic retinopathy; non-proliferative DR; proliferative DR. The eye with more severe form of the disease was considered. Twenty healthy controls were also included. Best corrected visual acuity (BCVA) was measured on logMAR scale. Retinal photoreceptor apoptosis was defined as disruption of retinal photoreceptor ellipsoid zone (EZ). Ellipsoid zone disruption was assessed using SD-OCT. Serum levels of total and ionized calcium were measured using standard protocol.
Results: EZ disruption was found to be positively associated with serum total calcium and ionized calcium. Also, EZ disruption was found to be positively associated with logMAR BCVA.
Conclusion: Increased serum ionized calcium induces retinal photoreceptor apoptosis resulting in increased EZ disruption in DR.

1. Introduction

Apoptosis or programmed cell death is characterized by specific morphological and energy-dependent changes in the cell.[1] Apoptosis is a vital component maintaining cellular homeostasis required for normal cell turnover, proper development and functioning of the immune system, embryonic development and chemical-induced cell death.[2]

Calcium, as a dynamic second messenger, is essential for biochemical processes in all eukaryotes. Calcium is released from the endoplasmic/sarcoplasmic reticulum upon cellular activation. This activates several enzymes, mainly phosphatases and kinases which regulate vital cellular processes like apoptosis and autophagy.[3]

Apoptosis is the final common pathway of photoreceptor cell death in retinal dystrophies and degeneration.[4] Retinal dystrophies and degenerations in humans and light-induced retinal degenerations in animal models demonstrate apoptotic photoreceptor cell death.[5]

Photoreceptor disruption can be visualized on spectral-domain optical coherence tomography (SD-OCT) as loss of integrity of the external limiting membrane (ELM), ellipsoid zone (EZ) and inter digitation zone (IZ).[6,7] In retinal degenerative diseases, optical coherence tomography (OCT) demonstrates that disorganization of outer retinal layers occur in a stepwise order: first at the IZ, followed by the EZ and finally the ELM.[8,9] Disruption of these structures have been reported as likely hallmarks of photoreceptor dysfunction or damage in a variety of retinal diseases.[10,11]

Spectral domain optical coherence tomography (SD-OCT) is a reliable, non-invasive imaging tool for *in-vivo* retinal histology.

2. Materials and methods

The study was performed in accordance to the tenets of the Declaration of Helsinki. It was a tertiary care center based observational cross

Conflict of interest: The authors declare no conflict of interest.

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https://doi.org/10.1016/j.jdiacomp.2018.12.003
1056-8727/

sectional study, where 60 consecutive cases and 20 healthy controls were included after obtaining informed voluntary consent. Cases were divided into patients with no diabetic retinopathy (No DR; n = 20), non-proliferative diabetic retinopathy (NPDR; n = 20) and proliferative diabetic retinopathy (PDR; n = 20) on the basis of Early treatment diabetic retinopathy study (ETDRS) classification.[12] Non-diabetic individuals presenting for refraction were included as controls.

Cases with ocular or systemic diseases which could affect the retinal vascular pathology (hypertensive retinopathy), or cases on medications or conditions causing altered calcium levels (Hyper or hypoparathyroidism, end stage renal disease, milk alkali syndrome, tuberculosis, sarcoidosis, malignancy, patients on thiazides diuretics or calcium channel blockers), any previous ophthalmic surgical or laser interventions, cases with signal strength 5 or below on OCT examination and cases taking any mineral supplements or antioxidants or medications causing altered calcium or magnesium levels in the body were excluded from the study. Best-corrected visual acuity was documented in logMAR scale (logMAR BCVA). Slit lamp biomicroscopy and dilated ophthalmoscopic examination were performed. Digital fundus photography and fluorescein angiography were performed using Zeiss fundus camera FF 450 Plus with pixel width of 0.0054 and image size 2588 × 1958 (Carl Zeiss Meditec AG, Jena, Germany). Spectral domain optical coherence tomography (SD-OCT) [Cirrus HD-OCT Carl Zeiss Meditech, Inc., CA, U.S.A]: macular cube 512 × 128 was used to assess photoreceptor ellipsoid zone (EZ). Retinal photoreceptor apoptosis was defined as disruption of EZ.

