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Tear Carnitine Analysis After Corneal Crosslinking in Keratoconus

Keratokonus'ta Korneal Crosslinking Sonrasi Gözyaşi Karnitin Analizi

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Abstract

Background: To investigate the changes that may occur after CXL treatment in carnitine levels, which have an important role in the cell's energy cycle.

Materials and Methods: Single eye of 25 patients who underwent crosslinking (CXL) therapy for keratoconus was included in this prospective, nonrandomized study. Patients were divided into 4 different groups as male, female, over 18 and under 18 before the treatment. Tears were collected with capillary tubes before, and at the 6th postoperative month without any anesthetic agent. From the tear samples, 27 carnitine ester parameters were measured by a mass spectrometer and an ultra high-performance liquid chromatograph.

Results: The average age of patients consisting of 11 (44%) men and 14 (66%) women was 18.28 ± 3.98 years (12-25). After CXL treatment, statistically significant changes were only detected in C12 (Dodecanoyl Carnitine) and C14 (Myristoyl Carnitine) levels. Among all carnitines, only C4 and C6 carnitine levels increased, but this increase was not statistically significant. Among the age groups, the highest difference was in carnitine derivative C6, and the least differing carnitine derivative was C51 and C5DC. The carnitine derivatives that differed most in the gender groups were C6 and C16, while the least differed were C5DC and C8:1. **Conclusions:** In cases where energy needs increase, such as inflammation, there may be a decrease in inflammation severity as carnitine levels decrease. The variation between pre and post CXL carnitine level measurements in keratoconus patients can be used as a useful marker to monitor this inflammation and intervene in the event of excessive inflammation.

Keywords: Acylcarnitine; Carnitine; Crosslinking; Keratoconus; Metabolomics.

ÖZ

Amaç: Hücrenin enerji döngüsünde önemli rol oynayan karnitin düzeylerinde CXL tedavisi sonrası oluşabilecek değişiklikleri araştırmak.

Gereç ve Yöntem: Bu prospektif, randomize olmayan çalışmaya keratokonus nedeniyle çapraz bağlama (CXL) tedavisi uygulanan 25 hastanın tek gözü dahil edildi. Hastalar tedavi öncesi erkek, kadın, 18 yaş üstü ve 18 yaş altı olmak üzere 4 farklı gruba ayrıldı. Gözyaşları ameliyat öncesi ve ameliyat sonrası 6. ayda herhangi bir anestezik madde kullanılmadan kılcal tüplerle toplandı. Gözyaşı örneklerinden 27 karnitin ester parametresi bir kütle spektrometresi ve bir ultra yüksek performanslı sıvı kromatografı ile ölçüldü. **Bulgular:** 11'i (%44) erkek, 14'ü (%66) kadından oluşan hastaların yaş ortalaması 18,28 ± 3,98 yıl (12-25) idi. CXL tedavisi sonrasında sadece C12 (Dodecanoyl Carnitine) ve C14 (Myristoyl Carnitine) düzeylerinde istatistiksel olarak anlamlı değişiklikler tespit edildi. Tüm karnitinlerden sadece C4 ve C6 karnitin düzeylerinde artış görüldü ancak bu artış istatistiksel olarak anlamlı değildi. Yaş grupları arasında en yüksek farklılık karnitin türevi C6'da görülürken, en az farklılık gösteren karnitin türevi ise C51 ve C5DC oldu. Cinsiyet gruplarında en fazla farklılık gösteren karnitin türevleri C6 ve C16 olurken, en az farklılık gösterenler ise C5DC ve C8:1 oldu.

