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ORIGINAL ARTICLE

L- CARNOSINE EYE DROPS AMELIORATES LENS PROTEINS CONFORMATIONAL CHANGES AND OPACIFICATION INDUCED BY Na SELENITE: AN EXPERIMENTAL STUDY IN RATS

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Abstract:

The purpose of the present study is to investigate the effect of topically applied L-carnosine eye drops on the lens proteins structure and cataract development in an experimental model of selenite induced cataract in rats. Forty-five rat pups aged 13 days were used. They were randomly divided into three equal groups as follows: Group I pups received saline eye drops twice daily (control). Group II pups, (untreated cataract model), received a single subcutaneous injection of sodium selenite (19μmol/kg). Whereas group III rat pups were injected with Na selenite and received one drop of 5% L-carnosine eye drops twice daily. Animals were subjected to ophthalmological examination weekly for three weeks, then sacrificed and their lenses were prepared for biochemical analysis of catalase, superoxide dismutase, reduced glutathione, and malondialdehyde (MDA). The lens soluble proteins were analyzed and monitored by Ultraviolet (UV) absorption spectra, fluorescence spectroscopy and fluorescence of 8-anilino-1-naphthalene-sulfonic acid (ANS). Results showed significant improvement in the tested biochemical parameters including the antioxidant enzymes, proteins structural alterations. Moreover, ophthalmological examination showed marked decrease in the severity and delay in the onset of cataract in the L-carnosine treated group. In conclusion, the results of the present study showed that L-carnosine eye drops markedly ameliorated the structural lens proteins changes, lens opacification and oxidative stress in selenite-induced cataract.

KEYWORDS:

Na selenite, cataract, oxidative stress, L-carnosine, lens proteins.

INTRODUCTION

Cataract is a worldwide leading cause of blindness. It is a major disease both in terms of number of people involved and of economic impact. The research into causative factors and mechanisms to prevent the development of cataract is essential, particularly in developing countries where cataract surgery is often inaccessible (Babizhayev, 2012). Many reports have shown that oxidative stress induced by free radicals, low antioxidants defense capacity and high lipid peroxidation is implicated in the pathogenesis of cataract (Harding, 1992; Guo and Yan, 2006 and Dizhevskaya *et al*., 2012). The oxidative stress of the lens had a

direct influence on the solubility of lens proteins, inducing their precipitation, followed by increase in the lens opacification. The antioxidant enzymes activity levels reflect the changes which took place in the cataract development (Babizhayev and Kasus-Jacobi, 2009). The usual oxidative stress lens parameters tested as an index for the degree of oxidation are: catalase (Cat), superoxide dismutase (SOD), reduced glutathione (GSH) and malondialdehyde (MDA).

SOD is an enzymatic antioxidant which provides defense by overcoming O_2 and converting it into H_2O_2 (Kaur *et al,* 2012). Whereas catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al.,* 2004). GSH is a low molecular weight, thiol containing antioxidant within mammalian cells. GSH protects cellular constituents, structural proteins and enzymes from oxidative damage by directly reacting with oxidants or as the substrate for GSH-peroxidase to scavenge peroxides (Lou, 2003).

Lipid peroxidation represents the oxidative tissue damage induced by hydrogen peroxide (H_2O_2), superoxide anions and hydroxyl radicals. Peroxidation results in structural alteration of cellular membranes, release of organelle contents, loss of essential fatty acids, and formation of cytosolic aldehyde and peroxide products. Malondialdehyde (MDA) is the major end product of the free radical reaction on membrane fatty acids (Donma *et al* 2002).

As a result of oxidative stress, lens proteins structure shows remarkable conformational changes. Lens proteins have a unique structure to maintain translucency and elasticity of the lens. They are mainly composed of crystallins, which are water-soluble proteins that compose over 90% of the proteins within the lens (Andley, 2006). The three main crystallin types found in the human eye are α-, β-, and γ-crystallins (Hoehenwarter *et al*., 2006). β- and γ-crystallins are found primarily in the lens, while subunits of α - crystallin have been isolated from other parts of the eye and the body. α-crystallin oxidative stress proteins belong to a larger super family of molecular chaperone proteins, and so it is believed that the α-crystallin proteins were evolutionarily recruited from chaperone proteins for optical purposes (Andley, 2006).

