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A New Approach for the Treatment of Diabetes and Nicotine Induced Cataract by Modulating Specific Ion-Channels Function in Rat Lens

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Authors' contributions

This work was carried out in collaboration between all authors. Author VRC has designed the study methodology and wrote the protocol. Author KG did experimental portion. Author SM managed the biochemical analyses and wrote the first draft of the manuscript. Author UMV did extensive literature survey. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Cataract is an eye disease characterized by a cloudiness of the normally transparent crystalline lens. Diabetes and smoking are the known risk factors for cataract development. It is well known fact that inorganic ions like Na^+, K^+, Ca^{++} and Cl^- play an important role for the maintenance of lens hydration and transmittance. Nicotine aggravates cataract formation in diabetic patients by disturbing the ionic balance in the lens, generation of free radicals and disturbing normal lens physiology by generation of free radicals. Based upon these observations we have screened few drugs like Lidocaine, Nifedipine and Phenylglycine against Streptozotocin (STZ) + Nicotine induced cataract.
Methodology: Diabetes was induced by administration of combination of STZ (single dose of 52 mg/kg *i.p.*) and nicotine (0.3 mg/kg *s.c*) for 22 consecutive days and simultaneously treated with

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ophthalmic preparation of test drug i.e. Lidocaine, Nifedipine and Phenylglycine at 1% and 2% to the right and left eye respectively. On 23rd day of the study, various parameters like measurement of various ions (Na^+, K^+, Ca^{++} and Cl^-), anti-oxidants (ascorbic acid, sulfhydral group, glutathione) and fructose content in rat lens were studied. We have also investigated the level of ascorbic acid in serum and monitored the blood glucose level at regular interval throughout the experiment.

Results: Accumulation of fructose in the lens cause malfunctioning of Na^+-K^+- ATPase pump which leads to accumulation of Na^+ ions inside the membrane that causes to accretion of water and osmotic swelling of the lens. By the treatment with lidocaine (1% and 2%) eye drop significantly ($P<0.001$) reduced the Na^+ content in both the eyes compared to disease control group.

Conclusion: In our study lidocaine has offered best protection against cataract. Phenylglycine has also shown protection but not as good as lidocaine, at the same time Nifedipine has not shown any protection.

Keywords: Cataract; diabetes; nicotine; Inorganic Ions; Ion-channel blocker.

1. INTRODUCTION

Cataract is a clouding of the lens inside the eye which leads to a decrease in vision. It is the most common cause of blindness and is conventionally treated with surgery. Visual loss occurs because opacification of the lens obstructs light from passing and being focused on to the retina at the back of the eye [1].

Cataract is the leading cause of blindness worldwide and accounts for an estimated 16 million cases of blindness, with approximately half of all cases originating in Africa and Asia [2]. In spite of the progress made in surgical techniques in many countries during the last ten years, and cataract remains the leading cause of visual impairment in all areas of the world (47.9%) except for developed countries.

The common cause of cataract is the normal aging process. Other conditions that can contribute to opacification of the lens include trauma, metabolic disorders (hereditary or acquired), infections (e.g., rubella), medications or congenital problems. The factors that may cause cataract progression, especially nuclear sclerotic cataracts includes smoking, alcohol and exposure to the ultraviolet radiation.

The possible pathophysiological mechanisms include activation of the polyol pathway causing increased hydration, non-enzymatic glycosylation with cross linking of proteins and calpain mediated proteolysis [3].

Ion-channels are pore forming proteins that regulate the flow of ions such as Na^+, Ca^{++}, Cl^- etc. Across a membrane and govern many physiological functions like nervous muscle contraction and hormonal secretions etc. Defects in ion-channels can result in many diseases like myriad disorder, such as heart arrhythmia, cystis fibrosis and many [4]. Ion-channels play important role in the maintenance of lens physiology specifically Na^+, Cl^- and Ca^{++} channel. But surprisingly till date very limited studies have been performed to understand the exact role of these channels in the prognosis and involvement in cataract pathogenesis.

Human data suggests that smokers are always higher risk for development of cataract formation than the non-smokers [5]. Similarly, chewing of tobacco by any other route has grater association for cataract development [6]. Moreover, chronically uncontrolled blood sugar levels is one of the secondary complication for cataract formation. An increased glucose level causes post-translational modifications of lens proteins by non-enzymatic reactions which causes fructose accumulation and subsequent clouding of the lens. By these considerations we have screened various types of ion-channel modulators i.e. Lidocaine, Nifedipine and Pheynylglycine at 1% and 2% ophthalmic preparations to the right and left eye respectively against STZ + Nicotine induced cataract model in male Wister rats by considering various lens and blood parameters like investigation of fructose content, antioxidant levels and inorganic ions in rat lens etc.

2. MATERIALS AND METHODS

2.1 Experimental lab Animal

One hundred and twenty nine (129) male Wistar albino rats of 180-250 gm body weight (13 weeks old) were procured from Teena labs Ltd. (Hyderabad) India.

Streptozotocine, Nicotine, Phenylglycine were purchased from Sigma Aldrich. Nifedipine was gifted by Cipla Pharmaceutical Pvt. Ltd. Mumbai. Lidocaine 2% injection purchased as marketed preparation Lox 2% manufactured by Neon Ltd. Mumbai. All the chemicals used in the experiment are of AR grade.

2.2 Instruments

Micro dissecting scissors, Ultra-Centrifuge (Remi Electrotechnik Ltd), Digital balance (Con tech Instrument Ltd), Tissue Homogenizer (Remi Electrotechnik Ltd), UV spectroscopy (Global Electronics), P^H meter (Global Electronics), Micropipette (Micropet).