Sonuç: İnflamasyon gibi enerji ihtiyacının arttığı durumlarda karnitin düzeyleri azaldıkça inflamasyon şiddetinde de azalma olabilir. Keratokonus hastalarında CXL öncesi ve sonrası karnitin seviyesi ölçümleri arasındaki farklılık, bu inflamasyonun izlenmesi ve aşırı inflamasyon durumunda müdahale edilmesi için yararlı bir belirteç olarak kullanılabilir

Anahtar kelimeler: Açilkarnitin, Karnitin, Crosslinking, Keratokonus, Metabolomics

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Highlights

- Carnitine, which plays an important role in the metabolism of fatty acids in the cell.
- A significant decrease in dodecanoyl carnitine and myristoyl carnitine levels was found secondary to the regression of inflammation after CXL treatment.
- Among all carnitines, only C4 and C6 carnitine levels increased.

Introduction

Corneal collagen crosslinking (CXL) treatment is the only treatment method known to stop or slow the progression of keratoconus down (1). After this treatment, which creates new covalent bonds between amino acids in collagen fibrils through reactive oxygen radicals, corneal biomechanical resistance and stability increase (2). After CXL treatment, changes occur in the fibroblasts in the corneal stroma, and these changes have been identified as markers of treatment efficacy (3). Although biochemical reactions occur mainly in the stroma during this treatment, some changes may occur in the ocular surface and tear film (4,5). In addition, the content of the tear film can also be an indicator of the metabolic functions of the cornea (3,6).

Pathological processes occurring in the cornea tissue may cause metabolome changes, leading to increased or decreased metabolites (7). Metabolomics, including carnitine, can be detected in many body fluids using the mass spectrometry (8). Carnitine, a branched, non-essential amino acid, is synthesized from essential amino acids lysine and methionine. Carnitine, which plays an important role in the metabolism of fatty acids in the cell, is a quarternary ammonium compound. Carnitine plays a role in the transport of fatty acids to mitochondria and β-oxidation by binding to long chain fatty acids with acyl residues (9,10).

Thus, this study aims to investigate the changes that may occur after CXL treatment in carnitine levels, which have an important role in the cell's energy cycle.

Material and Methods

Study design

The diagnosis of keratoconus was made according to the criteria defined by Rabinowitz et al. (11). Single eye of 25 patients who underwent crosslinking therapy for keratoconus was included in this prospective, nonrandomized study. The cases were selected from patients with keratoconus who were admitted to Harran University Faculty of Medicine cornea department.

Patients with an increase in Kmax more than 1 dioptre in the last two follow-up examinations performed at 6-month intervals, were evaluated as progressive keratoconus. The following patients were excluded from the study: the thinnest corneal thickness less than 400 μm, corneal scar and infection, uveitis, glaucoma, ocular trauma, history of contact lens use, autoimmune disease, pregnancy, lactation, previous ocular surgery, those with a topical / systemic immunosuppressive or steroid history.

As the surgical technique, conventional CXL (Dresden Protocol) treatment was applied (12). Following topical anesthesia with 0.5% topical proparacaine hydrochloride, 9.0mm central epithelial debridement was performed with 20% alcohol," as "keratectomy.

Riboflavin dropping was started, the solution containing 0.1% Riboflavin + 20% Dextran T500 (Collagex, Taipei, Taiwan) was dropped at intervals of 2 minutes for 30 minutes. Corneal thickness was measured with ultrasonic pachymeter before ultraviolet A (UVA) application and was confirmed to be over 400 μm. For the next 30 minutes, UVA was applied with LightLink-CXL (LIGHTMED, Taiwan) at 365nm and 3.0mW / cm2, and riboflavin was continued to be instilled at 2-minute intervals during the procedure. Contact lenses were placed after the procedure. Topical antibiotics were used 4 times a day for 1 week (0.05% moxifloxacin, moxai® abdi ibrahim). After the epithelium was closed, topical steroid treatment was started 4 times a day, and it was reduced after 2 weeks and continued for 3 months. (0.5% loteprednol etabonate, lotemax®, bausch+lomb)

Tears were collected with capillary tubes as defined by Posa et al (13) before and at the 6th postoperative month without any anesthetic agent. Collected tears were kept at -80˚C until the date of analysis. Patients were divided into four groups based on gender (male/female) and age (<18/≥18) before treatment."

