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Reactive Dicarbonyls in removed cataractous lens nuclei, aqueous humor and plasma of senile cataract patients

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Abstract:

Background:- Dicarbonyl compounds such as methylglyoxal and glyoxal have been identified as the predominant source for the formation of advanced glycation end products (AGE) in various tissues contributing to aging and cataract formation in the lens. The aim of the study was to compare the level of reactive dicarbonyls in removed senile cataractous lens nuclei, aqueous humor and plasma of diabetic and non-diabetic cases.

Methods: -. This was a cross sectional study done with sample size of 60 senile cataract cases. Half cases were known diabetic while other half non-diabetic. The reactive dicarbonyl levels were detected through UV spectrophotometry.

Results: - The mean dicarbonyls level in lens nuclei, aqueous humor and plasma were 888.07 ± 14.95 nmol/L, 2.85 ± 0.44 nmol/L and 2.71 ± 0.44 nmol/L respectively in diabetic individuals where as in non diabetic cases the respective mean levels were 490 ± 8.72 nmol/L, 1.80 ± 0.34 nmol/Land 1.78 ± 0.45 nmol/L ($p<0.001$).

Conclusion: - Diabetic cases were noted to have more browning of their cataractous nuclei. There was a significant elevation of dicarbonyls in the removed nuclei, plasma and in aqueous humor in diabetics.

Key words:-Dicarbonyl, lens nucleus, aqueous humour, diabetic, non-diabetic.

Introduction:

Dicarbonyl compounds such as methylglyoxal and glyoxal play an important role in the formation of advanced glycation end products (AGE) in various tissues including the human lens. The non-enzymic glycation of long-lived proteins through Maillard reaction has been implicated in several age-related and diabetes-related complications[1-3]. The complexity of the Maillard reaction arises partly from multiple fragmentation reactions of the sugar moiety that constitutes branch points in the reaction progress and establishes many parallel reaction pathways. Reactive intermediates produced by these processes are often α-oxoaldehydes which are up to 20,000-fold more reactive than glucose in glycation processes. α-oxoaldehydes react with proteins, nucleotides and basic phospholipids to form AGEs directly. The α-oxoaldehyde intermediates of the Maillard reaction represent major sources of damage to the proteome and genome[4].

Senile cataract that is usually related to age is now thought to be caused by excessive oxidative stress.[5,6] The reactive dicarbonyls like methylglyoxal and glyoxal usually react with lens protein and are responsible for browning of crystalline human lens and accumulation of AGEs[7]. The formation of AGE owing to glycation may alter the surface charge of the protein, leading to a conformational change that in turn may effect protein–protein and protein–water interactions which ultimately leads to decreased transparency of the human crystalline lens[8-11]. The aging of the human lens is associated with progressive changes in the physico-chemical properties of crystalline, which include aggregation, pigmentation, formation of disulphide and non-disulphide cross links and fragmentation of lens proteins and ability to form free radicals[12,13].

The pathophysiology behind senile cataracts is complex and multifactorial. Diabetes is considered a major risk factor for the development of cataract and it has been reported that cataracts reach maturity almost 10 years earlier in the presence of diabetes [14]. *In vitro* [15] as well as in *in-vivo* study [16, 17] has shown high levels of dicarbonyls resulting in diabetic related vascular complications.

However the studies comparing the levels of dicarbonyls in plasma, aqueous humor of the eye as well as on the removed cataractous nuclei of diabetic and non diabetic cases has not been reported in the literatures to our knowledge. Quantification of AGE has been difficult because commonly used methodologies, i.e. immunodetection assays or fluorescence measurements, reflect group reactivity and are not specific for chemically defined substances. Some investigators measured individual AGE compounds, e.g. pentosidine[18].

This study was undertaken to evaluate the level of dicarbonyls in the removed cataractous nuclei, in aqueous humor of the eye and in the human plasma of patients with senile cataract with diabetes or without diabetes by a simple spectrophotometeric method [20-22].

