

Effects of N-acetylcarnosine on Lens Opacities in Diabetic Rats with Cataract

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THE PRESENT study evaluates the effect of 1%N-acetylcarnosine\ (NAC) solution on lens protein in diabetic cataractous rat. Diabetes is induced in rats by intraperitoneal injection of streptozotocin (STZ) and maintained on hyperglycemia for a follow up periods of 1, 3, 5 and 7 weeks. The animals are classified as control, NAC treated group after STZ induced diabetic cataract and NAC treated group before and after STZ induced diabetic cataract. After the estimated periods, levels of soluble lens protein, refractive index (RI), total antioxidant capacity (TAC) and total oxidative capacity are estimated. Further, lens protein conformation changes are analyzed by FTIR spectroscopy. Diabetic cataract significantly decreases the protein level in soluble fraction of lens. Whereas the refractive index is decreased for all diabetic rat lenses. Moreover, diabetic cataract strongly enhances the enhancement of total oxidant capacity (TOC) and decrement in (TAC) with appearance of conformational change in protein assessed by FTIR spectroscopy. Treatment of cataractous lens with NAC before and after STZ induced diabetic cataract in rat suggests better improvement in lens clarity than treatment only after cataract induction. 1% NAC has potential as an in vivo antioxidant because of its ability to give effective protection against cataract and oxidative stress when applied with different techniques of ocular administration.

Keywords: N-Acetyl Carrosine (NAC), Streptozotocin (STZ), Diabetic cataract, Refractive index (RI), FTIR spectroscopy.

Diabetes and its complications have emerged as a major public health concern worldwide as global prevalence of diabetes is rising at an alarming rate. It is estimated that currently around 382 million people have diabetes globally and the number is projected to rise to 592 million in 2030⁽¹⁾. Chronic uncontrolled or poorly controlled diabetes can lead to micro and macrovascular complications⁽²⁾. Blindness due to cataract is an important long-term complication of diabetes. Cataract, characterized by cloudiness or opacification of the eye lens, is the leading cause of blindness all over the world and more so in the developing countries⁽³⁾. The prevalence of diabetic cataract is steadily rising due to increase in number of

people with diabetes all over the world. Studies indicated that the incidence of cataract is much higher in diabetic than in non-diabetic individuals^(3, 4). Though the etiology of cataract is not fully understood, oxidative damage to the constituents of the eye lens is considered to be the major mechanism in the initiation and progression of various types of cataracts, including diabetic cataract⁽⁵⁾. Crystallins are the major structural proteins in the vertebrate eye lens that account for about 90% of the total soluble protein⁽⁶⁾. There are three major crystallins, α , β and γ - belonging to two protein families. Structure, stability and short-range interactions of crystallins are thought to contribute to the lens transparency⁽⁶⁾. The long-term complications of diabetes, including diabetic cataract, are thought to be a result of the accumulation of tissue macromolecules that have been progressively modified by various post translational modifications (PTM). Being long lived proteins with slow turnover, crystallins are known to undergo extensive PTM including oxidation, mixed disulphide formation, deamidation, racemization, truncation, phosphorylation and glycation^(6, 7). Most of these PTM have been shown to occur with aging and are accelerated in clinical conditions such as diabetes. Among them, non-enzymatic glycation has been considered to be one of the mechanisms responsible for both age-related and diabetic cataracts^(8, 9).

Materials and Methods

Three-month old male Wister rats with average body weight of 190 ± 20 g are obtained from the animal house of Research Institute of Ophthalmology, Giza, Egypt. The animals are maintained at a temperature of $22 \pm 2^\circ\text{C}$, 50% humidity and 12 h light/dark cycle. Animal care and protocols are in accordance with and approved by Animal Ethics Committee. The animals are divided into 4 groups as following:

- Group 1. (The control rats): (n = 5 rats) received intraperitoneal injection of 0.1 M sodium citrate buffer, pH 4.4.
- Group 2. (STZ injected rats): (n = 20 rats) received a single intraperitoneal injection of STZ (35 mg/kg) in the same buffer. After 72 h of STZ injection, fasting blood glucose levels are monitored and animals with blood glucose levels >150 mg/dl are fasted overnight and selected for sacrifices after 1, 3, 5 and 7 weeks respectively.
- Group 3. (NAC after STZ injection): (n=20 rats) received daily administration of a topical 1% NAC (Profound product, UK) after a single intraperitoneal injection of STZ (35 mg/kg) and as the previous group the animals were selected for sacrifices after 1, 3, 5 and 7 weeks respectively.
- Group 4. (NAC before and after STZ injection): (n=20 rats) received a topical administration of 1% NAC for two weeks before STZ injection followed by daily administration of 1% NAC after STZ injection and treated in the same manner as the previous group.