2.3 Animal maintenance and Selection

The animals were maintained at a controlled temperature (22–25°C, 45% humidity) on a 12:12-h dark–light cycle. The animals were maintained under standard nutritional and environmental conditions throughout the experiment. All the experiments were carried out between 9:00–16:00 hours at ambient temperature. Nations CPCSEA guidelines were strictly followed and all the studies were approved by the Institutional animal ethical committee (IAEC), (Ref: CPCSEA /1657/ IAEC /CMRCP/Col 11-13/13) CMR College of Pharmacy, Hyderabad, India.

By observing health state and normal behavioral parameters for 10 days: healthy, male rats weighing in between 180-250 gm were selected for the study. Cataract was induced by STZ & Nicotine in all the rats except control group.

2.4 Pilot study for STZ dose Selection

Pilot study was conducted to select the dose of STZ to induce diabetes with minimum mortality and optimal rise of glucose level in the blood. Nine animals were selected and divided in three groups containing three animals in each group. They were administered with 60 mg/kg, 55 mg/kg, and 45 mg/kg of STZ by *i.p.* respectively. After 24 hrs, blood glucose level was recorded 400mg/dl, 250 mg/dl, 140 mg/dl in 60 mg/kg, 55 mg/kg and 45 mg/kg respectively. When there is sustained rise in glucose levels in blood (200-250 mg/dl) the possibility of developing cataract is high. Thus, 52 mg/kg dose of STZ was selected to induce cataract in the experimental animals.

2.5 Cataract Induction

Overnight fasted rats were used in the study to induce diabetes. STZ was administered at a dose of 52 mg/kg *i.p.* prepared in chilled normal saline solution. Basal glucose values were recorded before administrating STZ and 12 Hrs after administration of STZ [7]. The animals in which glucose level is above 200 mg/dl were included in the study while remaining were excluded. Next day i.e. 24 hrs after STZ administration nicotine treatment was initiated at a dose 0.3 mg/kg, *s.c.* for one week. There after the dose of 0.9 mg/kg was given for 3 days. Later the dose was gradually increased by 0.3 mg/kg body weight for every 3 days until a final dose of 2.1 mg/kg body weight is reaches. A total duration of 22 days is required to reach the maximum dose of 2.1 mg/kg Nicotine. This gradual increase in the dose of nicotine is to avoid acute toxicity in nicotine naïve animals [7]. Simultaneously test drugs i.e. Lidocaine, Nifedipine and Phynylglycine were applied eight hourly three times a day in the form of ophthalmic solution in two concentrations i.e. 1% and 2% to the right and left eye respectively for the period of 22 days. Body weight and glucose level of the rats was measured after regular intervals throughout the entire experiment.

Twenty-four hours (i.e. on 23rd day of study) after the administration of last test dose animals were anaesthetized under light ether anesthesia and blood was collected by retro orbital puncture using 10 µl × 20 mm (L) × 0.8 mm (2R) glass capillary into sterile EDTA-coated tube (3 mg/ml). Blood was kept in wet ice for 30 min, centrifuged for 5 min at 4000 rpm at 4°C (REMIMAK, India) and serum was aspirated to find out levels of ascorbic acid and glucose. On the 24th day of the study animals were sacrificed by high dose of the ether anesthesia and total eye ball was opened by a midline incision and finally lens was removed.

2.6 Animal Grouping

One twenty male Wistar rats weighing in between 200 to 250 gm were selected in this study. Animals were divided in five groups by of 24 animals in each. In each group 12 animals were used for estimation of antioxidant levels (i.e. Ascorbic acid, Fructose, Sulfhydryl group and Glutathione) with three for each antioxidant parameter. Remaining 12 animals were used for the estimation of inorganic content (Na^{++}, Ca^{++}, K^{+}, and Cl^{-}) three for each inorganic content. In

all test groups left eye and right eye was treated with 1% and 2% solution of eye drops respectively.

Animal's initial body weight, health status and glucose level of rats were checked and on random basis divided into 5 groups each group containing 24 animals. The animal grouping, treatment schedule is mentioned in Table 1.

2.7 Procedures for Test Drug Preparations

The doses of test drugs were selected based upon earlier pre-clinical experiments. Based on the available literature survey, we have selected "highest safe dose" for the treatment in our experiment.

2.7.1 Lidocaine

Lignocaine hydrochloride 2% injection was purchased from the local market by the brand name Xylocaine 2% injection (AstraZeneca Pharma India limited, Batch no. XZM073G) Lignocaine hydrochloride 2% solution was diluted with the sterile water for injection to prepare the concentration of 1% & and 2% injection. Further formulation was modified from injectable to ophthalmic preparation by adding methylparaffin as preservative and adding hydroxyl propyl methyl cellulose (HPMC K_4) thise as thickening agent to increase contact time with the eye lens. The preparation was stored in plastic PVC container to prevent any contamination.

2.7.2 Nifedipine

From the pure form of Nifedipine powder 1% and 2% sterile ophthalmic solution was prepared by suitably diluting it with sterile water for injection. In the diluted solution benzalkonium chloride was added as a preservative and to increase retention time of the ophthalmic solution with the rat lens HPMC K_4 thise was added as thickening agent.

2.7.3 Phenylglycine

From the pure form of Phenylglycine ophthalmic solution (1% and 2%) was prepared as described above.