Materials and methods:

Study design:

This is a cross sectional study done on diabetic and non-diabetic cases undergoing cataract operation. Sixty cases

(n=60) were recruited from the pool of patients with senile cataract undergoing routine cataract surgery by one surgeon (SS) at a tertiary care hospital in Benghazi, Libya during the period December 2006 and June 2007. Stratified sampling was done from the pool of diabetic and non-diabetic patients by choosing diabetic cases with odd entry number and non-diabetics with even number. For the diabetic group all the cases with more than 5 years of diabetes were included in the study. The cases with intra operative complications were excluded from the study. All cases recruited in this study had non-eventful cataract surgery. The local ethics committee approved the protocol and written informed consent was obtained from each patient after the nature of the study had been fully explained (in the native language). The sexes, weight, duration of diabetes, type of diabetes, and type of treatments received were also recorded. Physical examination including measurement of blood pressure was recorded. Individuals were classified as having diabetes mellitus if any of the following criteria were met [22], Fasting blood glucose levels of 7.0 mmol/l or more, random glucose levels of more than 11.1 mmol/l, current use of medications prescribed to treat diabetes (e.g. insulin or drugs).

Methods:

One ml of blood sample was taken from the blood samples collected for routine preoperative assessment in fasting state after 10-hour overnight fast. Samples were withdrawn by venous puncture and distributed equally into three tubes with no anticoagulant. Collection of 0.1ml of aqueous humor was done just before doing anterior capsulotomy, without modifying the surgical procedure. All the cases underwent manual small incision extra capsular cataract extraction through scleral tunnel incision. The removed nuclei were observed for the colour coding and were then sent for biochemical assay in the dry state on the same day. The nuclei were transferred in a sterile wide bore bottle without any preservative. The colours of the nuclei were categorized into 3 grades (Grade 1-yellow, Grade 2- brown and Grade 3-dark brown), which was a slight modification of CCRG (Cooperative Cataract Research Group of America) colour-coding of nuclei. The plasma, aqueous humor, and lens nuclei were frozen immediately at -19^0 C until they were assessed by a validated spectrophotometric procedure within 24 hours. [20-22].

Experimental Procedures:

The weight of individual nucleus was measured and frozen in 5ml of phosphate saline buffer of pH 7.4 at -19^0 C. Each nucleus was then homogenized individually in 20 ml of cold phosphate saline buffer of pH 7.4 by homogenizer with PCU (Process Control Unit) speed of 10,000 rpm for 2 minutes. The homogenized mixture was sonicated continuously using ultrasound bath for 5 minutes, cooled in ice (0^{0}C) for 5 minutes and centrifuged at 4000 rpm for 1 hr. One ml of the supernatant and 2.5 ml of 2, 4-dinitrophenylhydrazine were mixed in a closed test tube and heated in water bath at 42 ^{0} C for 40 minutes. Absorbance was measured at 432 nm using a double beam spectrophotometer[19]. Same procedure was followed for collected blood plasma and aqueous humor. This simple method is based on the chemical reaction between 2,4-dinitrophenylhydrazine with carbonyl moiety to form 2,4-dinitrophenylhydrazone[19] , which can be measured spectrophotometerically at the absorption maximum 432 nm, Glutathione, L-lactate, pyruvate and glucose did not interfere with the assay at expected biological levels[20].

Statistical analysis-

All the data were entered into Excel spread sheet and SPSS version 16.0 was used for data analysis. In descriptive statistics, mean, standard deviation, frequency and percentage were used. In inferential statistics, t-test, Kruskal-Wallis test and linear regression were used. T-test was used to compare the continuous outcomes between diabetes and non-diabetes groups. For age, we stratified the sample into diabetes and non-diabetes, and then non parametric test (Kruskal-Wallis test) was used for comparison of Diacarbonyl in lens nucleus in different age groups since the sample size for each group had less than 30. Linear regression analysis was used to control confounding variables. p value <0.05 was considered as significant.

This research followed the tenets of the World Medical Association Declaration of Helsinki on ethical principles for medical research involving human subjects.

Results:

In this study 36(60%) cases were male and 24 (40%) were female. Thirty patients had diabetes; the mean duration of diabetes was 6±1 years. The mean age of the non-diabetic patients was 51±10 years while the mean age of the diabetics was 54±10 years. The demographic characteristics of both diabetic and non-diabetic groups are shown in Table 1.

Table 1. Demographic characteristics of both Diabetic and non-diabetic groups

	Diabetic Group(n=30)	Non diabetic Group(n=30)
Age		
41-50 years	2(6.7%)	2(6.7%)
51-60 years	24(80%)	20(66.7%)
61-70 years	4(13.3%)	8(26.6%)
Sex		
Male	16(53.3%)	20(66.7%)
Female	14(46.6%)	10(33.3%)
Weight		
<70kg	10(33.3%)	12(40%)
>70kg	20(66.7%)	18(60%)
Mean BP in mmHg	132± 14/96± 8	128± 18/90± 10
Mean Diabetic years	6± 1	-
Mean Age in years	54± 10	51± 10