Slit lamp examination and lens collection

Eyes are examined for lens opacity using a slit lamp microscope (Kowa SL15, Portable slit lamp, Tokyo, Japan). The eye balls are enucleated and lenses were dissected.

Whole tissue lysate preparation

A tissue samples from the lenses are accurately weighed and part from it is used for FTIR spectroscopy for lens protein. The rest of the lenses tissue samples are homogenized using cell homogenizer (type Tübingen 7400, Germany), in a 10-fold volume of 20 mM ice-cold tris-HCl buffer, pH 7.4. The homogenate is centrifuged for 15 min at 10,000 rpm in a bench centrifuge (Avel centrifuge MF 20, France). The resultant supernatant is used for total protein content, refractive index (RI), total antioxidant capacity (TAC) and total oxidative capacity (TOC). All chemicals reagents were of the highest purity available and purchased from Sigma Chemical Co., St. Louis, MO., USA.

Total protein concentration

Lens protein concentration is determined according to the method of Lowry *et al.*⁽¹⁰⁾. The developing color was measured at 750 nm with a spectrophotometer (type Thermo Fisher Scientific Evolution 600 UV-Vis, Modison, WI 53711, USA).

Measurement of the total antioxidant capacity (TAC)

The total antioxidant capacity is determined by using a colorimetric method performed by the reaction of antioxidants in the lens protein supernatant with a defined amount of exogenously provided hydrogen peroxide (H₂O₂). The TAC is determined at 505 nm by an enzymatic reaction and the results are expressed in terms of mM/g tissue⁽¹¹⁾.

Measurement of the total oxidant capacity (TOC)

The TOC levels of the lens are determined using a colorimetric method⁽¹²⁾. Therefore, the color intensity at 510 nm, is related to the total number of oxidant molecules present in the sample. The results are expressed in terms of mM/g tissue⁽¹³⁾.

FTIR spectroscopy analysis for lens protein

FTIR spectra of different lenses samples are recorded by means of a Thermo Nicolet iS5 FTIR spectrometer, USA, in the range 4000:1000 cm⁻¹ at room temperature. The spectrometer is operated under a continuous dry nitrogen gas purge to remove interference from atmospheric carbon dioxide and water. The data is baseline corrected and smoothed to eliminate the noise before Fourier transformation. One hundred scans are taken for each interferogram at 2 cm⁻¹ resolution. Lenses are weighed, lyophilized and then mixed with KBr powder (98 mg KBr: 2 mg lens) to prepare the KBr disks for FTIR analysis. The spectrum of experimental group is normalized and analyzed for the following spectral regions: 4000:3000 cm⁻¹ (NH-OH region), 3000:2800 cm⁻¹ (CH stretching region) and the fingerprint region (1800:1000 cm⁻¹), which includes the amide I band (1800:1600 cm⁻¹). The average of spectra for each group is obtained using Origin Pro 7.0 software.

Statistical Analysis

Statistical analysis is performed using students' t-test. The results are expressed as the mean \pm standard deviation (SD). Statistical significance was assumed at a level of $P < 0.05$ ⁽¹⁴⁾.

Results

Total protein and refractive index

The total soluble lens proteins as a function of the STZ injected group, group treated after STZ injection with 1% NAC and group treated with 1% NAC before and after STZ injection are shown in Table 1. There are highly significant decreases ($P < 0.01$) in the soluble lens protein content for the diabetic rats after one and three weeks. Moreover, a very highly significant decrease ($P < 0.001$) in lens protein after 5 and 7 weeks of diabetes induction. In addition, the group treated with 1% NAC after induction of diabetes in rats showed highly significant decrease in lens protein ($P < 0.01$) after 1, 3, 5 and 7 weeks respectively. Furthermore, groups treated with 1% NAC before and after induction of diabetes with STZ showed high significant decreases in soluble lens protein ($P < 0.01$) after 1, 3 and 5 weeks and significant decrease ($P < 0.05$) after 7 weeks of treatment. Furthermore, Table 1 shows the direct relationship between the refractive index (RI) and the protein content for all groups.

