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Research Article

PROTECTIVE EFFECT OF AMINO ACIDS ON EYE LENSES AGAINST OXIDATIVE STRESS INDUCED BY HYDROGEN PEROXIDE

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ABSTRACT

The present study aims to investigate the effect of amino acids on mammalian eye lenses against oxidative stress *in vitro*. Oxidative insult by hydrogen peroxide (H_2O_2) cause glutathione (GSH) depletion and cross-linking of the endogenous proteins within the lens of the eye that lead to reduced total soluble protein (TSP). Antioxidant property of amino acids is reported in food and oil industry. However, Limited information is available on effect of independent amino acids on lens in cataract and the prospectives of topical application. Present study describes that presence of certain amino acids surrounding lens in an *in vitro* cataract model have preventive role against oxidative stress. Total soluble protein (TSP) and glutathione (GSH) were taken as markers for the study. Paired isolated lenses of goat were used to evaluate the effect of Glycine or L –Tryptophan or L-tyrosine or L-Phenylalanine or L-Histidine or L-Lysine or L-Arginine or L-Cysteine or L-Glutamic acid or L-Aspartic acid or L-Proline at 1 mM concentration against oxidative stress produced by 1 mM H_2O_2 solution. After 24 h of incubation at 37^{0}C, the lenses treated with control (H_2O_2 solution) and test solutions (H_2O_2 solution containing amino acids) were taken out and estimated for TSP and GSH content and compared statistically determined by paired t-test, $p< 0.05$ was considered significant. The results indicate significant protective role of selected amino acids in H_2O_2 induced cataract model *in vitro*. The study may open new prospective in cataract prevention by topical application of amino acids in the form of eye drops.

Key words: Amino acid, Eye lens, Antioxidant, Cataract.

INTRODUCTION

Cataract is an ocular condition characterized by opacification of the crystalline lens in the eye which leads to blurring of vision. The natural lens is a crystalline structure composed of water and proteins arranged in a precise structure to create a clear passage for light to pass through it, but with aging, the lens becomes opaque, thus reducing the amount of light reaching to the retina[1, 2]. Cataract is a leading cause of blindness globally. Nearly 19 million people are blind due to cataract in the world and was estimated to affect 20 million people world wide and accounted for 47.8 % of total blindness in the world .The age-related prevalence of cataract in India is three times that of the United States [3, 4]. Age is the single most important risk factor for the occurrence of cataract. Apart from aging, various risk factors such as nutritional deficiencies or inadequacies, diabetes, sunlight, environmental factors, smoking, and lack of consumption of antioxidants are known to increase the risk of cataract[5, 6, 7]. Though the etiology of cataract is not fully understood, oxidative damage to the constituents of the eye lens is considered to be a major mechanism in the development of cataract [8, 9]. Hydrogen peroxide (H_2O_2) induces similar oxidative stress as occurs in cataract. Oxidative insult by hydrogen peroxide (H_2O_2) cause glutathione (GSH) depletion and cross-linking of the endogenous proteins within the lens of the eye that leads to reduced total soluble protein (TSP) content in cataract lens as compared to normal one[10-13]. The lens contains a high concentration of soluble amino acids upon which in part its transparency is dependent. Free amino acids present in the lens, can be broadly divided into two categories: proteogenic amino acids (amino acids from which proteins are synthesized) and non-proteogenic amino acids (these amino acids are not protein constituents). Free amino acids play an important role as precursors of lens proteins, hence, the study of lens free amino acids provides a most suitable subject for study. Studies on rats indicated that deficiency of certain amino acids lead to cataract[14]. Cataract lenses have been found to have imbalance in lenticular levels of certain amino acids when compared to normal. It is speculated that an amino acid transporter system may be upregulated in patients with cataract and may have some role in cataract development[13].With maturation of the cortical cataract water content, wet weight and sodium concentration of the crystalline lens have been shown to be significantly increased, while, protein, free amino acid, glutathione and potassium levels were decreased. The reduced levels of amino acids in the lens may lead to the decreased synthesis of lens proteins while glutathione content reduced during protecting mechanism of lens against oxidative stress [15].

Marcuse reported that tryptophan and histidine had good antioxidant effects in emulsions[16]. Karel et al found that histidine, lysine, cysteine had an antioxidant effect in freeze dried model[17]. Antioxidant and antiatherogenic effect of L-arginie is reported[18]. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate contains free amino acids is cited in the literature[19].Addition of Amino Acids and Peptides in antioxidant system resulted in protection of lipids from oxidation [20]. Antioxidant effects of amino acids are reported in food and oil industry. However, information on effect of individual amino acids on eye lens against oxidative stress is sparse. The present study aims to investigate the effect of selected amino acids on goat eye lenses against H_2O_2 induced cataraact *in vitro* by estimating the content of GSH and TSP in lenses. Any substance that render the glutathione and amino acid level high against stress may have preventive effect on cataract.