Table 1. Dose of treated drug for cataract diseased albino Wistar rats

Group no	Purpose	Treatment & Dose	Dose	
			L. Eye	R. Eye
Group I	Normal control	Normal saline (NS)	2 drops of NS, three times in a day, for 22 days	2 drops of NS, three times in a day, for 22 days
Group 2	Disease control	Streptozotocine, 52 mg/kg, *i.p* once on first day of study Nicotine 0.3 mg/kg *s.c.* for 22 days Normal Saline	2 drops of NS, three times in a day, for 22 days	2 drops of NS, three times in a day, for 22 days
Group 3	Lignocaine Hcl	Streptozotocine, 52 mg/kg, *i.p* once on first day of study Nicotine 0.3 mg/kg *s.c.* for 22 days Lidocaine	1% of sol, 2 drops, three times in a day, for 22 days	2% of sol, 2 drops, three times in a day, for 22 days
Group 4	Nifedipine	Streptozotocine, 52 mg/kg, *i.p* once on first day of study Nicotine 0.3 mg/kg *s.c.*, for 22 days Nifedipine	1% of sol, three times in a day, for 22 days	2% of sol, three times in a day, for 22 days
Group 5	Phenylglycine	Streptozotocine, 52 mg/kg, *i.p* once on first day of study Nicotine 0.3 mg/kg *s.c.*, for 22 days Phenylglycine	1% of sol, three times in a day, for 22 days	2% of sol, three times in a day, for 22 days

2.7.4 Nicotine preparation

Pure nicotine was purchased from the local supplier. 5 mg of nicotine was dissolve in 10 ml of water for injection to make final concentration of 0.5 mg/ml. In the solution benzalkonium chloride was added as preservative. All preparation was done in sterile conditions and the formulation was given by *s.c.* route.

2.7.5 Streptozocin (STZ) preparation

Desired concentration of STZ was prepared by diluting it with cold 0.1M citrate buffer. Citrate buffer was prepared fresh by adding 20 ml of 0.1 M sodium citrate with 30 ml of 0.1 M citrate acid to produce 0.1 M Citrate buffer and PH was adjusted to 4.0 by using 1 N NaOH. Final Solution was filtered and stored in a sterile conical tube on ice.

2.8 Procedure of Removable Lens

2.8.1 Micro dissection

Initially rats were sacrificed by cervical dislocation. Micro dissecting scissors were used to remove the eyeballs from the eyes of 13 weeks old etherized rats. A small incision was made on the posteriorly and pressed on the opposite side of the eye ball. The eye ball was transferred into a 16 ml capacity petri dish containing 5 ml of warm sterile suspension medium. Using 0.1 mm tipped tweezers, any adhering tissue from the lens was cleaned and transferred them to a second 60 ml petri dish containing 5 ml of sterile suspension medium to reduce contamination. The posterior sight of the lens was identified and a small tear is made in the capsule. The capsule is then peeled downwards and pressed into the bottom of the dish which was covered with solidified black color wax. This process was repeated around the entire lens. The lens fiber mass was removed by rocking it sideways. The peripheral of the tissue was cut away and the explants were incubated at 37°C for 8 hrs. Finally isolated lens were transferred to a test tube for bio-chemical estimations (Refer Fig. 1).

2.9 Biochemical Parameters

2.9.1 Estimation of ascorbic acid in rat lens

Estimation of ascorbic acid was done as per the method prescribed by Roe & Kuther [8]. Briefly, rat lens were homogenised in 5 ml of Norit reagent. To 2 ml diluted homogenate 0.5 ml of 2,4, DNPH was added followed by a drop of 10% thiourea. The sample tubes were kept in a boiling water bath for 15 minutes, then cooled in ice for 15 minutes. After cooling, 2.5 ml of 85% H_2SO_4 was added to each tube, and the tubes were left for 30 minutes for incubation. The absorbance of the sample solution was read at 540 nm in a spectrophotometer. The standard tube was run with four ml. of ascorbic acid solution (10 µg/ml), and the blank tube with 4 ml of TCA (6%) instead of tissue homogenate. The ascorbic acid content in each sample was calculated, using the standard concentration and optical density, expressed in µg/100 g. wet lens tissue wt. or µg/ml. Results are mentioned in Table 2.

2.9.2 Estimation of fructose in rat lens

Estimation of fructose was done as per the method prescribed by Foreman et.al. [9].

Briefly, lens samples were homogenized in 5 ml of perchloric acid (PCA) (5%) 2 ml of the lens homogenates was taken into a 10 ml test tube containing 1ml of 0.1% resorcinol and 3ml of 30% HCl. The samples tubes were warmed in water bath at 80°C for 1hr. After warming, all the tubes were cooled and absorbance was recorded at 410 nm in spectrophotometer. Simultaneously 2 ml of 5% (PCA) was prepared and used as blank.

The concentration of fructose in each sample was calculated using standardized conversion factor (cot θ = 0.90; Eq. F = 123.46). Results are mentioned in Table 2.

2.9.3 Estimation of sulfhydryl group in lens

Estimation of ascorbic acid was done as per the method prescribed by Foreman et al. [9]. Sedlak and Lindsey (1968). Briefly, lens were homogenized in 8 ml of 0.0lM EDTA at 4ºC. 0.5 ml of the lens homogenate was taken and mixed in a 15 ml test tube containing 1.5 ml of 0.2 M tris buffer (PH 8.2) and 0.1 ml of 0.01M DTNB. The mixture was made up to 10 ml by adding of absolute methanol. Occasionally this mixture was shacked for every 15 min and centrifuged at 3000 rpm at room temperature for 15 min. then absorbance was recorded at 412 nm in UV spectrophotometer. Results are mentioned in Table 2.