TABLE 1. Soluble lens protein content and the refractive index for different groups.

Groups	Periods	Total protein		Refractive index
		Mean \pm SD mg/g tissue wet wt.)	Percentage change	
Control	-	458 \pm 7	-	1.3580
STZ	1w	340 \pm 5**	-25.8%	1.3380
	3w	330 \pm 13**	-27.9%	1.3370
	5w	290 \pm 5***	-36.7%	1.3365
	7w	185 \pm 0***	-59.6%	1.3360
NAC after STZ	1w	350 \pm 3**	-23.6%	1.3400
	3w	366 \pm 7**	-20.1%	1.3435
	5w	375 \pm 8**	-18.1%	1.3470
	7w	380 \pm 5**	-17.0%	1.3495
NAC before & after STZ	1w	365 \pm 5**	-20.3%	1.3500
	3w	375 \pm 8**	-18.1%	1.3530
	5w	384 \pm 5**	-16.2%	1.3550
	7w	415 \pm 9*	-9.4%	1.3565

Note: % change with respect to the control group.

*: Significant ($P < 0.05$), **: Highly significant ($P < 0.01$), ***: Very highly significant ($P < 0.001$).

Total antioxidant capacity

Table 2 shows the total antioxidant capacity of rats lens protein after STZ induced cataract and after treatment with 1% NAC. The TAC of control lens was (0.83±0.03) mM/g tissue while the diabetic group injected with STZ shows high significant decrease in TAC (P<0.01), then after one week with a mean value of (0.40±0.03) mM/g tissue and percentage change of -49.2% with respect to the control group. After 3, 5 and 7 weeks the value of TAC indicates very high significant decrease (P<0.001) with mean values of (0.22±0.04) mM/g tissue, 0.19±0.06 mM/g tissue and (0.18±0.05) mM/g tissue respectively.

TABLE 2. Total antioxidant capacity (TOC) and total oxidative capacity for various groups.

Groups	Periods	Total antioxidant capacity (mM/g tissue)		Total oxidative capacity (m M/g tissue)	
		Mean±SD	Percentage change	Mean±SD	Percentage change
Control	-	0.83±0.03	-	0.46±0.01	-
STZ	1w	0.40±0.03**	-49.2%	0.70±0.01***	52.2%
	3w	0.22±0.04***	-73.5%	0.83±0.04***	80.4%
	5w	0.19±0.06***	-77.1%	0.91±0.04***	97.8%
	7w	0.18±0.05***	-78.3%	1.03±0.02***	124%
NAC after diabetes	1w	0.49±0.03**	-41.0%	0.65±0.04**	41.3%
	3w	0.24±0.02***	-71.1%	0.59±0.01**	28.3%
	5w	0.44±0.05**	-45.0%	0.55±0.03**	19.6%
	7w	0.57±0.06**	-31.3%	0.52±0.03*	13.0%
NAC before & after diabetes	1w	0.53±0.03**	-36.1%	0.60±0.02***	30.4%
	3w	0.37±0.03***	-55.4%	0.55±0.02**	19.6%
	5w	0.48±0.03**	-42.2%	0.50±0.01*	8.7%
	7w	0.76±0.04*	-8.4%	0.48±0.01*	4.0%

The group treated with 1% NAC after STZ induced cataract shows a high significant decrease in TAC (P<0.01) with a mean of 0.49±0.03 mM/g tissue after 1 week and high significant decrease (P<0.001) after 3 weeks with a mean value of 0.24±0.02 mM/g tissue. In addition, TAC of lens protein indicates moderate increase after 5 to 7 weeks of treatment with 1% NAC with mean values of 0.44±0.05 mM/g tissue and 0.57±0.06 mM/g tissue and percentage decrease of -45.0% and -31.3% with respect to the control group. The group treated with 1% NAC before and after STZ induced diabetic cataract showed reduction in TAC after 1 and 3 weeks of treatment followed by gradual improvement after 1, 3, 5 and 7 weeks with mean values of 0.53±0.03, 0.37±0.03, 0.48±0.03 and 0.76±0.04 mM/g tissue. The percentage decrease in TAC after 1, 3, 5 and 7 weeks are -36.1%, -55.4%, -42.2% and -8.4% respectively, relatively to the control group.