In addition to providing refractive properties to the lens for focusing the image, it is believed that the molecular chaperone function of α-crystallin is essential in preventing the light scattering and maintaining lens transparency and thereby prevention of cataract. By now, it is fairly acknowledged that decreased chaperone-like activity of α-crystallin is associated with various types and stages of cataract (Babizhayev, 2012).

The pharmacological targeting of safeguarding the natural antioxidant defense systems (e.g., GSH, Cat, and SOD) may aid the development of new therapeutic strategies that could evade the need for cataract surgery and revive lens transparency of the cataractous lenses.

Many antioxidant compounds were studied and applied either systemically or topically for that purpose. However, topical application is considered a more easy and economic way for combating cataract in susceptible individuals.

L-carnosine is a powerful non-enzymatic free-radical scavenger and a natural antioxidant as well. Its systemic administration was reported to have many beneficial effects in ocular diseases (Quinn et al., 1992). L-carnosine and its ophthalmic prodrug N-acetylcarnosine (NAC) are part of group of active peptides (Babizhayev et al., 1996). Carnosine (β-alanyl-L-histidine) was first identified in 1900 in beef extract, hence its name (carno, carnis: meat) (Babizhayev and Kasus-Jacobi, 2009)). NAC, like its parent compound, carnosine, occurs naturally throughout the human body. Both compounds are found primarily in the heart and skeletal muscles and in the brain (Quinn et al., 1992). Growing evidences and discussions of recent patents demonstrated the ability of topical ocular N-acetylcarnosine (L-carnosine prodrug) eye drops to act as a pharmacological chaperone, to decrease oxidative stress and ameliorate oxidative and excessive glycation stress-related eye diseases (Babizhayev, 2011). Moreover, the ocular pharmacokinetic of carnosine 5% eye drops following topical application in rabbits was tested. L-carnosine eye drops were likely to be absorbed into aqueous humor efficiently and accumulated in lens (Tianyang *et a*l., 2010).

The effect of L- carnosine on delaying cataract formation may be through anti-glycation of protein, protection of proteins against cross-links and DNA damage. It operates as a universal aldehyde and reactive oxygen species (ROS) scavenger in both aqueous and lipid environments, consequently preventing ROS-induced damage to biomolecules (Babizhayev, 2012). However, further studies of L-carnosine on its biological features, ocular pharmacokinetics and the mechanisms of delaying the progression of cataract may provide a new strategy for preventing cataract. The present study invites attention to the potential ability of L-carnosine-eye drops to postpone or prevent the development of cataract in experimental model of selenite induced cataract in rats.

MATERIALS AND METHODS

Nine-day-old rat pups (Wistar strain) (obtained from the laboratory animal research house,

Research Institute of Ophthalmology) were used in this study. The pups were housed with parents in large spacious cages, and the parents were given food and water ad libitum. The animal room was well ventilated, and a regular 12 h: 12 h light/dark cycle was maintained throughout the experimental period. The experiment was performed in accordance to the ARVO rules for use of animals in ophthalmic and vision research.

Rat pups were randomly divided into three groups comprising 15 pups each ; Group I, control group, received saline eye drops twice daily .Group II, cataract-untreated, which received a single subcutaneous injection of sodium selenite (19 µmol/kg body weight) on the 10th postpartum day. Animals in this group received one drop of the vehicle solution, twice daily. Vehicle eye drops was prepared without adding L-carnosine. Group III pups, were injected Na selenite and received one drop of 5% carnosine eye drops, , twice a day. L- carnosine eye drops (5%, pH 7.4) were prepared in 25mmol/l sodium phosphate buffer pH 7.4, containing 2.527 g of sodium dihydrogen phosphate dihydrate, 1.36g of disodium phosphate dodeca hydrate, and 800 ml double distilled water.

Ophthalmic examination:

When the pups first opened their eyes (approximately 16 days after birth), a slit-lamp biomicroscopic examination was performed on each eye of each rat pup to provide a morphological evaluation of any lenticular opacification. Prior to performing the examination, mydriasis was achieved by tropicamide eye drops 1% (Alcon Egypt). Direct ophthalmoscope (Wech Allen) and hand held slit lamp (Carl Zeiss) were used to detect the presence of cataract. At the end of the experimental period (postpartum day 30), the degree of lenticular opacification was graded and photographed using Topcon fundus camera. The degree of opacification was graded according to Geraldine et al, using slip lamp to score on a 4-point scale: 0 (extremely clear), 1 (diffuse scattered nuclear opacities), 2 (formation of pinpoint cataracts), and 3 (mature dense opacities involving the entire lens) (Geraldine et al., 2006); at the end of the experimental period animals were anaesthetized using diethyl ether, and lenses were dissected out by the posterior approach. Lenses were immediately blot dried on a blotting paper and weighed and kept in clean glass vials at -20°C till analyzed.