Laboratory analysis

Chemicals and Reagents

The reagents used were the internal standard set of Labeled Carnitine Standards-Set B from Cambridge Isotope Laboratories (UK). Mobile phase modifiers of formic acid and Acetonitrile were used in combination with high performance liquid chromatography (HPLC) gradient grade methanol (J.T. Baker, Center Valley, PA, USA) and deionized water (Millipore Simplicity UV water purification system, Waters Corporation, Milford, MA, USA)

LC-MS / MS carnitine determination

From the tear samples, 27 carnitine ester parameters were measured by a Shimadzu-8040 triple quad mass spectrometer (MS / MS) (Shimadzu-8040) and a Shimadzu Nexera X2 ultra high-performance liquid chromatograph (UHPLC). The carnitine profile has been studied by modifying the neonatal screening method developed by LaMarca and Azzari (19,20). Filter paper (Whatman filter paper 10538018) cut into 3.2 mm discs was placed in 96 well plates. 5µl of tear samples were then placed in each well and allowed to dry at room temperature overnight. Sample extraction was performed using a methanol mixture of approximately 66.6% and 33.3% relative volume / volume and 300 µL extraction solution of 3 mmol / L hydrate hydrazine aqueous solution, respectively. The extract solution included internal standards and several stable heavy isotope analogs of carnitine and acylcarnitines. The extracted sample was injected into Shimadzu LCMS-8040. Mass spectral data for amino acids were obtained using a neutral loss scan of 46 Da in positive mode (CE-15V). Mass spectral data for acylcarnitines were obtained using 85 m / z precursor ion scanning in positive mode (CE-25V). Percent recovered in each analyte was determined by comparison to an internal standard for each analyte. Standard concentrations were in the range 7.6-152 µmol / L for acylcarnitines. Spiked samples containing different concentrations of analyte were used as daily control quality tests.

Analysis Condition

2.2 minutes run in FIA Flow 0.070 uL / min (A: Water + 0.05% of Formic Acid, B: Acetonitrile, A / B: 30% / 70%) Column Oven 30 ° C, Desolvation Line 300 where 40 µL sample is injected ° C, Temperature 500 ° C, Nebulizing Gas 3 L / min, and Drying Gas 20 L / min. All data collected were reprocessed using Shimadzu Neonatal Software, which automatically calculates the concentration of each compound**.**

Statistical analysis

The data were analyzed using SPSS for Windows version 22.0 software (IBM SPSS Inc, Chicago, IL, USA). Shapiro-Wilk test was used to analyze the normal distribution. Paired sample t-test was applied using mean ± standard deviation value for normally distributed data. Wilcoxon test was performed using median and interquartile (IQR) value for non-normally data. A value of $p < 0.05$ was accepted as statistically significant.

Ethical Approval

This study approval was obtained from the HarranUniversity Faculty of Medicine, Ethics Committee (number: HRU/20.08.07 date: 27.04.2020). Informed consent was obtained from all patients. All procedures were carried out in accordance with the Declaration of Helsinki.

Results

The average age of patients consisting of 11 (44%) men and 14 (66%) women was 18.28 ± 3.98 years (12-25). Twelve (48%) of the patients were under the age of 18 and 13 (52%) were over 18 years. Of those under the age of 18, 7 were female and 5 were male. In the investigation for twenty-seven carnitine derivatives, tear levels of 15 carnitine derivatives (free carnitine and 14 acyl carnitine) could be measured. Distribution of levels of all carnitine derivatives before and after CXL treatment is given in Table-1. Accordingly, after CXL treatment, statistically significant changes were only detected in C12 (Dodecanoil Canitine) and C14 (Myristoil Canitine) levels. A decrease was detected in both carnitine derivatives (p: 0.045, 0.038, respectively).

