·Basic Research·

Changes of the thiol levels in the corneas of the diabetic rats: effect of carnosine, aspirin and a combination eye drops

Qiong Shi, Hong Yan

Department of Ophthalmology, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, Shaanxi Province, China

Correspondence to: Hong Yan. Department of Ophthalmology, Tangdu Hospital, Fourth Military Medical University, Xi'an 710038, Shaanxi Province, China. yhongb@fmmu.edu.cn

Received:2010-07-19 Accepted:2010-08-16

DOI:10.3980/j.issn.2222-3959.2010.03.07

Shi Q, Yan H. Changes of the thiol levels in the corneas of the diabetic rats: effect of carnosine, aspirin and a combination eye drops. *Int J Ophthalmol* 2010;3(3):211-215

Abstract

• AIM: To investigate the effect of carnosine (Car), aspirin (Asp) and a combination of Car and Asp eye drops on the change of the thiol contents from glutathione (GSH) and protein in the corneas of the diabetic rats.

• METHODS: All the animals were randomly divided into five groups. The normal control group received injections of vehicle only. Diabetes was induced by injection of streptozotocin (STZ) in the Sprague-Dawley (SD) rats. The untreated group rats received only the vehicle solution (the placebo). One treated group rats were treated by instillation of one drop of 10g/L Car eye drops, another were treated by 0.5g/L Asp eye drops and the last group were treated alternately by Car 10g/L and Asp 0.5g/L eye drops for a period of 8 weeks. At the end of 8 weeks, the animals were killed and the thiols contents in the corneas were investigated.

• RESULTS: About 15.6% of the rats (blood glucose measured <14mmol/L) were rejected. In the corneas, the levels of thiols were declined in the untreated, Asp-treated group and combination-treated group, but they went up in Car-treated group. The levels of thiols in the Car-treated groups were much higher than that in the untreated group, and there was statistically significant difference between them (P<0.05).

• CONCLUSION: The results indicated that diabetes decreases the levels of thiols (from GSH and proteins) in the cornea. The Car eye drops in our study may protect the cornea against the oxidative damage caused by diabetes. And the combination eye drops also may have a certain protection for the diabetic corneas.

• KEYWORDS: carnosine; aspirin, eye drops; diabetic rats; glutathione; thiol

INTRODUCTION

Diabetes was found to be ineluctably connected with increased oxidative stress both in diabetic humans and hyperglycaemic animals [1]. Among the number of mechanisms proposed as a pathogenic link between hyperglycaemia and diabetic complications, oxidative stress and the Maillard advanced glycation remain as tenable hypotheses. Irreversible advanced glycation end products (AGEs) were shown to be formed via a sequence of glycation and oxidation reactions[2].

Hyperglycaemia would not only generate more reactive oxygen species (ROS) but also attenuate endogenous antioxidative mechanisms through glycation of scavenging enzymes and depletion of low molecular antioxidants, for example, glutathione. Shifts in redox balances due to derangement in energy metabolism of carbohydrates and lipids also contribute to the overt oxidative stress in diabetic individuals [3]. However, the role of oxidative stress in diseases including diabetes and the related complications is controversial[4-6].

Glutathione (γ-glutamyl-cysteinyl-glycine; glutathione (GSH)) and the related enzymes belong to the defence system protecting the eye against chemical and oxidative stress. GSH is involved in many cellular functions including scavenging free radicals and other reactive species, regulation of DNA and protein synthesis, signal transduction, cell-cycle regulation, proteolysis, immune response and cytokines, as well as in various metabolic pathways[7,8].