Preparation of lenses for analysis

Lenses from each group were homogenized in ten times their mass of 50mM phosphate buffer (pH 7.2), and centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant obtained was stored at - 70°C in aliquots until used for the analysis

ANALYSIS OF ANTIOXIDANT ENZYMES

Catalase

Catalase (CAT) activity was determined by the method of Sinha (1978). Dichromatic acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2), with the formation of perchloric acid as an unstable intermediate. The green color developed was read at 590 nm against blank on a spectrophotometer. The activity of catalase was expressed as units/mg protein (one unit was the amount of enzyme that utilized 1 mmol of H_2O_2/min).

Superoxide dismutase

Superoxide dismutase (SOD) activity was determined by the method of Nandi and Chatterjee (1988). The degree of inhibition of pyrogallol auto-oxidation by the supernatant of the lens homogenate was measured. The change in absorbance was read at 470 nm against blank every 60 s for 3 min on a spectrophotometer. The enzyme activity was expressed as units/mg protein (one unit was considered to be the amount of enzyme that inhibited pyrogallol auto-oxidation by 50%).

Reduced glutathione (GSH)

GSH content was estimated by the method of Ellman (1959) as modified by Xu et al. (1992). Each lens was homogenized in 1 ml of 0.1 M phosphate buffer, and was centrifuged at 5,000 rpm for 15 min at 4°C. To the supernatant of the lenticular homogenate 10% trichloroacetic acid (0.5ml) was added, followed

by re-centrifugation. To the protein-free supernatant, 4 ml of 0.3 M Na_2HPO_4 (pH 8.0) and 0.5 ml of 0.04% (wt/vol) 5,5- dithiobis-2-nitrobenzoic acid were added. The absorbance of the resulting yellow color was read spectrophotometrically at 412nm. A parallel standard was also maintained. The results were expressed in μmol/g wet weight.

Lipid peroxidation product (malondialdehyde, MDA)

The extent of lipid peroxidation was determined by the method of Ohkawa et al. (1979). Briefly, 0.2 ml of 8.1% sodium dodecyl sulphate, 1.5 ml of 20% acetic acid (pH 3.5), and 1.5 ml of 0.81% thiobarbituric acid aqueous solution were added in succession. To this reaction mixture, 0.2 ml of the lenticular homogenate was added. The mixture was then heated in boiling water for 60 min. After cooling to room temperature, 5 ml of butanol : pyridine (15:1 v/v) solution were added. The mixture was then centrifuged at 5,000 rpm for 15 min. The upper organic layer was separated, and the intensity of the resulting pink color was read at 532 nm. Tetramethoxypropane was used as an external standard. The level of lipid peroxide (MDA) was expressed as nmoles/g wet weight.

Protein carbonyl measurement

Protein carbonyl content of soluble protein was measured using the 2,4 DNPH method and was expressed as the ratio of moles of DNPH reduced (average E_{365nm}=21.0 $mM^{-1}cm^{-1}$)/mg of protein according to Uchida *et al.* (1998).

Protein measurement

Lens protein was assayed using the method described by Lowry et al. (1951) by using bovine serum albumin as a standard.

SDS-PAGE analysis of soluble lens protein

The subunit profile and cross-linking of soluble proteins were analyzed on 10% polyacrylamide gels in the presence of SDS under reducing conditions as described by Laemmli (1970). Proteins were separated on a mini vertical slab gel electrophoretic apparatus. Four weighed lens samples from different groups were homogenized in 0.5ml of Phosphate buffered saline (PBS) solution (pH 7.4), centrifuged at 12,000g for 10 minutes. The supernatant was designated as the water-soluble protein fraction. Proteins were dissolved in sample buffer in the ratio 1:1 and incubated in a boiling water bath for two to three minutes and chilled for one minute, then brought to room temperature. The sample was used immediately. Standard SDS molecular weight markers were used. After electrophoresis, the gel was developed, using coomassie brilliant blue.