On horizontal and vertical SD-OCT scans, photoreceptor ellipsoid zone disruption was graded into three categories (Fig. 1)[13]:

- Grade 0: Intact photoreceptor ellipsoid zone
- Grade 1: Focal disruption (photoreceptor ellipsoid zone disruption indicating subfoveal localized involvement)
- Grade 2: Global disruption (photoreceptor ellipsoid zone disruption indicating generalized involvement within the macular cube)

Blood samples from study subjects were drawn by aseptic vein puncture. Blood was collected without stasis and transferred into tubes containing 3.89% trisodium citrate (in the ratio of 9:1) for separation of plasma. Serum was separated and levels of glycated hemoglobin (HbA1c) and total calcium were measured on autoanalyzer using standard protocol. Serum ionized calcium was measured by electrolyte analyzer using ion selective method. Serum vitamin D (25-hydroxy vitamin D) levels were measured using chemiluminescence microparticle microassay method.

Data is summarized and presented as Mean ± SE. Chi square ($\chi 2$) test analyzed the difference in gender distribution between the groups. One way analysis of variance (ANOVA) was done to compare the values of serum total calcium and ionized calcium between the study groups - control, NPDR and PDR. Pearson's correlation coefficient (r) was found to analyze the correlation of the continuous variables serum total and ionized calcium with logMAR BCVA. Univariate linear regression analysis was done taking logMAR BCVA as dependent variable with serum total calcium and ionized calcium as independent variables. Binary logistic regression was done taking EZ disruption as dependent variable and serum total calcium and ionized calcium as independent variable. All analyses were performed using SPSS software (window version 21.0).

3. Results

The mean age was 52.7 ± 8.16 years, 54.2 ± 7.34 years, 53.55 ± 6.99 years and 50.25 ± 7.69 years respectively in the control, No DR, NPDR and PDR groups. The demographic characteristics of the study groups are illustrated in Table 1. Comparing the mean age of four groups, ANOVA revealed similar age among the groups and was not