Total oxidative capacity

Table (2) indicates very high significant enhancement in total oxidative capacity for the STZ induced diabetes group ($P < 0.001$) with mean values of 0.70 ± 0.01 , 0.83 ± 0.04 , 0.91 ± 0.04 and 1.03 ± 0.02 mM/g tissue after 1, 3, 5 and 7 weeks respectively. Moreover, the TOC for the group treated with NAC after STZ induced diabetic cataract shows high significant increase in TOC ($P < 0.01$) after 1, 3 and 5 weeks with mean values of 0.65 ± 0.04 , 0.59 ± 0.01 and 0.55 ± 0.03 mM/g tissue. The percentage increase indicates gradual improvement with values of 41.3%, 28.3%, 19.6% and 13.0% after 1, 3, 5 and 7 weeks respectively. Furthermore, the group treated with NAC before and after diabetes induced cataract shows a better improvement in TOC than the previous group with percentage changes of 30.4%, 19.6%, 8.7% and 4.0% after 1, 3, 5 and 7 weeks respectively.

FTIR spectroscopic analysis

The FTIR results are analyzed for the following spectral regions: $4000: 3000 \text{ cm}^{-1}$, $3000: 2800 \text{ cm}^{-1}$, and $1800: 1000 \text{ cm}^{-1}$, which correspond to NH-OH stretching region, CH stretching region, and fingerprint region, respectively. The control pattern reveals the presence of 11 bands discernible at 3434 ($_{\text{Str}}\text{O-H}$), 3283 ($_{\text{Sym}}\text{O-H}$), 3069 ($_{\text{Str}}\text{C-H}$, N-H), 2966 ($_{\text{Str}}\text{C-H}$), 2875 ($_{\text{Str}}\text{C-H}$), 1646 (Amide I), 1543 (Amide II), 1457 (CH_2 bend), 1399 ($_{\text{Sym}}\text{COO}$), 1318 ($_{\text{Str}}\text{C-O}$), and 1237 ($_{\text{asym}}\text{PO}_2$).

Figure 1 shows the change that has occurred as a result of injury with cataract after intervals of 1, 3, 5, and 7 weeks without treatment by 1% NAC compared with control groups. In the STZ induced diabetes group, some of these bands gradually disappeared like 3283 ($_{\text{Sym}}\text{O-H}$) band. In addition, changing occurs in band width like 3434 ($_{\text{Str}}\text{O-H}$), 1646 (Amide I), 1543 (Amide II), and 1318 ($_{\text{Str}}\text{C-O}$) bands. Meanwhile, there is no noticeable change in the remaining bands.

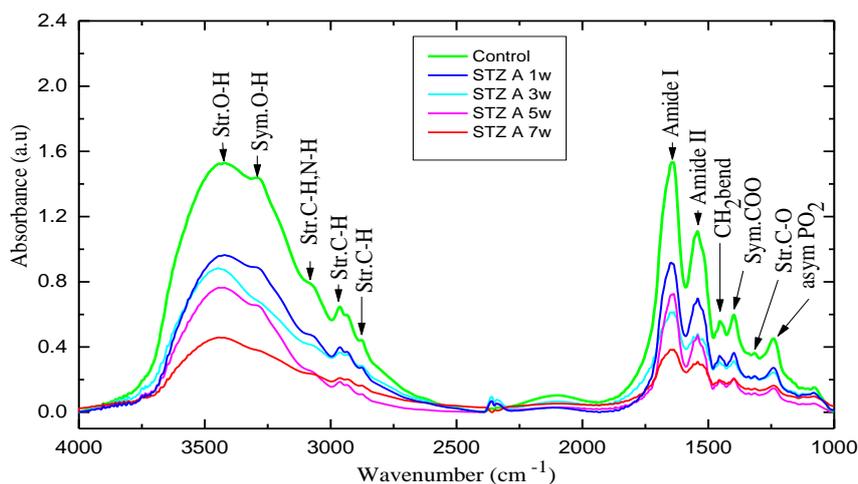


Fig. 1. FTIR spectra for the group of control lens and STZ induced diabetic cataract for different periods.