Structural alterations

To understand the mechanism for structural and conformational changes of lens soluble proteins, we monitored secondary and tertiary structural states of crystallins by Ultraviolet (UV) spectra and fluorescence spectroscopy.

UV spectroscopy

UV spectroscopy is used to quantify protein and DNA concentrations as well as the ratio of portion to DNA concentration in a solution. A sample of 0.5 ml of the soluble fraction of lens protein was aspired using an aspiration syringe, and diluted to 1 ml with phosphate buffer solution (pH 8.2) in a quartz cuvet to study the UV absorbance of the lens soluble protein. Measurements were taken by using an Uvikon 930 spectrophotometer (KONTRON INSTRUMENTS, Milan, Italy).

Fluorescence measurements

Fluorescence measurements were performed using a Jasco spectrofluorometer (FP-750). For all measurements, 0.15mg/ml protein in 20mM sodium phosphate buffer, pH 7.2 was used. Intrinsic tryptophan fluorescence was recorded by exciting at 280 nm and following the emission between 300-400nm.

Fluorescence of 8-anilino-1-naphthalene-sulfonic acid (ANS) in the presence of soluble lens protein fraction.

For this, lens protein was incubated with 50µM ANS for 30 min at room temperature and the fluorescence of protein bound dye was measured by excitation at 390 nm and following the emission between 450 and 600nm. . The spectra were corrected with appropriate protein and buffer blanks.

Statistical analysis

Values were expressed as the mean ± SE. Differences between groups were assessed by one-way analysis of variance (ANOVA) and paired sample t test using the Statistical Package for Social Sciences (SPSS) software package for Windows (version 10.0). A value corresponding to $p<0.05$ was considered statistically significant.

RESULTS

Ophthalmic examination

Ophthalmic examination showed that 100 % of rats in group II (selenite induced cataract), had nuclear cataract. The cataract started on the third day after injection of selenite and increased rapidly in intensity on the following days to the extent of dense white nuclear cataract of score 3 on the seventh day with anterior cortical opacity. In contrast, 40 % of rats in group III, in which selenite was injected and carnosine eye drops (5%) was applied, showed faint nuclear opacification of scores 1 and 2 which started on the seventh day after selenite injection with no detectable increase in intensity on the following days. The lenses of remaining rats (60 %) in the treated group and the control group remained clear with no visible opacification (score 0) Fig. (1).

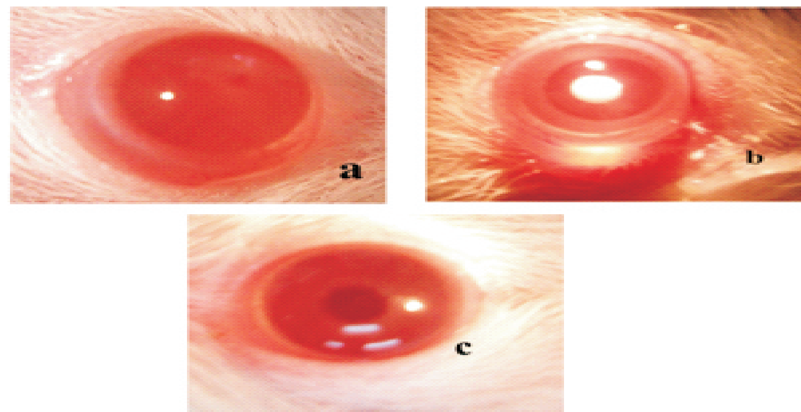

Fig. 1: Anterior segment photography.

(A: A clear normal lens, group I; B: A mature nuclear cataract, group II; C: A clear nearly transparent lens, group III).

Biochemical changes

Table (1) shows that the antioxidant enzymes (CAT and SOD), GSH and total soluble protein decreased significantly in lenses of group II (untreated selenite rats) ($p<0.001$, <0.05, and <0.001 respectively), as compared to control group. Meanwhile, in Group III they improved significantly ($p<0.001$, as compared to group II) with no significant difference from the control group. The carbonyl groups and MDA levels in group II increased significantly ($p<0.001$, as compared to the control group). In group III, there was a remarkable decrease in carbonyl group and MDA levels relative to group II.