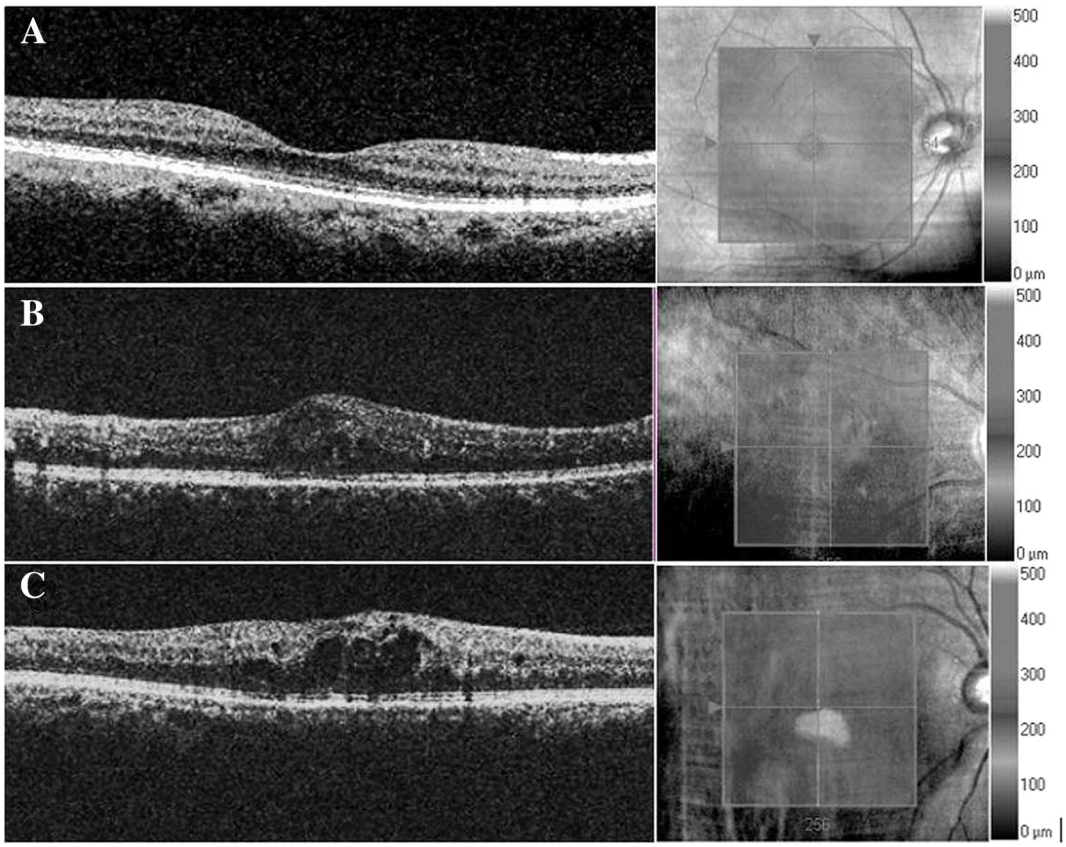

Fig. 1. Spectral domain optical coherence tomography of macula along with corresponding ILM-RPE overlay map illustrating grades of ellipsoid zone disruption. A - No disruption B - Focal disruption C - Global disruption.

Table 1
Demographic characteristics of the study groups.

Demographic characteristics	Control (n = 20)	No DR (n = 20)	NPDR (n = 20)	PDR (n = 20)
Mean age (years)	52.7	54.2	53.55	50.25
Sex:				
Female	8 (40%)	5 (25%)	13 (65%)	12 (60%)
Male	12 (60%)	15 (75%)	7 (35%)	8 (40%)

statistically significant (F = 1.045, $p = 0.378$). Mean serum levels of total calcium were 8.4 ± 0.46, 8.93 ± 0.59, 9.25 ± 0.40 and 9.15 ± 0.88 (mmol/dL), while mean serum levels of ionized calcium were 1.13 ± 0.06, 1.12 ± 0.07, 1.17 ± 0.07 and 1.18 ± 0.07 (mg/dL) respectively in the control, No DR, NPDR and PDR groups. The mean levels of HbA1c were 5.69 ± 0.21, 6.45 ± 0.21, 7.48 ± 0.46 and 9.55 ± 0.96 (grams/dL), and mean logMAR visual acuity was 0.11 ± 0.15, 0.41 ± 0.37, 0.83 ± 0.51 and 0.89 ± 0.56 respectively, among the study groups. The mean serum levels of vitamin D (25-hydroxy vitamin D) were found to be 23.21 ± 1.30, 21.31 ± 1.61, 16.78 ± 1.78 and 13.01 ± 1.10 (ng/dL). Mean serum levels of total and ionized calcium in the four groups are depicted in Figs. 2 and 3.

Pearson's correlation analysis revealed a statistically significant positive correlation of logMAR BCVA with serum total calcium (r = 0.24, $p = 0.03$) and serum ionized calcium (r = 0.32, $p = 0.004$). (Table 2).

On univariate linear regression analysis with logMAR BCVA as dependent and HbA1c as independent variable, a significant negative association was found between logMAR BCVA and HbA1c. (r = 0.49, $p < 0.001$).

On univariate linear regression analysis with logMAR BCVA as dependent variable and serum total calcium and ionized calcium as independent variables, a significant increase in logMAR BCVA was observed with increase in serum total calcium (r = 0.241; $p = 0.03$) and serum ionized calcium (r = 0.315; $p = 0.004$).

Binary logistic regression with EZ disruption as dependent variable and serum total calcium and ionized calcium as independent variables, the probability of EZ disruption was found to be positive with serum total calcium and ionized calcium. All test results were statistically significant. (Table 3).