Figure 2 illustrates the output change occurs after treatment with 1% NAC for 1, 3, 5 and 7 weeks compared with control pattern. There are several changes occur in overall bands after 1 week and these changes begin to improve gradually after 3 and 5 weeks. Moreover, after 7 weeks, most of these changes are disappeared except for the band width in 3434 (Str-O-H).

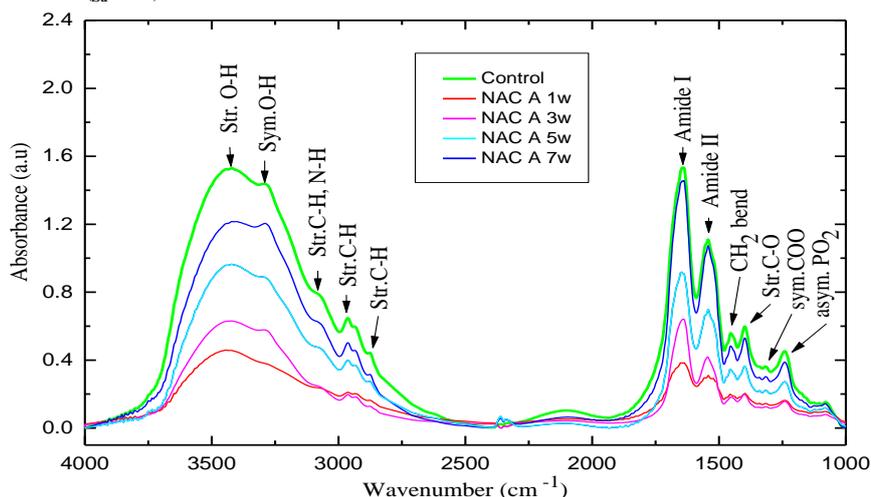


Fig. 2. FTIR spectra for the group of control and lenses treated with 1% NAC after STZ induced diabetic cataract.

Figure 3 shows FTIR spectra of the control rats and STZ induced diabetic cataract in rats treated with 1% NAC two weeks before cataract and after 1, 3, 5 and 7 weeks after cataract. The treatment with 1% NAC before and after induction of cataract shows better improvement than the previous group that take 1% NAC only after induction of cataract. There is an obvious similarity between control pattern and the pattern which dealt with 1% NAC before and after injury with cataract.

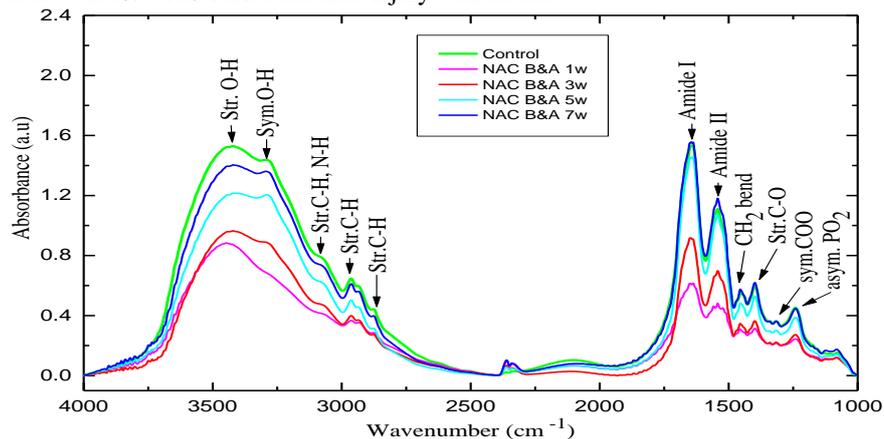


Fig. 3. FTIR spectra for the control and lenses treated with 1% NAC before and after STZ induced diabetic cataract.