2.9.4 Estimation of glutathione in rat lens

Determination of glutathione concentration in the samples was carried out by the method of Grunert and Philips [10]. Briefly, isolated lens

sample was homogenised in 3 ml of metaphosphoric acid (3%) and 1 ml of double-distilled water. The blood samples (0.5 ml) was haemolysed in one ml of double-distilled water and 2.5 ml. of metaphosphoric acid (3%). The homogenised samples were saturated with NaCl granules. It was mixed well, centrifuged at 5000 rpm for 15 mins at 4-8°C. Two ml of the supernatant was collected. After equilibration at 20°C for 5-10 minutes, 1ml of sodium nitroprusside solution was added immediately followed by 1 ml of sodium carbonate-sodium cyanide (1:1) solution. The optical density of the resulting colour was measured soon after the addition of sodium carbonate-sodium cyanide solution at 520 nm on a Colorimeter. Two ml. of metaphosphoric acid (3%) saturated with sodium chloride was used as the blank.

The glutathione concentration in each sample was calculated using reduced glutathione as the standard. The standardised conversion factor was (cotθ = 0.3. Eq.F = 1111.14). Results are mentioned in Table 2.

2.10 Elemental Analysis (Estimation of Inorganic Ions in Rat Lens)

The elemental analysis of the lens was done by the method prescribed by Duncan and Bushell [11]. Briefly, Wistar rat lens were extracted by the posterior approach, blotted on filter paper, moistened with deionized water and ethylene glycol-bis-(β amino ethyl ether) N,N'-tetra acetic acid (1 mM). The blotted lens was further processed for analysing K^+, Na^+, Ca^{2+} and Cl^-. The concentration of these elements was expressed in percentage.

2.10.1 Determination of potassium and sodium

The concentration of K^+ and Na^+ in the lens samples was determined by Flame Photometry.

The weighed lens samples was digested to dryness alternately in 3 ml concentrated $HN0_3$, and then in one ml H_2O_2. Residual salt (ash) was dissolved in HCl (1%) containing 0.2% lanthanum chloride and made up to 50 ml. This solution was used for K^+ and Na^+ analysis was done by using flame photometry. Results are mentioned in Table 3.

2.10.2 Determination of Ca^{++} and Cl^-

The weighed lens sample was digested to dryness alternatively in 3 ml of concentrated HNO_3 and in 1 ml H_2O_2. Residual salt (ash) was dissolved in HCl (1%) containing 0.2% lanthanum, and made up to 50 ml. This solution was used for Ca^{++} and Mg^+ analysis by using the atomic absorption spectrophotometer. Results are mentioned in Table 3.

2.10.3 Determination of serum Ascorbic acid levels

Serum Ascorbic acid levels was calculated on the 23rd day of the study 2 hours after the last drug administration by the method prescribed by Roe and Kuether, [8] and calculated against its standard curve absorption values. For results refer Fig. 2.

2.10.4 Determination of Serum glucose levels

Random serum glucose level was checked for all groups of the animals except for the normal control group for every three days interval for thirty days by using commercial Accu-Chek blood glucose meter (see results in Fig. 3).

2.11 Statistical Analysis

Results are expressed as the mean ± S.E.M. Significance was evaluated using one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software. Values are significant at *#P<0.05, **##P<0.01 and ***###P<0.001.

3. RESULTS AND DISCUSSION

Cataract affected around 50 million people world-wide and reduced visual fields and compromised the quality of life. Many hereditary and environmental risk factors are recognized for the development of cataract like glaucoma, ocular inflammation, trauma, aging, ultraviolet rays, smoking and diabetes. Clinical study revealed that diabetes and tobacco consumption (by any route) are major risk factors for the development of cataract. Taking all together, uncontrolled diabetes patients having smoking habit are the highest risk for the development of cataract. Increased glucose levels causes post-translational modifications of lens proteins by non-enzymatic reactions and nicotine accentuates free radical formation causes fructose accumulation and subsequent clouding of the lens. Many pre-clinical studies are carried out to induce lens clouding by using Streptozotocin (STZ) in rats.

Table 2. Effect of Lidocaine, Nifedipine and Phynylglycine Eye drop (1% and 2%) on the Ascorbic acid, Fructose, Sulfhydryl group and Glutathione content of rat lens against STZ and Nicotine induced diabetic cataract

Group	Treatment	Biochemical parameter							
		Left (Eye)				Right (Eye)			
		Ascorbic acid (µg/100 mg wet wt)	Fructose (µg/mg wet wt)	Sulfhydral group (µM/G Wet Wt)	Glutat hione µg/mg wet wt	Ascorbic acid (µg/100 mg wet wt)	Fructose (µg/mg wet wt)	Sulfhydral group (µM/G Wet Wt)	Glutat hione µg/mg wet wt
I	Control 0.9% Nacl	2.42±0.004	6.02±0.033	57.19±0.29	2.76±0.60	2.49±0.040	6.06±0.037	57.62±0.013	2.71±0.06
II	Nacl+STZ+Nicotine	1.80±0.049###	6.19±0.033##	35.44±0.28###	1.17±0.038###	1.82±0.044###	6.23±0.037##	35.39±0.24###	1.17±0.04###
III	Lidocaine+Nacl+STZ +Nicotine	2.31±0.086***	6.11±0.023	51.22±52.82*** ###	2.48±0.098***#	2.51±0.020***	6.13±0.011	52.32±0.39*** ###	2.57±0.070***
IV	Nifedipine+Nacl+STZ+ Nicotine	1.70±0.029###	6.16±0.024##	30.70±0.39*** ###	1.47±0.032*## #	1.59±0.075###	6.24±0.033##	30.15±0.50*** ###	1.53±0.164###
V	Phynylglycine+Nacl+STZ+Nicotine	2.03±0.046*##	6.10±0.015	43.90±1.13*** ###	1.94±0.026*** ###	2.08±0.095*##	6.15±0029	47.93±0.40*** ###	1.82±0.075** ###