4. Discussion

Apoptosis is referred to as type I programmed cell death while autophagy as type II programmed cell death.[14] Autophagy is a

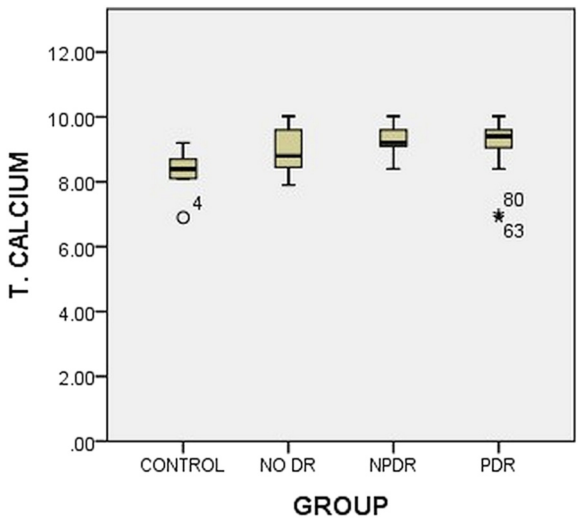

Fig. 2. Box and whisker plots illustrating mean serum levels of total calcium among study groups. No DR = No diabetic retinopathy, NPDR = Non-proliferative diabetic retinopathy, PDR = proliferative diabetic retinopathy.

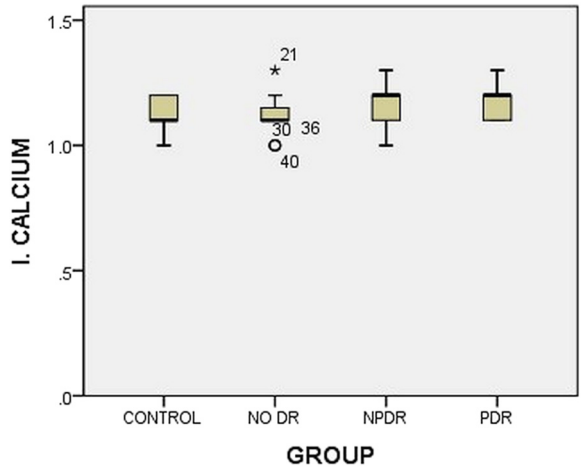

Fig. 3. Box and whisker plots illustrating mean serum levels of ionized calcium (I. calcium) among study groups. No DR = No diabetic retinopathy, NPDR = Non-proliferative diabetic retinopathy, PDR = proliferative diabetic retinopathy.

physiological process promoting cell survival. It provides the raw material and energy for cell regeneration under normal circumstances. Dysregulated autophagy under diseased conditions may promote cell death through protein accumulation and organelle dysfunction. The term "autophagic cell death" describes a form of programmed cell death morphologically distinct from apoptosis and is presumed to result from excessive levels of cellular autophagy.[15]

This study highlights the association of serum ionized calcium ($[Ca^{2+}]i$) with retinal photoreceptor apoptosis in the three groups of DR for the first time. In the present study, increased serum levels of ionized calcium were found to be associated with increased EZ disruption on SD-OCT and increased severity of DR.

The calcium ion (Ca^{2+}) is a major second messenger required for cellular coordination in all higher cells. There is ten thousand fold difference between cytosolic and extracellular $[Ca^{2+}]i$. Thus, $[Ca^{2+}]i$ influx or release from intracellular stores produces large fluctuations of free cytosolic $[Ca^{2+}]i$.[16] The diffusion of $[Ca^{2+}]i$ is much slower than other intracellular messengers.[17] Thus it is able to regulate a variety of different functions in different parts of the cell.[18] Slight alterations in calcium signaling can lead to apoptosis or autophagy through various mechanisms.[3]

Published literature has highlighted the role of ionized calcium in retinal photoreceptor homeostasis. The role of ionized calcium in photoreceptors is compartmentalized. In the outer segment (OS), ionized calcium controls photo transduction and in the inner segment (IS), ionized calcium regulates cellular metabolism, enzyme release, cytoskeletal dynamics, gene expression and cell death.[19]

$[Ca^{2+}]i$ homeostasis is crucial to maintain normal photoreceptor function. Influx and outflux of ionized calcium are maintained through various processes in the photoreceptor. While $[Ca^{2+}]i$ influx is curtailed by keeping the resting membrane potential of the photoreceptor IS just below the threshold for regenerative activation of $[Ca^{2+}]i$ channels, the outward current flowing through Cl^- and K^+ channels acts to clamp the membrane potential *via* fluxes of Cl^-.