It was observed that there was some decrease in free carnitine levels after CXL treatment, but this decrease was not significant ($p = 0.346$). Among all carnitines, only C4 and C6 carnitine levels increased, but this increase was not statistically significant (p: 0.0707, 0.086, respectively), **(Figure-1), (Table-1).**

Table 1. The acylcarnitines in the tear samples of the two studied groups quantified using the LC-MS/MS method

Acylcarnitines	m/z	Formula	Status	Pre-CXL (n:) Mean ± SD	Post-CXL (n:) Mean ± SD	Fold change	p
C0 (free carnitine)	218.20>103.00	C7H15NO3	↓	14.81±8.90	12.65±6.21	-1.17	0.346
C2 (acetyl carnitine)	260.20>85.00	C9H17NO4	↓	1.72±1.09	1.59±1.20	-1.08	0.656
C3 (propionyl carnitine)	274.20>85.00	C10H19NO4	↓	1.12±0.69	0.91±0.60	-1.23	0.287
C4 (butyryl carnitine)	288.20>85.00	C11H21NO4	↑	0.38±0.20	0.40±0.19	0.95	0.707
C4DC (methylmalonyl carnitine)	374.30>85.00	C11H19NO6		Ø	Ø		
C5 (isovaleryl carnitine)	302.20>85.00	C12H23NO4	↓	1.68±1.15	1.41±1.35	-1.19	0.360
C5:1 (tiglyl carnitine)	300.20>85.00	C12H21NO4	↔	0.03±0.02	0.03±0.02	0	0.841
C5OH (isovaleryl carnitine)	318.20>85.00	C12H23NO4	↔	0.03±0.01	0.03±0.02	0	0.452
C5DC (glutaryl carnitine)	388.30>85.00	C12H21NO6	↔	0.04±0.02	0.04±0.02	0	0.959
C6 (hexanoyl carnitine)	316.20>85.00	C13H25NO4	↑	0.06±0.04	0.09±0.05	0.66	0.086
C6DC (adipoyl carnitine)	344.20>85.00	C13H23NO6	↔	Ø	Ø		
C8 (octanoyl carnitine)	342.20>85.00	C15H29NO4	↓	0.02±0.01	0.01±0.01	-2	0.151
C8:1 (octenoil carnitine)	302.20>85.00	C15H27NO4	↓	0.07±0.06	0.05±0.06	-1.4	0.367
C8DC (suberyl carnitine)	430.40>85.00	C15H27NO6		Ø	Ø		
C10 (decanoyl carnitine)	372.30>85.00	C17H33NO4	↔	0.01±0.01	0.01±0.008	0	0.092
C10:1 (decenoil carnitine)	370.30>85.00	C17H31NO4		Ø	Ø		
C10DC (sebacoyl carnitine)	458.40>85.00	C17H31NO6		Ø	Ø		
C12 (dodecanoyl carnitine)	400.30>85.00	C19H37NO4	↓	0.02±0.01	0.01±0.009	-2	0.045
C14 (myristoyl carnitine)	428.40>85.00	C21H41NO4	↓	0.02±0.01	0.01±0.008	-2	0.038
C14:1(tetradecenoyl carnitine)	426.40>85.00	C21H39NO4		Ø	Ø		
C14:2(tetradecadienoyl carnitine)	424.40>85.00	C21H37NO4		Ø	Ø		
C16 (palmitoyl carnitine)	456.40>85.00	C23H45NO4	↓	0.06±0.04	0.04±0.02	-1.5	0.457
C16:1 (hexadecenoyl carnitine)	454.40>85.00	C23H45NO4		Ø	Ø		
C18 (stearoyl carnitine)	484.40>85.00	C25H49NO4		Ø	Ø		
C18:1 (oleoyl carnitine)	482.40>85.00	C23H45NO4		Ø	Ø		
C18:2 (linoleyl carnitine)	480.40>85.00	C23H45NO4		Ø	Ø		
C18:1OH (hydroxoleoyl carnitine)	498.40>85.00	C25H49NO4		Ø	Ø		