MATERIALS AND METHODS

All materials used in present work were of analytical grade. Fresh whole eye balls of goat or sheep were collected form local butcher shop (Sanjeet Naka, Mandsaur) immediately after slaughtering and preserved in cold Dulbecco's phosphate buffer saline (DPBS, pH 7.4) in order to prevent any sort of deterioration.

Estimation of soluble protein and Glutathione content in Goat lens

An incision on goat eyeball was made (on scleral portion) and lens was taken out carefully with intact capsule and was washed with normal saline. The lenses of the same animal were incubated for 24 h at 37^0 C in either of following solution:

i. Mg free Tyrode medium 10ml containing 1mM H_2O_2 only.
ii. Mg free Tyrode medium 10ml containing 1mM H_2O_2 with solution of amino acid 1 mM.

Following the specified period of incubation the lenses were taken out from the solution and washed with normal saline. Each lens was decapsulated and homogenized in 4ml of 0.9% normal saline. The homogenates ware centrifuged at 6000 rpm for 1hr. The supernatant was analyzed for glutathione content[21] and protein concentration[22].

Protein Content Estimation

Under alkaline conditions, copper complexes with protein. When folin phenol reagent (phospho-molybdic-phosphotungstic reagent) is added, the Folin-phenol reagent binds to the protein. Bound reagent is slowly reduced and changes color from yellow to blue. The intensity of color depends on the amount of aromatic amino acids present and will thus vary for different proteins.

(I) Preparation of Solutions

(i) Lowry A solution was prepared by adding Na2CO3 in 0.1 M NaOH to have 2% concentration w/v. Lowry B solution was prepared by adding $CuSO_4$ in distilled water to produce 1% w/v solution. Lowry C solution is 2% w/v solution sodium potassium tartrate ($NaKC_4H_4O_6 \bullet 4H_2O$) in water.

(ii) Lowry stock reagent was preparing by adding 49 ml Lowry A, 0.5 ml Lowry B and 0.5 ml Lowry C to have 50 ml of reagent

(iii) Folin's Reagent: Phenol reagent - 2N (Folin - Ciocalteau reagent). It was Diluted 1:1 with distilled water before use. Bovine serum albumin in water was taken as standard solution.

(II) Method

To 1ml of test solution 5ml of the alkaline solution was added. These were mixed thoroughly and allowed to stand at room temperature for 10 min. To this solution 0.5ml of diluted Folin-ciocalteau reagent was added rapidly with immediate mixing. After 30 minutes absorbance was read at 750 nm in a UV-Visible spectrophotometer (Shimadzu) using appropriate blank and concentration was determined by plotting standard curve (100 to 1000 µg /ml)of BSA in normal saline in the same manner described above using known concentrations .

Estimation of Glutathione (GSH) in Isolated Eye Lenses of Goat

(I)Preparation of Solutions

(i) Preparation of 1 m M hydrogen peroxide solution

Required amount (1.76 ml) of Hydrogen Peroxide 6 % w/v was taken and diluted 1000 times with tyrode solution/ test substance solution.

(ii) Preparation of 5, 5'- Dithiobis 2-nitrobenzoic acid (DTNB) solution

This reagent is prepared by dissolving 4 mg of DTNB in 10 ml of 1% solution of Trisoidium citrate.

(iii) Preparation of Trichloroacetic acid (TCA) Solution

TCA 10 % solution was prepared by dissolving 10 g of TCA in water in a100 ml of volumetric flask and volume was made up to 100 ml with distilled water.

(iv) Preparation of Disodium Hydrogen Phosphate (DHP) Solution

Disodium Hydrogen Phosphate solution (0.3M) was prepared by dissolving 3.84 g of DHP in water in 100 ml of volumetric flask and volume was made up to 100 ml with distilled water.

(II) Method

Two ml of homogenate mixed with 0.5 ml of 10 % TCA and a protein free supernatant was obtained. One ml of TCA supernatant was mixed with 1 ml of 0.3 M of DHP and 0.5 ml of DTNB. The absorbance of this solution was taken in a UV-Visible spectrophotometer at 410 nm against blank prepared similarly but instead of lens homogenate distilled water was used. The GSH content was estimated by preparing standard curve of known concentrations in the manner similar described above at 410 nm.