Discussion

Diabetic cataract, leads to the opacification of the lens and considered as a complication of diabetes mellitus, which accounts for approximately 42% of all causes of blindness⁽¹⁵⁾. Chronic hyperglycemia and diabetes mellitus duration are major risk factors for this diabetic complication⁽¹⁶⁾.

STZ, a glucosamine nitrosourea compound obtained from *Streptomyces achromogenes*, is a diabetogenic agent extensively used to induce insulin-dependent diabetes mellitus and to study diabetic complications⁽¹⁷⁾. In the present study, because high dose single injections of STZ are used, it is possible that the toxicity to the lenses in a short time come from STZ per se.

L-Carnosine is generally found in mille molar concentrations in several mammalian tissues, potentially exhibiting different metabolic activities. Published data suggest that L-carnosine has excellent potential to act as a natural antioxidant with hydroxyl radical and singlet oxygen scavenging and lipid peroxidase activities⁽¹⁸⁾.

1% NAC solution topically administered to rabbit eyes penetrated into the eye and the native form of L-carnosine accumulated in aqueous humor within 15 to 30 min of administration⁽¹⁹⁾.

The present study evaluates the ability of a topical solution of 1% NAC on lens opacities in diabetic rats with cataract to prevent, or partially reverse, lens cataract. Hence, the investigation of the crystalline levels for the STZ induced cataract group, provide some important clues about their implication in diabetic cataract. The decreased levels of crystallins after 1, 3, 5 and 7 weeks of diabetes by values of -25.8%, -27.9%, -36.7% and -59.7% can be explained by a couple of factors. The altered protein synthesis or increased post translational modifications might be responsible for decreased levels of crystallins in diabetic cataractous lens. Some of the previous studies reported the decreased crystalline synthesis in galactose induced cataract rat model (Kador *et al.*)⁽²⁰⁾ supported the first possibility. Nevertheless, the hyperglycemic condition makes the crystallins highly susceptible to degradation^(21, 22) and crystallins have been long shown to be targeted by multiple types of post translational modifications in the lens. Hence, these findings strongly suggest that, decreased crystallins might be associated with multiple types of post translational modifications. Consequently, the refractive index is significantly decreased, this may lead to impairment of vision due to light scattering. Obviously, the present data show direct relation between the protein concentration and the RI.

After treatment with 1% NAC for 7 weeks the concentration of soluble lens protein and refractive index are obviously improved (-17.0% with respect to the control). These improvement were better in the group treated with 1% NAC before and after diabetes (-9.4%).

Oxidative stress is one of the molecular mechanisms involved in the development of diabetic cataract⁽¹⁵⁾; the accumulations of protein carbonyl (PC), malondialdehyde (MDA), and oxidized sulfhydryls are all consequences of oxidative stress. Kyselova *et al.*⁽²³⁾ observed a time dependent increase in PC and a decrease in the content of protein sulfhydryl in the lenses of STZ-induced diabetic animals in a cataract development experiment.

In addition, Boscia *et al.*⁽²⁴⁾ quantified the level of protein oxidation in human lenses as a function of age and different types of cataract, and came to the conclusion that idiopathic senile, diabetic, and myopic cataractogenesis appeared to be dependent on oxidative damage to lens proteins, and that this damage occurred earlier in myopic and diabetic patients.

In the present study TOC for diabetic rats' lenses is significantly increased during the follow up intervals of 7 weeks (Table 2), and is about 124% greater than the control group. These results suggest that, STZ enhances the oxidative stress in the lenses of induced diabetic rat. A major observation associated with oxidative stress is the drop of TAC that indicates severe cataracts (78.3%). Numerous studies postulate that oxidative stress to the lens mediated by reactive oxygen species and lipid peroxides produced in the crystalline lens can initiate the process of cataractogenesis^(25,26). In addition, it is established that superoxide anion radical, hydroxyl radical, hydrogen peroxide, singlet oxygen and lipid peroxides can be generated by photochemical reactions in the lens surroundings, triggering the development of cataract⁽¹⁸⁾. Furthermore, peroxide damage to the lens plasma membranes may lead to disturbance of their permeability to ions, loss of thiol groups of the membrane-bound crystallins, and the appearance of new fluorophores, together with large protein aggregates and low solubility in the substance of the lens.