Abbreviations: * p<0.05 and **p<0.001 values for the comparison of the variables between two groups were calculated according to the Mann-Whitney U test. (m/z: mass of main ion and product ion)

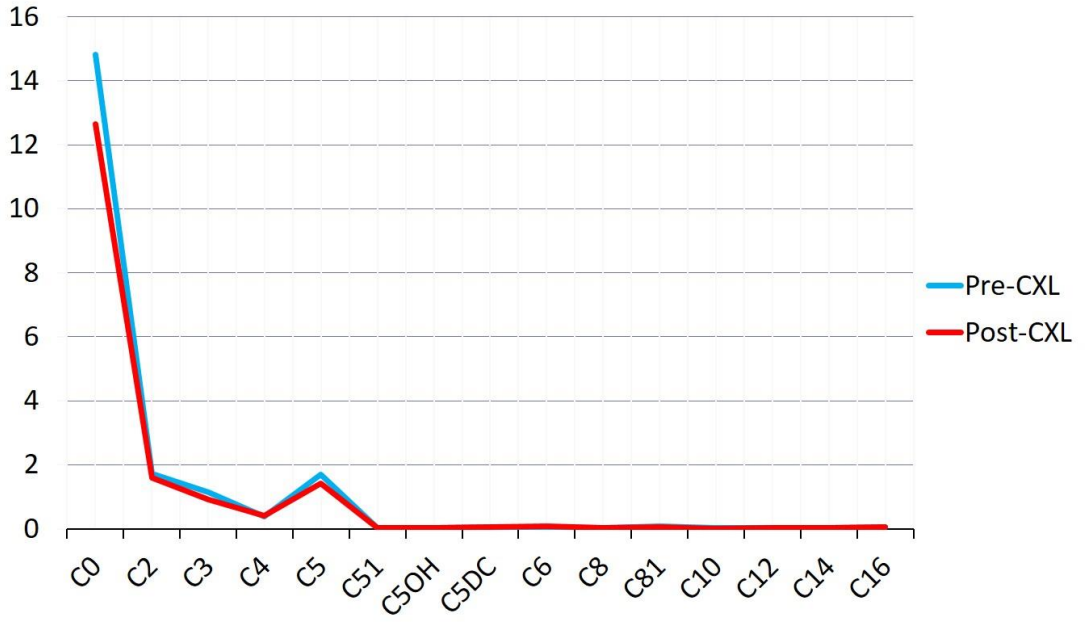

Figure 1. Tear acylcarnitine levels pre- and post- crosslinking

While the C6 level increased statistically significantly only in the group under the age of 18 (0.048 μg / ml increase, p: 0.032), it was not significant even though there was an increase in the group over the age of 18 (0.007 μg / ml increase, p: 0.691). Among the age groups, the highest difference was in carnitine derivative C6, and the least differing carnitine derivative was C51 and C5DC **(Figüre-2)**.