RESULTS AND DISCUSSION

Oxidative insult by H_2O_2 (free radicals) cause cross-linking of the endogenous proteins (i.e. they cause glycosylation) within the lens of the eye and consequently cause reduction in TSP content along with GSH depletion. An antioxidant effect of amino acids is reported in food and oil industry. Oxidative stress is considered as major cause for cataract development. Defeciency of certain amino acids may lead to catract progression. Amino acids play a significant role in lens metabolic activities. However, information on effect of individual amino acids on eye lens against oxidative stress is sparse. The present study aims to investigate the effect of selected amino acids on goat eye lenses against H_2O_2 induced cataraact *in vitro* by estimating the content of (Glutathione reduced) GSH and TSP (total soluble protein Content) in lenses.

Glycine is the simplest amino acid among all. The results (Table1) showed that glycine at 1 mM concentration protected the lens form oxidative stress caused by 1 mm hydrogen peroxide. The amount of total soluble protein was higher (0.3933 mg per ml of lens homogenate) in the lens treated with glycine as compared to control (0.3083 mg/ml) as shown in Table 1. Glutathione content was also rendered higher by presence of 1mM glycine in solution surrounding the lens on oxidative stress. The GSH content was 0.0021 mg/ml in control lens while 0.0038 mg/ml in glycine treated lens (Table 2). Statistical analysis revealed that TSP and GSH content was significantly more ($p<0.05$) in glycine treated lens compared to control. The presence of L-tryptophan surrounding the lens facing oxidative stress in form of hydrogen peroxide protected the lens from oxidative damage. The results revealed elevated protein content in treated lens (0.3783 mg/ml) compared to control (0.305 mg/ml). GSH level of 0.0047 mg/ ml in treated lens was observed as compared with control lens i.e 0.0019 mg/ml of lens homogenate (Table 1 and 2). The L-tryptophan deficiency is known to cause cataract in rats. This amino acid is a precursor of some naturally found anticataract agents. The data indicated that L-tryptophan is acting as strong antioxidant that significantly ($p<0.05$) protected the TSP and GSH against oxidation.

The data of L-tyrosine in antioxidant study on goat lens against hydrogen peroxide induced oxidative stress are shown in Table 1 and 2. Total soluble protein and GSH content in control was 0.2933 and 0.0018 mg/ml respectively while that with treated lens was 0.4133 and 0.0034 mg/ml respectively. The data suggested the protective role against oxidative damage of lens against hydrogen peroxide. L-tyrosine rendered TSP significantly higher ($p<0.05$) compared to control. The GSH content in L-tyrosine treated lens was also higher than control; however was not significant.

The result of studies with L-phenylalanine suggested indirect involvement in lens protection against oxidative stress. The data are shown in Table 1 and 2. The TSP content in lens treated with L-phenylalanine was 0.4083 mg/ml of lens homogenate as compared to control which was 0.2716 mg/ml after 24 hours of incubation. GSH content in treated and control lens was 0.0024 and 0.003 mg/ml respectively. TSP content was significantly higher ($p<0.05$) in treated lens than control. GSH content was more in treated lens but was statistically non significant than control as determined by paired t test.

L-histidine also showed protection for soluble protein content and GSH content Table 1 and 2. Both TSP and GSH content were significantly more ($p<0.05$) in lens treated with 1 mM L-histidine as compared to control. The data suggested that L-histidine played significant role in preventing the eye lens against oxidative stress.

L-lysine also prevented the TSP and GSH content against oxidative stress .Data are presented in Table 1 and 2. The protection was significant ($p<0.05$) in lens treated with L-lysine as compared to control.

L-arginine is an amino acid having a positively charged guanido moiety. This moiety is found responsible in lipid peroxidation prevention in one antiatheroscleratic study. The amount of soluble protein and GSH was 0.2933 and 0.0013 mg/ml respectively in control lens while that was 0.42 and 0.0036 mg/ml in lens treated with 1mM L-arginine (Table 1 and 2). The result suggested that L-arginine exerts strong antioxidant activity and by virtue of this strong action significant ($p<0.05$) protection was revealed on the lens proteins and on GSH from becoming deactivated due to oxidative stress.

Table 1: Effect of amino acids on total soluble protein content of goat eye lenses kept under H_2O_2 induced oxidative stress

Amino Acid	Total Soluble Protein Content (mg/ml of lens homogenate)	
	Control	Test
Glycine	0.3083 ±0.0158	0.3933±0.0231*
L-Tryptophan	0.3050±0.0325	0.3783±0.0268*
L-Tyrosine	0.2933±0.0092	0.4133±0.0434*
L-Phenylalanine	0.2716±0.0187	0.4083±0.0130*
L-Histidine	0.2983±0.0083	0.3966±0.0066*
L-Lysine	0.3400±0.0050	0.4330±0.0083*
L-Arginine	0.2933±0.0233	0.4200±0.0057*
L-Cysteine	0.3133±0.0101	0.4500±0.0189*
L-Glutamic Acid	0.2786±0.0210	0.3416±0.0220*
L-Aspartic Acid	0.2800±0.0132	0.3153±0.0101
L-Proline	0.2886±0.0094	0.4383±0.0224*

Values are mean±SEM of three experiments in each group. * indicates significant ($p<0.05$) when compared with control lens as determined by paired t test.