*All of the data obtained from the experimental groups have been compared to disease control and normal control groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software. Values are significant at *#P<0.05, **##P<0.01 and ***###P<0.001. # Comparison of disease control and all test groups with normal control group. * Comparison of test groups with disease control group.*

Table 3. Effect of Lidocaine, Nifedipine and Phynylglycine Eye drop (1% and 2%) on the Na^+ Ca^{++} K^+ and Cl^- content of rat lens against STZ and Nicotine induced diabetic cataract

Group	Treatment	Inorganic ion % dry weight							
		L(eye)				R(eye)			
		Na++	Ca++	K+	Cl-	Na++	Ca++	K+	Cl-
I	Normal control 0.9% NaCl	0.196±0.007	0.004133±0.001	0.56±0.060	13.50±0.17	0.18± 0.006	0.003±0.000	0.56±0.009	13.47±0.12
II	NaCl+STZ + Nicotine	0.199±0.007###	0.004867±0.001	0.52±0.006###	17.733±0.12###	0.19±0.001###	0.0050±0.001	0.52±0.006###	17.467±0.12###
III	Lidocaine+NaCl + STZ+Nicotine	0.195±0.006***	0.004767±0.001	0.56±0.005***	12.47±0.23***##	0.199±0.003***	0.0044±0.000	0.56±0.007***	12.70±0.11***##
IV	Nifedipine+NaCl + STZ+Nicotine	0.200±0.004###	0.005667±0.001	0.52±0.005###	10.63±0.14***###	0.20±0.006###	0.0055±0.001	0.52±0.007###	10.70±0.057***###
V	Phynylglycine+NaCl +STZ+Nicotine	0.19±0.0038###	0.0046±0.0006	0.51±0.007###	12.50±0.15***##	0.204±0.007###	0.004±0.0006	0.51±0.008###	12.47±0.18***###

*All data obtained from the experimental groups have been compared to disease control and normal control groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software. Values are significant at *#P<0.05, **##P<0.01 and ***###P<0.001.# Comparison of disease control and all test groups with normal control group; * Comparison of test groups with disease control group.*