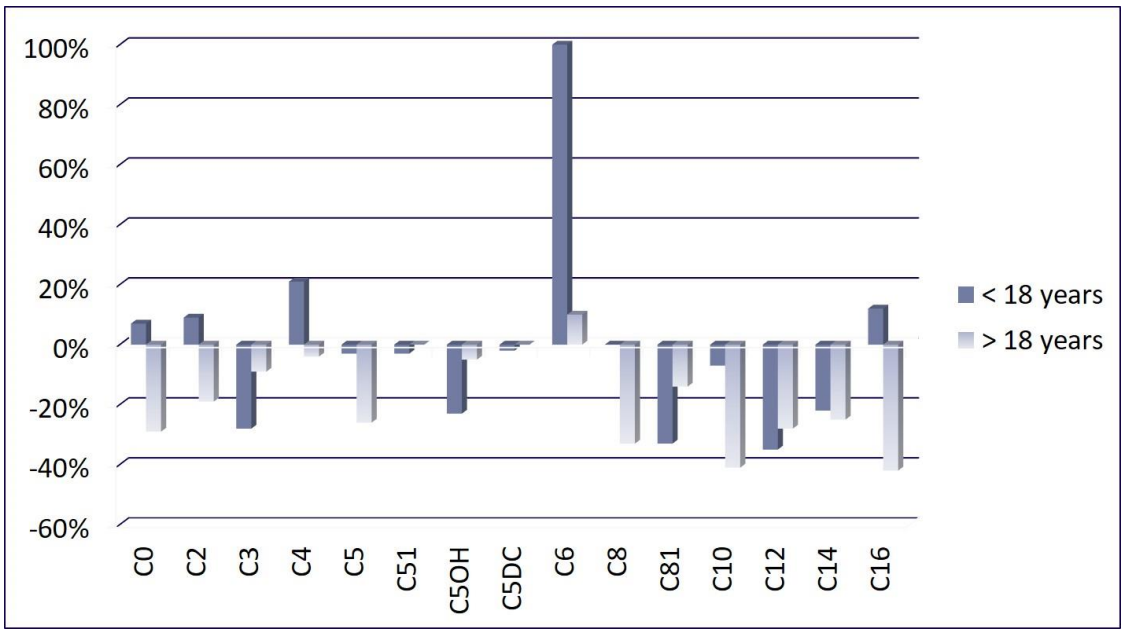

Figure 2. Distribution of the difference in tear acylcarnitine levels before and after crosslinking by age groups

Considering the overall change in carnitine derivatives, the change in male patients was greater than that of female gender **(Figure 3).** The carnitine derivatives that differed most in the gender groups were C6 and C16, while the least differed were C5DC and C8: 1. C6 and C16 levels were higher in males than females.
It was observed that postoperative corneal scar developed in one case that did not cause vision loss. In this case, a significant increase was observed in all carnitine derivatives except for C14 (0.01 μg / ml) and C16 (0.04 μg / ml).

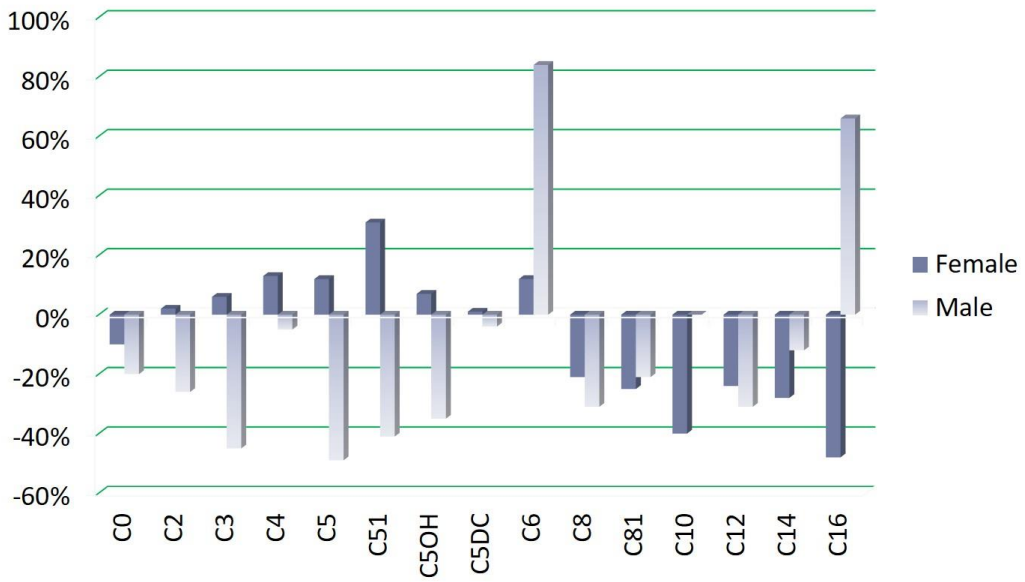

Figure 3. Distribution of difference in tear acylcarnitine levels before and after crosslinking by gender