Table 2: Effect of amino acids on glutathione content of goat eye lenses kept under H_2O_2 induced oxidative stress

Amino Acid	Glutathione content (mg/ml of lens homogenate)	
	Control	Test
Glycine	0.0021±0.0003	0.0038±0.0020*
L-Tryptophan	0.0019±0.0002	0.0047±0.0001*
L-Tyrosine	0.0018±0.0002	0.0034±0.0005
L-Phenylalanine	0.0024±0.0009	0.0030±0.0002
L-Histidine	0.0016±0.0003	0.0035±0.0003*
L-Lysine	0.0020±0.0002	0.0036±0.0002*
L-Arginine	0.0013±0.0001	0.0036±0.0002*
L-Cysteine	0.0018±0.0002	0.0045±0.0002*
L-Glutamic Acid	0.0020±0.0002	0.0028±0.0001*
L-Aspartic Acid	0.0015±0.0002	0.0026±0.0000*
L-Proline	0.0017±0.0001	0.0030±0.0002*

Values are mean±SEM of three experiments in each group. * indicates significant ($p<0.05$) when compared with control lens as determined by paired t test.

L-cysteine is known for its antioxidant activity and is one of the constituent amin acid of glutathione. The amino acid protected the soluble protein content GSH content significantly ($P<0.05$) as compared to control. Data presented in Table 1 and 2.

The investigation on L-glutamic acid showed its protection potential on goat eye lens against oxidative stress. The amino acid showed significant protection for protein and GSH content of treated lens as compared to control. The data is presented in Table1 and 2. L-aspartic acid showed significantly higher GSH content ($p<0.05$) than control. The soluble protein content was 0.28 mg/ml in control while that was 0.3153 mg in treated lens (Table 1 and 2). The results suggested the involvement of aspartic acid in direct oxidation process that prevented the GSH from being consumed due to oxidative stress. The protective effect on lens soluble protein however was statistically non significant as determined by paired t test. L-proline protected the lens proteins and GSH content significantly ($p<0.05$) indicating the protective role of this amino acid on aggregation of proteins and GSH depletion due to oxidative stress. The data are presented in 1 and 2.

All amino acids showed significant protection for GSH depletion against free radicals produced by H_2O_2 except L-tyrosine and L-phenylalanine. All amino acids protected the lens protein against aggregation due to oxidative stress significantly compared to control except L aspartic acid. The protective effect of amino acids seems to be due to their free radical scavanging property and direct involvement in protecting the lens against oxidative stress. The percentage of TSP and GSH protected by amino acids against H_2O_2 induced oxidative stress is shown in Figure 1. Maximum % TSP content was protected by L-proline (34.15 %) while that with GSH was by L-arginine (63.88%) as compared to control lens kept under oxidative stress without amino acids.

On the basis of the above studies it could be stated that amino acids possess antioxidant activity and their presence surrounding the eye lens may be helpful in cataract delaying or prevention. Increase intake of the studied amino acids could facilitate the protection of lens against oxidative stress. However, in vivo studies are needed to comment more in this aspect.

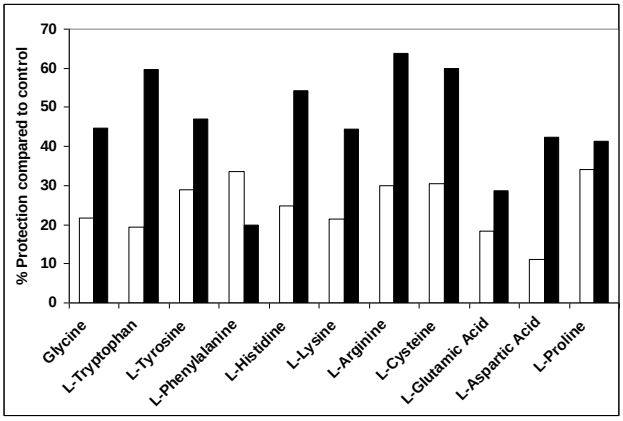

Figure 1: Protection (%) of total soluble protein and glutathione content treated with amino acids as compared to control lens kept with H_2O_2 only. □ % Gluthione protected, ■ % TSP protected

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