Table 1: Levels of catalase, SOD, Carbonyl group, MDA, reduced glutathione and total soluble protein in lenses of rat pups in studied groups.

Parameters \ Groups		Control	Selenite induced cataract	Selenite induced cataract + L-carnosine
Catalase (U/ g protein)	Range Mean±SE	18.51-20.73 19.91±0.47	14.11-16.66 14.92±0.6[a]	18.51+20.37 18.97±0.47[b]
Superoxide dismutase (U/ mg protein)	Range Mean±SE	2.66-2.96 2.85±0.071	1.15-1.79 1.53±0.157[a]	2.45-2.72 2.58 ±0.055[b]
Carbonyl group (nmol/mg protein)	Range Mean±SE	1.66-1.69 1.67±0.075	2.89-3.01 2.95±0.025[a]	1.73-1.79 1.76±0.0125[b]
Malondialdehyde (nmoles/g wet wt).	Range Mean±SE	61.11-66.66 62.49±1.38	72.77-77.77 73.6±1.38 [a]	61.21-64.56 65.27±1.38 [b]
Reduced glutathione (µmoles/g wet wt)	Range Mean±SE	8.33-9.69 8.67±0.34	3.72-7.61 5.6±0.79 [a]	7.69-8.33 7.92±0.15 [b]
Total protein (mg/g wet wt).	Range Mean±SE	277.77-284.44 281.11±0.93	172.55-178.79 175.67±1.8[a]	264.92-271.92 268.3±1.95[ab]

a: $p<0.05$ vs. control group; b: $p<0.05$ vs. selenite untreated group

Structural alternations (UV spectra and intrinsic tryptophan fluorescence)

UV spectral changes of lens soluble proteins in group II (selenite induced cataract) suggested conformational changes at the tertiary-structural level comparative to the control group. These changes were improved in group III (selenite induced cataract + L-carnosine) (Fig. 2). There was loss of intensity of intrinsic tryptophan fluorescence in group II with respect to the control group, which confirmed the altered tertiary structure of lens soluble protein. Treated group (group III) restricted the decrease in spectra with respect to group II (Fig. 3).

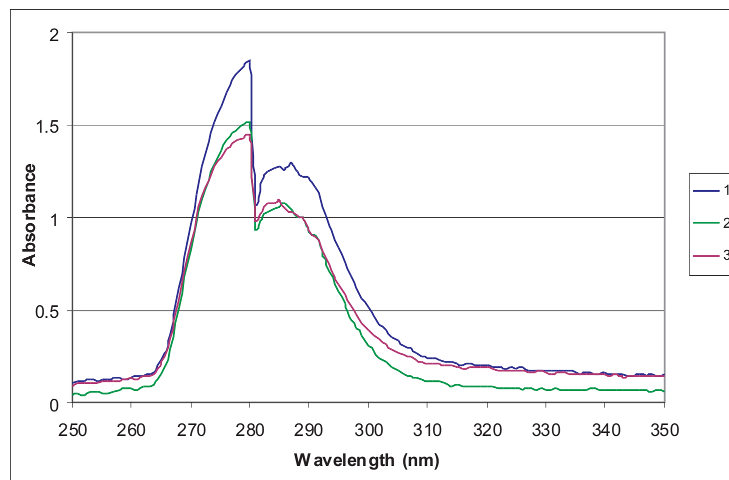

Fig. 2: UV absorption spectrum of lens soluble protein in different groups. Trace 1, control; Trace 2, selenite induced cataract; Trace 3, selenite induced cataract+ L- Carnosine.