In cornea, GSH plays an important role in maintaining normal hydration level, in protecting cellular membrane integrity, and degrading xenobiotic agents[9]. The transparent

and refractive properties of the mammalian cornea are essential for proper image formation on the retina. Simultaneously, cornea functions as a protective barrier for the eye. Because the corneal surface comes in direct contact with various kinds of environmental stress, multiple defence systems are required to reduce chemical and oxidative stress, to confer mechanical strength, and to provide an immunologic surveillance system. When the cornea is under oxidative stress (e.g., in the presence of H_2O_2), a rapid turnover of endothelial GSH via glutathione reductase and the hexose monophosphate shunt is required. However, unlike the rat lens, the synthesis of glutathione in rat cornea forms a minor portion of the L-cysteine metabolic products[10]. Glycation has an important role in altering corneal biochemistry during diabetes and ageing. Corneal layers have been found glycated in diabetic patients, and AGEs detected, which correlate with morphological alterations in human cornea; age-related cross-linking occurs largely on the collagen component of the cornea (stroma and lamina)[11]. The presence of an imbalance in the redox system under such circumstances has been proved by the decreased GSH level in galactose-fed guinea pigs, as well as a reduced level and cellular uptake of GSH by cornea with age[12].

Carnosine (beta-alanyl-L-histidine) is a dipeptide, which has recently attracted much attention as a naturally occurring antioxidant and transition-metal ion sequestering agent [13]. The anti-ageing effect of carnosine had been demonstrated in many studies *in vitro* and *in vivo* [12,14,15]. According to the report, aspirin (acetylsalicylic acid) could protect patients with diabetes mellitus against cataract [16]. Experimental studies then showed that aspirin protected lens proteins against a variety of chemicals relevant to cataract formation [15]. Aspirin protected diabetic rats against cataract [15,17], and was associated with a ptoective effect in epidemiological studies[18,19].

Therefore, the purpose of this study was to investigate whether the thiol (glutathione-SH and protein-SH) contents are changed in parallel in cornea of diabetic rats, and whether carnosine and aspirin eye-drops may prevent any changes, and if the combination was better than carnosine or aspirin eye drops individually.

MATERIALS AND METHODS

Materials Streptozotocin (STZ) and carnosine (Car) were obtained from Sigma Chemical Company (Beijing, China). Sprague-Dawley rats were provided by Animal Laboratories of Fourth Military Medical University (Xi'an, China). Protein and enzyme quantification kits were obtained from Jiancheng Biology Company (Nanjing, China). Glucotrend 2 was from Roche Diagnositic Limited Company (Xi'an, China). Tes-Tape was from Zhujiang Biochemistry Reagents (Guangzhou, China). All other chemicals and solvents were of analytical grade and were obtained from local companies.

Methods

Animals One hundred and two male Sprague-Dawley (SD) rats, one-month old, initially weighing 135-180g were selected in the study. All the animals were fed standard diet ad libitum and randomly assigned to five groups. The animals were housed in five individual cages in a room. All the animals had free access to drinking water. Group A rats (normal control, n=12) received an injection of 0.02mol/L citrate buffer (pH 4.5,65mg/kg body weight, intraperitioneally) as a vehicle, however, the experimental groups (Group B-E) received an intraperitoneal injection of 1% STZ in citrate buffer at a dose of 65mg/kg body weight (Yan *et al*[14], 2008). After 96 hours, random blood glucose levels were measured. The animals having blood glucose levels <14mmol/L (n =14) were excluded from the experiment and the rest were distributed into the following groups: B (diabetic rats untreated, n=21), C (diabetic rats treated with Car eye drops 1% only, n=17), D (diabetic rats treated with Asp eye drops 0.05% only, n=21), E (diabetic rats treated alternately with Car 1% and Asp 0.05% eye drops, n=17).

Experimental design Car eye drops (1%, pH 7.4) were prepared by dilution of 10 ml of the 25mmol/L sodium phosphate buffer (pH7.4, containing 2.527g of di-hydrated sodium dihydrogen phosphate, 1.36g of dodeca-hydrated dibasic sodium phosphate, and 800mL double distilled water). Animals from group C and D were treated respectively by instillation of Car eye drops 10g/L, Asp eyedrops 0.5g/L only, one drop, twice a day for 8 weeks. Rats from group E were combining treated alternately by instillation of Car 10g/L eye drops and Asp eye drops 0.5g/L, one drop, twice daily for 8 weeks. However, the animals from group B just received the instillation of the vehicle solution, one drop, twice a day. Blood was collected from the caudal end of rats for glucose estimation with Glucotrend 2 every two weeks. The urine glucose was monitored weekly.