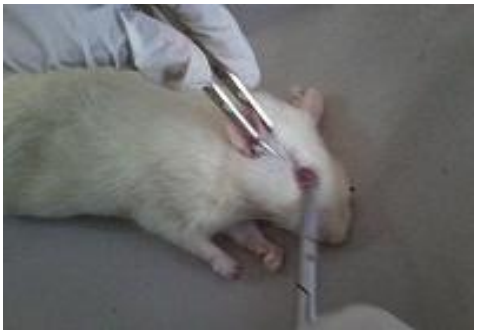

Fig. 1a. Removal of rat eye fibrous tissue

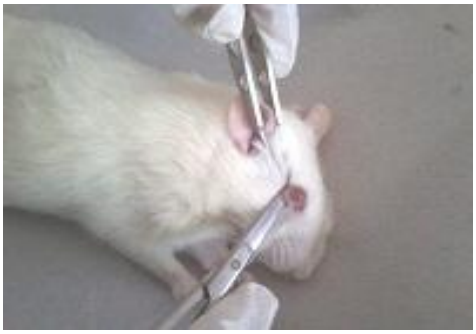

Fig. 1b. Removal of total eye lid

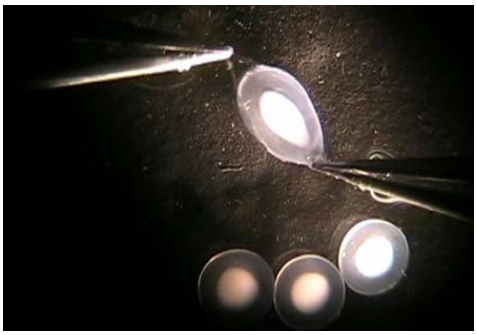

Fig. 1c. Small tear is made in the capsule

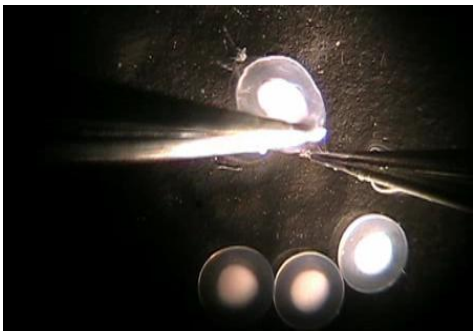

Fig. 1d. Eye boll peeled to words the wax

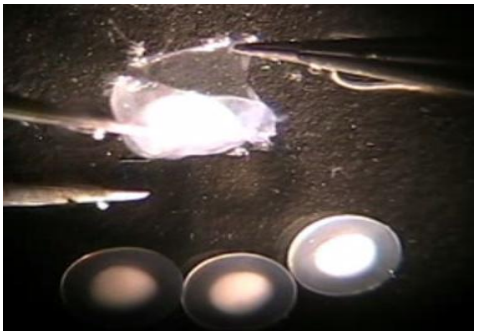

Fig. 1e. Initial **s**eparation of lens from the eye ball

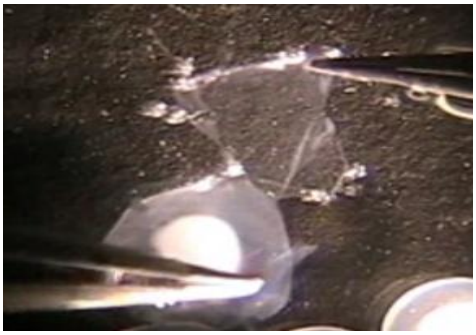

Fig. 1f. Separation of lens from the eye ball

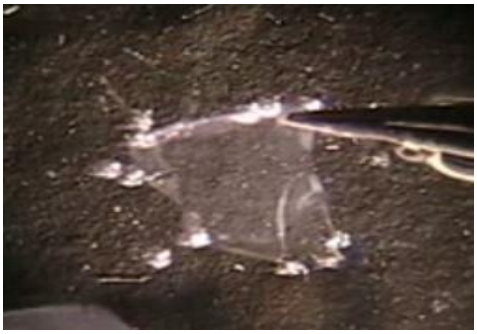

Fig. 1g. Blotting of Separated lens on wax tray

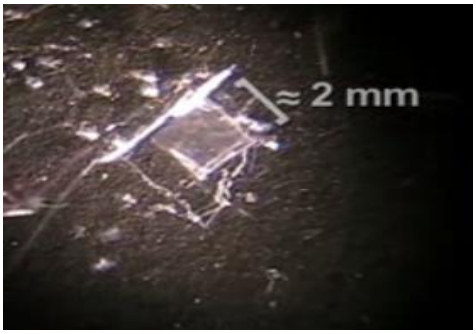

Fig. 1h. Cutting of eye lens in a specific dimensions

Fig. 1. Procedure for the removal of rat eye lens

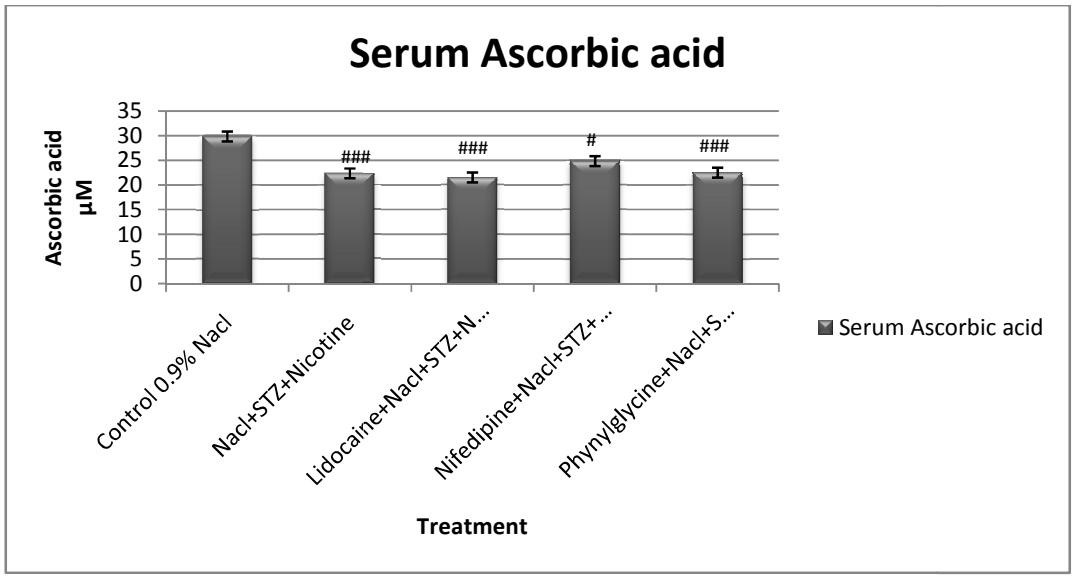

Fig. 2. Effect of nicotine + STZ on serum ascorbic acid level in male rats treated with various test drugs lidocaine, nifedipine and phynylglycine eye drops (1% and 2%) for its anti-cataract activity

*All the data obtained from the experimental groups have been compared to disease control and normal control groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software. Values are significant at *#P<0.05, **##P<0.01 and ***###P<0.001.# Comparison of disease control and all test groups with normal control group* Comparison of test groups with disease control group.*