The nuclei colour grading in non-diabetic group showed less nuclear browning than those removed from diabetic patients (Figure 1)

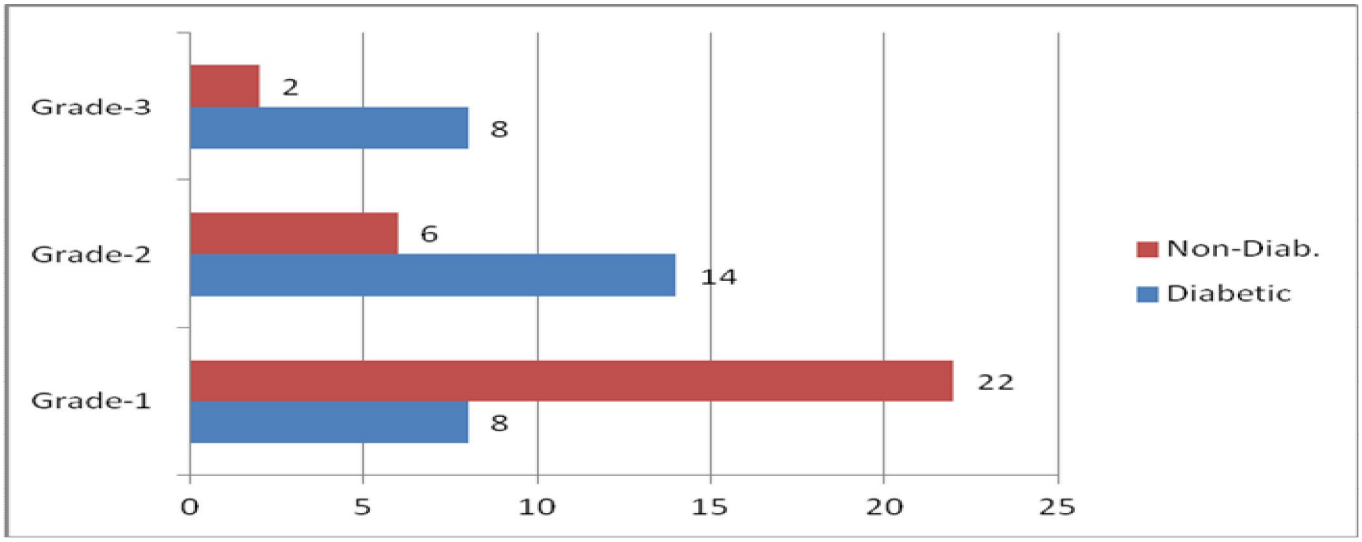

Figure 1. Colour Coding of removed nuclei

Table 2 shows the concentration of dicarbonyl in removed cataractous nuclei,aqueous humor and plasma in both groups. There was statistically significant difference of diacarbonyl concentration between the two groups. The average dicarbonyl concentration in removed nuclei of cataractous lenses in diabetic group was 888.07 ± 14.95 nmol/L while the average dicarbonyl concentration in non-diabetics was 490 ± 8.72 nmol/L. The mean concentration of dicarbonyl in aqueous humor in non diabetic cases was 1.80 ± 0.34 nmol/L compared to 2.85 ± 0.44 nmol/L in the diabetic patients. The average concentration of dicarbonyl in the plasma of non-diabetics was 1.78 ± 0.45 nmol/L compared to 2.71 ± 0.44 nmol/L in diabetics.

Table 2.Dicarbonyl in lens nucleus, Dicarbonyl in aq. humour and Dicarbonyl in plasma among Diabetes and Non Diabetes patients(Unit: nmol/L)

Variables	Diabetes	Non-Diabetes	t	95% CI	p-value
	Mean ±SD	Mean ±SD			
Diacarbonyl in lens nucleus	888.07 ± 14.95	490 ± 8.72	125.807	391.14 – 403.86	0.001
Dicarbonyl in aq.humour	2.85 ± 0.44	1.80 ± 0.34	10.322	0.84 – 1.25	0.001
Dicarbonyl in plasma	2.71 ± 0.44	1.78 ± 0.45	8.039	0.69 – 1.15	0.001

We stratified the data into diabetes and non-diabetes and Kruskal-Wallis test was used to compare the dicarbonyl in lens between different age groups. Table III showed the dicarbonyl in lens nucleus between different age groups among diabetes and non-diabetes patients. There were significant different of dicarbonyl concentration between different age groups among diabetes patients (p-value 0.016) and -diabetes patients (p-value <0.001).