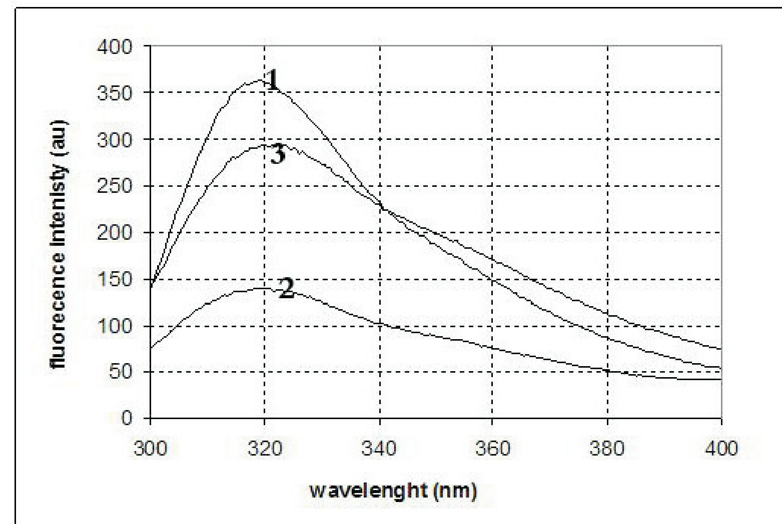

Fig. 3: Intrinsic tryptophan fluorescence spectra of lens soluble protein. Trace 1, control; Trace 2, selenite induced cataract; Trace 3, selenite induced cataract + L-Carnosine.

ANS fluorescence spectra

At a saturating ANS concentration, the fluorescence intensity of ANS (associated with modified lens soluble protein) in group II (selenite induced cataract) was lower when compared with native protein (group I), which correlated well with the decreased soluble protein in group II. Also, the improvement in Group III (treated group) was reflected as increased ANS binding as compared to protein in group II (Fig. 4).

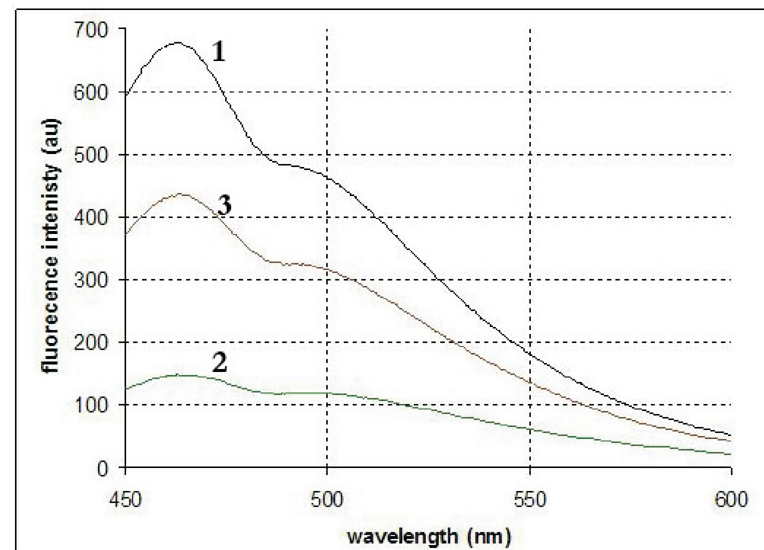

Fig. 4: ANS study fluorescence spectra of lens soluble protein in vivo. Trace 1, control; Trace 2, selenite induced cataract; Trace 3, selenite induced cataract +L-Carnosine.

SDS gel electrophoresis.

SDS-page profile of water soluble protein in Fig. (5) indicated that there were aggregation bands at 44.5, 11, and 113 kDa in group II. These bands in group III (selenite induced cataract +L-carnosine) were found to be similar to that of the normal bands (group I). Fig. (5) showes that there were an aggregation band at 44.5, 11, and 113 kDa in group II (black arrows). These bands in group III (selenite induced cataract +L-carnosine) were found to be similar to that of the normal bands (group I). (Line M; the molecular weight

marker, Line 1; control, Line 2; selenite induced cataract, Line 3; selenite induced cataract + L-carnosine).

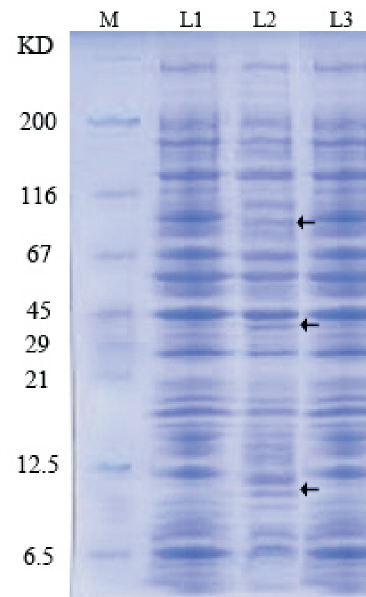

Fig. 5: SDS-page profile of water soluble protein.