Table 2
Illustrating Pearson's correlation analysis between study variables (n = 80).

Variables	Log MAR BCVA	HbA1c	Total calcium	Ionized calcium
Log MAR BCVA	1			
HbA1c	0.499**	1		
Total calcium	0.243*	0.298**	1	
Ionized calcium	0.324**	0.285**	0.406**	1

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table 3
Illustrating binary logistic regression between EZ disruption and serum levels of total and ionized calcium.

Biochemical parameters (independent variable) (x)	Odd's ratio	*p* value	95% C.I.	
			Lower	Upper
Total calcium	3.218	0.004	1.446	7.162
Ionized calcium	38,446	0.004	26.416	55,957,113.62

K^+ and sets the upper limit to which local $[Ca^{2+}]i$ can rise. This prevents both $[Ca^{2+}]i$ spiking and $[Ca^{2+}]i$ -mediated toxicity.[20]

Ionized calcium is required to sustain the release of transmitter in photoreceptor IS. But the need for tonic depolarization in darkness creates a potentially lethal physiological trap. Activation of IS $[Ca^{2+}]i$ triggers a self-regenerative cycle of $[Ca^{2+}]i$ influx and depolarization resulting in impaired photoreceptor function and death.[21] Increased intracellular $[Ca^{2+}]i$ causes apoptosis by directly activating Bax and Bak, the pro-apoptotic genes, or indirectly by sequestering anti-apoptotic Bcl-2 family members.[22] The role of these proteins in the regulation of apoptosis at mitochondria and endoplasmic reticulum has been elucidated.[23] Increase in $[Ca^{2+}]i$ results in altered cellular homeostasis at the level of retinal photoreceptors denoted as retinal photoreceptor EZ disruption on SD-OCT.

The role of autophagy in regulating maintaining the structural integrity and physiological functions of retinal photoreceptor cells and contribution to retinal diseases, namely, retinitis pigmentosa and Leber's congenital amaurosis has been recently documented.[24] Direct evidence that calcium signaling stimulates autophagy has also been elucidated. The physiological process of OS internalization also requires $[Ca^{2+}]i$ dependent mechanisms. Endoplasmic reticulum calcium mobilization induces autophagy when stimulated by agents such as vitamin D.[25] Dysregulated autophagy in the early stage of the disease results in cell regeneration in the outer retinal layers. During the later stage of the disease increased ionized calcium leads to enhanced dysregulated autophagy causing cell death which is reflected *in-vivo* as retinal photoreceptor EZ disruption.

Increased levels of intracellular $[Ca^{2+}]i$ may occur during inherited retinal degenerations, retinal diseases and injuries and may play a key role in apoptotic cell death in humans and animals. The role of elevated ionized calcium in photoreceptor cell death in animal models has been studied. Photoreceptor rescue has been suggested to be a result of reduced influx and lower intracellular levels of $[Ca^{2+}]i$ and consequent stabilization of ionized calcium homeostasis.[26]

Thus, various signaling pathways causing cell death involve $[Ca^{2+}]i$ either directly, or indirectly. Photoreceptor functioning requires $[Ca^{2+}]i$ homeostasis. Disturbance in this homeostasis may lead to activation of several signaling pathways with resultant apoptosis and autophagy of photoreceptors. Also, increased intracellular levels of $[Ca^{2+}]i$ following calcium mobilization in Vitamin D deficiency may accentuate the photoreceptor cell death process.

The increase in logMAR visual acuity is associated with increased EZ disruption.[27–29] Similar pattern of photoreceptor apoptosis has been observed in response to primary and recurrent retinal detachment (RD) in human and animal models, suggesting that photoreceptor cell apoptosis may be one of the causes of reduced vision after RD.[30]

Regular serum ionized calcium monitoring will help in maintaining cellular homeostasis and thus predicting retinal photoreceptor apoptosis in cases with DR.

5. Conclusion

Calcium homeostasis is essential for retinal photoreceptor integrity. *In-vivo* histology of the retina by SD-OCT demonstrates that retinal photoreceptor apoptosis, denoted by EZ disruption, is associated with increased levels of ionized calcium in diabetic retinopathy.

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