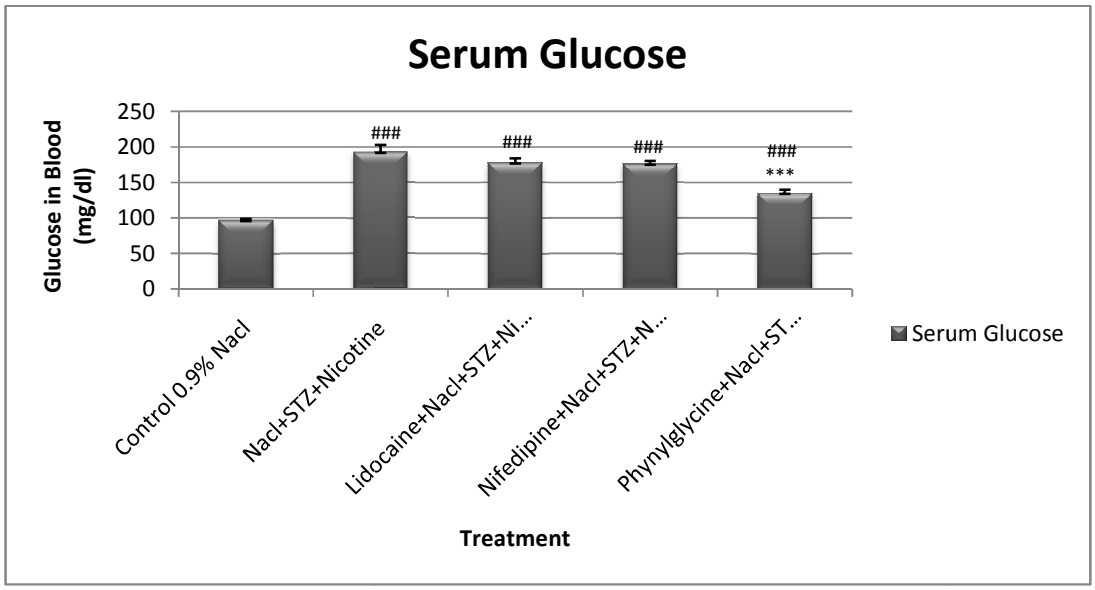

Fig. 3. Effect of nicotine + STZ on serum glucose level in male rats treated with various test drugs lidocaine, nifedipine and phynylglycine eye drops (1% and 2%) for its anti-cataract activity

*All the data obtained from the experimental groups have been compared to disease control and normal control groups. The data was analysed statistically by one-way ANOVA followed by Dunnett test using Graph pad prism version 5.0 software. Values are significant at *#P<0.05, **##P<0.01 and ***###P<0.001.# Comparison of disease control and all test groups with normal control group* Comparison of test groups with disease control group.*

Hyperglycemic environment results in greater susceptibility of ocular tissue to further damage from environmental factors and stress.

It is well known that nicotine and diabetes are independent etiological factors for the cataract and moreover diabetic patients who consume tobacco in any form are on greater risk for the development of cataract. Based upon these clinical observations we have selected nicotine + STZ induced model to induce cataract in male Wistar rats by considering various lens and blood parameters.

Oxidative stress is an initiating factor for the development of cataract and describes the events leading to lens opacification. The toxic species of oxygen formed in lens milieu contributing free radicals for the pathology of cataract include superoxide anion, lipid hydroperoxides, OH^- radicals and H_2O_2. Elevated H_20_2 levels can cause lens opacification compared to other free radicals so H_2O_2 is the major oxidant involved in cataract formation. Endogeneous antioxidant like reduced glutathione is present in high concentration in lens, superoxide dismutase and catalase keep the level of free radicals below toxic levels. In cataractous lens the concentration of SOD and Catalase are decreased [12]. Hence, with the use of antioxidants cataract formation can be prevented.

In the normal, young human lens, there is no oxidation of the cytosolic protein and no oxidation in the membrane fraction. All thiols are buried in the interior of the macromolecular structure. In normal lens, some membrane protein oxidation is apparent at the age of 60-65, but still there is no oxidation of cytosolic protein. Only about 50% of the protein thiols remain buried at the age of 60-65 years. In cataract, the picture is dramatically different that all the thiols are exposed and massive oxidation of thiol to both protein and mixed disulfides (probably with GSH) as well as cystic acid is observed.

Based upon this pathological role of the free radicals in cataract we have considered measured of anti-oxidant levels of the lens and in blood.

In the present study, disease control group has shown significant ($P<0.0001$) decrease in all antioxidant levels as per the expected outcome and earlier findings. Lidocaine 1% and 2% have shown significant increase in the sulfhydral group ($P<0.001$) and glutathione ($P<0.05$) content when compared with disease control group. Ascorbic acid content is also increased but was insignificant. Increase in the oxidative stress of the lens is because of nicotine which is synergized with high glucose level.

Nicotine is low molecular weight and highly lipid soluble alkaloid easily cross many barriers like blood-brain barrier (BBB) and skin with great ease [13]. Nicotine is a major composition of many tobacco products affects immune response and mediates inflammatory cell signaling cascades [14]. Moreover, after prolong use nicotine has been shown to decrease insulin sensitivity consumed by as tobacco product or gum insulin [15].

Nicotine causes oxiadative stress and forms reactive oxygen species (ROS), superoxide and hydrogen peroxide [16]. In hyperglycemic rats, because of excess glucose poylols accumulation takes place and causes swelling of lens fibers. Nicotine generated ROS interact with rat lens proteins and lipids causing additional damage to the already weaken lens fibers leading to severe. Cataract. Furthermore, free radical reduces the cellular antioxidant defense and inhibits the level of antioxidant enzymes [17]. So, studies revels that dietary intake of antioxidants such as riboflavin, vitamins C, Vit E and carotene have protective effect against cataract formation [18]. Systemic hyperglycemia and diabetes in rats have been associated with development of lens cortical lesions. Additionally, in our study increased level of glutathione, sulfhydryl group and ascorbic acid demonstrated protective properties in the context of nicotine-induced oxidative damage by the lidocaine in a dose dependent manner due to its antioxidant property [19]. Surprisingly, Nifedipine also has proven antioxidant property. But in our study it has not shown any protection against free radicals and ROS generation.