DISCUSSION

The present study investigated the possible role of topically applied L-carnosine-eye drops in preventing and treating selenite induced cataract in rats. Cataract was induced by a single subcutaneous injection of sodium selenite in a dose of 4mg/kg to rat pups. Selenite was suggested to inactivate the Na- K-ATPase by oxidation of sulfhydyl groups in the lens epithelium followed by impaired calcium homeostasis, elevated calcium in the nucleus and activation of calpain (Shearer *et al.*, 1997). Calpain is a calcium dependent intracellular cysteine protease; it is the major proteinase in the lens. Proteolysis of crytallins by calpain would lead to abnormal interactions between the crystallins ending in insolubilization of lens proteins and final lens opacities (Shearer *et al.*, 1997). Slit lamp examination of the untreated group showed marked opacities of their lenses reaching 100% of the lenses on the 7th day after selenite injection.

At the present work, the untreated group eyes showed a significant increase in the lenlicular MDA level, which indicated an increase in the oxidative stress or a decrease in the antioxidant defense mechanism. In cases of oxidative stress induced cataract, lipid peroxidation was suggested to be the real cause of destruction of the plasma membrane of lenticular fibres with subsequent oligomerization of lens crystallins. In addition, reduction in the level of antioxidants (catalase, SOD and GSH) was detected in cataractous group lenses suggesting the consumption of these antioxidants for removal of peroxides, which agreed the results of previous studies (Kaur *et al*., 2012).

Monitoring secondary and tertiary structural states of crystallins by Ultraviolet (UV) spectra and fluorescence spectroscopy revealed markedly affected soluble proteins in the lenses of untreated model rats (group II). These changes were found to be conformational changes at the tertiary-structural level. There was loss of intensity of intrinsic tryptophan fluorescence in group II with respect to the control group, which confirmed the altered tertiary structure of lens soluble protein. Moreover, lowered fluorescence intensity of ANS and SDS gel electrophoresis changes indicated a decrease in lenticular soluble proteins levels. These protein changes were explained by Lou and Dickerson (1992). They reported that oxidative stress modifies protein disulfide formation in the human lens and results in cross-linkage and insoluble large-molecular protein precipitants.

In the present study, topical application of L-carnosine eye drops to the treated group showed a significant delay in cataract progression as examined by the slit lamp. Moreover, a remarkable improvement in the oxidative stress enzyme levels, preserved lens protein structure and restored soluble proteins levels were noticed biochemically. This could be explained by various mechanisms as the role of L- carnosine is a mulifactorial integrated process. It was reported that N-acetylcarnosine (an ophthalmic prodrug of L- carosine) considerably suppressed UV induced aggregation of βL-crystallin, so it was supposed that L-carnosine could inhibit βL-crystallin aggregation via formation of a protein-dipeptide

complex that prevents macromolecular conformational changes and ensuing proteins aggregation. (Dizhevskaya *et al*., 2012)

Additionally, L- carnosine has the ability to protect lens from oxidative stress by chelation of transition metals such as copper and iron (Aydogan, 2012). L-carnosine can bind to the Cu^{2+} ions; the copper -carnosine complex exhibited a superoxide dismutase (SOD)-like activity (Kohen et al., 1991). Moreover, Carnosine can inhibit lipid peroxidation (LPO) in membranes by reacting with the necessary LPO products such as aldehydes (Aydogan, 2012).

Another additional role supporting the effects of L- carnosine against cataract is its ability to act as a competitive inhibitor of the non-enzymatic glycosylation of proteins. Therefore, L- carnosine could prevent and reverse the formation of the advanced glycation end-products (AGEs) which play a big role in lens proteins aggregation and cataract formation. AGEs are formed via a non enzymatic reaction between reducing sugars and amino groups, N-terminal groups of proteins, DNA and even free amino acids. AGEs do not only increase in diabetics but it has also been found to be an age related process. Glycation generates marked conformational changes in the lens proteins leading to aggregation and covalent cross linking of crytallins losing their transparency (Babizhayev and Kasus-Jacobi, 2009).

In conclusion, the present work investigated the protective potential of L-carnosine eye drops against Na selenite induced cataract in rat pups. L-carnosine eye drops were found to preserve the lens protein structure against oxidative stress with a marked delay in the onset of cataract. Multiple mechanisms were implicated in the therapeutic potential of L-carnosine. The results of the present study provide a substantial basis for further evaluation of L-carnosine and other antioxidants in the form of eye drops as a new strategy for the treatment and prevention of cataract.

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