Table 3.Dicarbonyl in lens nucleus between different age groups among Diabetes and Non Diabetes patients

Variables	Diabetes			Non - Diabetes		
	N (%)	Mean Rank	p-value	N (%)	Mean Rank	p-value
≤ 50	2 (6.7)	15.50	0.016	2 (6.7)	1.50	<0.001
51 – 60	24 (80.0)	13.56		20 (66.6)	12.85	
> 60	4 (13.3)	27.13		8 (26.7)	25.63	

Linear regression of dicarbonyl concentration in both diabetic and non diabetic group indicates significant increase of dicarbonyl level with advancement of age, where as there was no statistical significance on gender to the level of dicarbonyls in nuclei of both the groups.

Table 4: Linear regression of Dicarbonyl in lens nucleus

	Diabetes			Non-Diabetes		
	B	95% CI for B	p-value	B	95% CI for B	p-value
Age	2.385	1.66 – 3.11	<0.001	1.481	1.16 – 1.80	<0.001
Gender	- 5.917	-13.67 – 0.84	0.084	-1.413	-4.84 – 2.02	0.405

Discussion:

Diabetes mellitus is a major health problem in Libya [23]. Diabetes has been associated with rapid progression of senile cataract. One of the prominent characteristics of human and experimental cataract is the massive increase in water insoluble protein polymers. These polymers are characterized by fluorescent brown coloration [26]. The measurement of AGEs in human diabetics is difficult to carry out because of the lack of a recognized standard and due to the wide variety of AGEs. In the present study the dicarbonyl compounds have been identified as predominant source for the formation of AGEs in various tissues including the lens[28] that was determined spectrophotometerically.

Dicarbonyls have previously been shown to accumulate and react with proteins and amino acids in various tissues with age independent of diabetes[28,29]. In this study, it was found that the relationship between dicarbonyl level in removed nuclei and age is linear (Table IV). Due to small sample size we have categorized age groups into 10 years interval. Similarly we have not documented BMI status, instead categorized into above 70kg and below 70 kg of weight which may be a limitation to this study. We also observed that in the diabetic group, a significantly higher dicarbonyl concentrations was present compared to the nondiabetic group in all body fluids and tissues included in this study (plasma, cataractous nuclei, aqueous humor).This result is consistent with previous reports[30-32] which state that the role of AGEs formed by dicarbonyl compounds in diabetic and nondiabetic cataractogenesis is potentially important because it induces both the structural and functional implications[33] .

It's evident from the present study that the nuclei removed from diabetic cases were more pigmented in comparison to those removed from non-diabetic individuals. This is due to high level of dicarbonyls in nuclei of lenses, which were responsible for browning of lens[7]. The α-crystalline (small heat shock protein) usually functions like molecular chaperone that help in maintaining the transparency of the eye lens. But non enzymic browning of α-crystalline which is done by reactive carbonyls lead to alterations in secondary/ tertiary structure that results in formation of very high molecular weight aggregates due to cross-linking of subunits. This has also been stated that the dicarbonyl induced changes to α-crystallin are relevant to the molecular changes that might occur in the diabetic eye lens as the greater tendency of reactive dicarbonyls to induce intermolecular cross-linked protein aggregates, which could increase the scattering of light[26]. Many other conditions, like environmental conditions, oxidative stress [34,35], metabolic pathways of carbohydrates, e.g., glycolysis [36] and lipids[37] etc can result in the formation of various AGEs by a variety of chemical reactions.

The other risk factors like UV light and diabetes, age and smoking[38] have also been stressed in relation to cataractogenesis apart from oxidative stress. It has been clearly seen in our study that the concentration of dicarbonyls in cataractous nuclei were higher than in plasma and aqueous humor of respective cases in both diabetics and non-diabetic groups. This further explains the role of dicarbonyls in inducing oxidative stress and progress of senile cataract.

Conclusion:

More browning of cataractous nuclei were detected in diabetic cases in comparison to non-diabetics. The levels of dicarbonyls in removed cataract nuclei from both diabetics and non-diabetics were found to be several folds higher than the levels in plasma and aqueous humor of respective cases. The dicarbonyl level in nuclei of diabetic cases were significantly higher in comparison to that of non diabetic individuals (p=0.000).

These results support the hypothesis that AGEs, which are produced by the action of reactive dicarbonyls on various proteins, may have an important role in degenerative changes in human crystalline lens particularly in diabetic patients. The pharmacological agents like antioxidants that reduce the oxidative stress produced by diacarbonyls might help in preventing cataract formation or reducing cataract progression.

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