Discussion

Fatty acids are used as an energy substrate in all tissues except the brain (14). Carnitine is an amino acid derivative that facilitates the introduction of long chain fatty acid into mitochondria and provides a substrate for oxidation and energy production. A decrease in carnitine levels in the cell is expected with inflammation and increased energy consumption of the cell (15). Although the pathogenesis of keratoconus has not been clarified, metabolomic changes in tears have been demonstrated in pre and post cxl comparisons (16). In another study investigating the activity of metabolomics related to energy production in keratoconus and healthy corneas, increased carnitine synthesis activity was reported in corneas with keraconus (17). As a matter of fact that, significantly reduced carnitine levels in keratoconus corneas were detected in a study comparing keratoconus corneas to post mortem corneas (18).

The most important function of biological fluids circulating in the body is the transport of nutrients to tissues and removal of metabolic waste. On the other hand, apart from the function of carnitine in energy metabolism, it has been reported that carnitine enhances the activity of the enzyme that scavenge free radicals in the tear, the catalase and glutathione peroxidase (19). Also, lower GSH levels were reported in tear samples taken from patients with KC has been demonstrated (20). In the study by Snytnikova et al comparing aqueous humor in eyes with keratoconus and post-mortem normal cornea, carnitine concentrations in aqueous humor were shown to be decreased in the group with keraconus (18). In a study investigating the relationship between tear film carnitine and dry eye, patients with dry eye disease have been shown to be significantly lower carnitine levels in tear fluid compared to controls and suggested using solutions containing carnitine to reduce damage to the ocular surface in these patients (21). As a result of active transport of ocular tissues through the cell membrane, the reduction of carnitine levels in the tear film has been shown (22, 23). In addition, inflammation negatively affects carnitine metabolism in the cells. Previous studies have reported that plasma carnitine levels are reduced in inflammatory processes in particular (24). Therefore, we think that increased carnitine levels in the tear film after CXL in the current study are also compatible with these results and that CXL may be effective in suppressing possible inflammation. In asthmatic patients, whose pathophysiology is similar to keratoconus, where inflammatory mechanisms play a role, serum myristoyl carnitine and dodecanoyl carnitine were found to be significantly higher in asthmatic patients than in healthy controls (25). Similarly, in our study, a significant decrease in dodecanoyl carnitine and myristoyl carnitine levels was found secondary to the regression of inflammation after CXL treatment.

In the cornea, keratocytes or fibroblasts play a central role in mediating the corneal response after injury. After injury, the keratocytes next to the wound undergo apoptosis, while those farther away from the wound transform into fibroblasts and / or myofibroblasts. At the end of these processes, scar tissue develops (26). Therefore, high carnitine concentrations in the tear film in the case with corneal scar development in this study can be explained by the increase in energy demand due to the increase in the activity of keratocytes, and an increase in carnitine transport through tear film to compensate this need. With a similar approach, in the study which we previously investigated carnitine levels

in pterygium tissue, where energy metabolism was accelerated due to premalignant cells and chronic inflammation, we demonstrated increased carnitine levels compared to normal conjunctival tissue (27). Galbis-Estrada et al, by prescribing oral nutraceutical supplements in dry-eyed patients, it was concluded that the teardrop metabolomic profile in patients with dry eye disease can be modified by appropriate oral supplements containing antioxidants and essential fatty acids (28). With this approach, we believe that it would be useful to investigate whether it would be beneficial to disrupt carnitine production in order to minimize or prevent scar tissue that may develop after CXL.

Study limitations

The study has some limitations. One of them is that no tear samples were collected from the healthy population and no comparison was made with the healthy population. Another is the relatively low sample size, especially the small number of scars developed patients. A longer postoperative follow-up period could provide more robust insights into carnitine level variations over time.

Conclusion

The results of this study showed that the change between carnitine level measurements before and after CXL in KC patients may be a useful marker for monitoring this inflammation and intervening in case of excessive inflammation.

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