By contrast, after 7 weeks both groups treated with 1% NAC after inducing diabetes or treated with this agent before and after inducing diabetes shows improvement in TAC by -36.1% and -8.4% respectively, with comparison to control.

Furthermore, the FTIR spectra of lens protein is changed in the STZ induced diabetes group, such as the frequencies as well as the bandwidths of amide I and amide II. This change is occurred to their band widths suggesting that an initial variation has occurred in the lenses of the STZ-induced diabetic mice. Furthermore, modified crystallins become insoluble by aggregation and cross-linking in rats lenses during cataractogenesis. Moreover, the treatment with 1% NAC before and after induction of cataract shows gradual and better improvement than the group that take 1% NAC only after induction of diabetic cataract.

Conclusion

- The present work reports that, chronic hyperglycemia leads to drop in the levels of crystalline and altered protein of lens and its synthesis. This, might affect
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the aggregation and insolubilization of crystallins under chronic hyperglycemic conditions which ultimately resulting in the development of cataract.

- In the lense of STZ diabetic mice after irrigation by 1% NAC will tolerated when applied as topical antioxidant. The drug has an efficient protection against oxidative stress by turnover into L-carnosine (natural antioxidant).
- 1% NAC should be expected to penetrate through the cornea gradually, thus prolonging the active therapeutic concentration of *hydrophobic L-carnosine* in the aqueous humor of the treated eye.

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(Received 23/ 6/2015;
accepted 18/10/2016)

تأثيرات N-acetylcarnosine على عتامة عدسة العين في الفئران المصابة بالمياه البيضاء نتيجة لمرض السكري

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و*قسم الفيزياء الحيوية - معهد بحوث امراض العيون - الجيزه - مصر .

يعتبر مرض السكر أحد أكثر الأمراض إنتشارا في جميع انحاء العالم ويتوقع العلماء أن عدد المصابين بهذا المرض قد يصل إلى حوالي ٥٩٢ مليون مريض بحلول عام ٢٠٣٠. يتعرض الكثير من مرضى السكر في جميع أنحاء العالم الى الإصابة بمرض عتامة عدسة العين (Cataract) وقد دلت الكثير من الدراسات على أن مرض عتامة عدسة العين يحدث نتيجة إنخفاض في تركيز مضادات الاكسدة الكلوية (TAC) يقابله زيادة في تركيز المواد المؤكسدة الكلوية (TOC).

وقد أجريت هذه الدراسة لتقييم تأثير أحد مضادات الأكسدة وهو (carnosine) %١ (N-acetyl) على بروتين عدسة العين للفئران المصابة بمرض السكر. تم إصابة الفئران بمرض السكر عن طريق حقنها بمادة الإستربتوزيتوسين (STZ) والحفاظ على ارتفاع مستوى السكر في الدم طوال اجراء الدراسة. وقد أجريت هذه الدراسة على ثلاثة مجموعات بخلاف المجموعة الضابطة وتم تقسيم كل مجموعة من الفئران لحقنها بمضاد الاكسدة %١ NAC لفترات محددة ٧،٥،٣،١ أسابيع لكل مجموعة وذلك لمعرفة مدى تأثير مضاد الأكسدة.

وقد أوضحت النتائج أن مضاد الاكسدة %١ NAC يقي عدسة العين من العتامة التي تصيب الفئران مرضى السكر وتزداد فاعلية مضاد الأكسدة عندما يتم إعطاؤه قبل وبعد الإصابة بالمرض وقد إقتربت قيمة تركيز البروتين في عدسة العين من مستوى المجموعة الضابطة. كما أظهرت النتائج زيادة في قيمة معامل إنكسار عدسة العين من ١,٣٥٠٠ عند بداية العلاج حتى وصلت إلى حوالي ١,٣٥٦٥ في نهاية فترة العلاج مقارنة بقيمته في المجموعة الضابطة (١,٣٥٨٠). كما حدث تحسُن تدريجي في شفافية العدسة بعد العلاج بمضاد الأكسدة عند إعطاؤه قبل وبعد الإصابة بمرض السكرى .