Ion-channels are a diverse group of pore-forming transmembrane proteins that selectively conduct ions and play physiological roles in most cell types including neurons, skeletal muscle, smooth muscle and cardiac muscle [20]. Inherited mutations in genes encoding ion-channels have been associated with a large number of human diseases like epilepsy, febrile seizures, Dent's disease and cardiac arrhythmias. The disorders are called "channelopathies" [21]. Expression of ion-channels in heterologous systems allows for investigation of inherited ion- channel defects at

the single protein and cellular level to directly identify the disease-associated alterations in ion-channel function [21]. The lens volume is a balance of two opposing forces: a) the normal permeability characteristic of the lens membranes; b) is the efficient cation pump that continually extrudes Na^+ ions and concentrates K^+ ions. The intraocular fluids bathing the lens contain a high level of the Na^+ and low K^+. While the cations in the lens have the opposite composition of high K^+ and low Na^+. Thus, if allowed to come to equilibrium Na^+ would enter and K^+ would leave the lens. Because the lens membranes are impermeable to proteins the situation which allows for free exchange of cations would eventually lead to a donnan type of swelling [22]. In the lens, however, the cation pump mechanism linked to active metabolism normally prevents from free swelling.

Lens as a pump leak system in which the levels of cations are regulated by a balance between active uptake and passive diffusional processes. Na^+ enters principally across the posterior surface by passive diffusional uptake and then diffuses anteriorly reaching the epithelium where it is actively extruded. In contrast, K^+ is actively transported into the lens at the anterior epithelial surface, diffuses posteriorly and leaves the lens by diffusing across the posterior capsule. These ions are constantly entering and leaving the lens and the forces that move the cations are the differences in chemical potential of the lens and the active transport mechanism which is primarily located in the epithelium. Maintaining the pump-leak balance is crucial to preserve the viability of the lens.

In diabetes induced cataract lens swelling may seen due to exposure of more glucose molecules. In lens, glucose is converted to sugar alcohol by the enzyme aldose reductase. The sugar alcohol is unable to further metabolize and penetrate the lens membranes accumulate in lens fibers. The hypertonicity it creates is immediately corrected by an influx of water. The osmotic change that occurs initially with sugar alcohol accumulation does not seriously alter the state of viability. In the early stages of diabetes cataract formation the process is reversible. However, when the lens is maintained in a swollen state for a prolonged period, other changes occur, such as the loss of free amino acids and redistribution of cations. Redistribution of cations is may be due to malfunctioning or altered kinetics of Na^+, K-ATPase pump [23]. Actually, the lens attempts to maintain its normal distribution of cations by accelerating the pump mechanism to compensate for the increase in permeability to cations. Eventually, however, the increase in cation permeability cannot be compensated and thus Na^+ and Ca^+ ion level increases while K^+ and Cl^- ion levels are drastically reduced. Overload of Ca^{++} ions in the lens membrane causes activation of calpains which ultimately activates many proteolytic enzymes which digests lens membrane proteins. It is a well known fact that K^+ and Cl^- ions are involved in maintaining the lens hydration and transmittance loss of these ions from the lens cause lens swelling and opacification [24].

Based upon all these observations and the critical role of the inorganic ions, we have investigated the concentration of various inorganic ions like Na^+, Ca^{++}, K^+ and Cl^- in the rat lens. In the present study we have used three test drugs i.e. Lidocaine as Na^+ channel blocker, Nifedipine as Ca^{++} channel blocker and phenylglycin as Cl^- channel opener. By treatment with Lidocaine eye drop has shown significant ($P<0.001$) decrease in the Na^+ and a significant ($P<0.001$) increase K^+ content in the rat lens compared to the disease control group. But Nifedipine and phenylglycin have not shown any protection at either dose levels. As described earlier, K^+ ions are important for maintaining lens hydration and transparency. Thus the presence of these ions in their normal limits might have shown protection against cataract formation. In the present study, there was significant increase in lens fructose content in the diseases control group when compared to normal control group. But, treatment with lidocaine eye drop (1% and 2%) the fructose content was reduced significantly in disease control group more than normal control group.

In STZ + nicotine induced cataract nicotine aggravates the diabetes by reducing insulin sensitivity and increasing oxidative stress on the eye lens leading to development of cataract. Under hyperglycemic conditions glucose is reduced to sorbital by the enzyme aldose reeducatase by polyol pathway. Recently the conversion of sorbitol to fructose via sorbital dehydrogenase has been suggested to contribute to redox imbalance in diabetic tissues. This finding led to conclusion that major culprit for diabetic cataract is fructose accumulation [25]. Thus, in the present study we have considered measurement of fructose level in rat lens. Accumulation of fructose in the lens might have compromised functioning and working of

Na^+-K^+- ATPase which causes accumulation of Na^+ inside the membrane leading to hypertonicity (accumulation of water) and osmotic swelling of the lens. In our study, lidocaine have shown considerable protection by blocking sodium channels in the lens and preventing efflux of Na^+ ions from lens to intraocular fluid.

Apart from the Na^+ and K^+, Cl^- ions also play a vital role for maintaining lens integrity and hydration. Thus Cl^- ions is measured in the lens. Thought behind the use of phenylglycine is that earlier literature reveals the use of tamoxifen for the treatment of breast cancer lead to the development of cataract in many patents. Tamoxifen is a potent Cl^- channel blocker which might elect specific side effects when administered therapeutically. Thus, phenylglycine was used as test compound a 'Cl^- channel opener' reduces accumulation of Cl^-ions in the rat lens and prevents lens opacification.

4. CONCLUSION

In our study lidocaine have shown considerable protection by blocking Na^+ channels in the lens and preventing efflux of Na^+ ions from lens to intraocular fluid. Moreover, lidocaine has already proven anti-oxidant property that has tackled oxidative stress by neutralizing free radicals. In the present study, Phenylglycine also has offered protection against cataract by opening Cl^- channel on lens membrane but anti-cataract activity was less potent than that of lidocaine.

CONSENT

Not applicable**.**

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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