In the eighth week, the animals were killed following cervical dislocation under ether anaesthesia, the eyes were enucleated, and the corneas were dissected and kept at -70℃ until further analysis. The thiol contents and the water-soluble protein contents were analyzed in the soluble fraction of the homogenate.

Biochemical Estimations

Protein determination Protein concentration was

determined by the Coomassie brilliant blue method using the protein assay kit from Jiancheng Company (Nanjing, China)[20]. Two lenses in each rat were ground in 9g/L neutral normal saline (1:9) and homogenized by hand in the ice-water mixture to make the 10% homogenate, and then centrifuged (11 500g) in Eppendorf tubes. Clear supernatant was used for protein determination, which was according to the method described with the kits.

Thiol determination Thiols were measured using the DTNB method at 25°C and 412nm [21,22]. The clear supernatant liquid used for protein determination was taken by suction from the centrifuged Eppendorf tube, and the thiol content was determined according to the description of kit. Thiols react with dithio-dinitrobenzoic acid to give a yellow compound, which has a high absorption of light at 412nm which was measured. Through this colorimetric method the content of thiol (from GSH and protein) in each cornea was determined.

Statistical Analysis One-way ANOVA was used for testing statistical significance between groups of data. Statistical analysis of stage of urine glucose were performed by using Wilcoxon Rank Sum Test. Individual pair difference was tested by means of Duncan's multiple-range test. P<0.05 was considered significant.

RESULTS

Forteen rats were no respond to STZ (blood glucose measured <14mmol/L) at 96 hours after receiving an intraperitoneal injection, two rats (respectively from Group D and E) with the blood glucose level below 14mmol/L in the 3rd week, and the animals that died before the end of the experiment were excluded leaving 51 rats in the study.

There was an increase in blood glucose level in Group B-E compared with Group A, and the treatment did not reverse the changes in blood glucose levels. The results indicated that the treatment of Car or Asp and combination eye drops had no effect on the blood glucose level of STZ-induced diabetic rats. In comparison with the normal group, the urine glucose in all the diabetic groups increased.

The levels of thiols appeared to be decreased in the untreated diabetic rats. Compared with the normal control group, the level of thiols was increased in the Car-treated group (Group C), whereas, in the other groups they were decreased, and there was significantly statistical difference between the Asp-treated group and the normal control group (Group D *vs* Group A, P=0.016). Moreover, the thiols contents in the Car-treated group were higher than that in the untreated and Asp-treated groups, and there was significantly statistical difference (Group C *vs* Group B, P= 0.003, Group C *vs* Group D, P<0.001) (Figure 1).

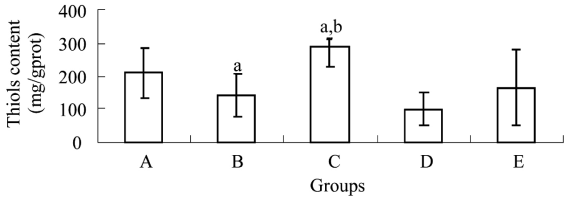

Figure 1 Effect of carnosine, aspirin and the combination eye drops on thiols content in the cornea The asterisk indicates significantly different from Group B; [a]P<0.05. The sharp (hash mark) indicates significantly different from Group D; [b]P<0.001. A was group of normal control, B was group of diabetic rats untreated, C was group of diabetic rats treated with carnosine eye drops 1% only, D was group of diabetic rats treated with aspirin eye drops 0.05% only, E was group of diabetic rats treated alternately with carnosine 1% and aspirin 0.05% eye drops

DISCUSSION

Glutathione (L-g-glutamyl-L-cysteinylglycine) is the principal nonprotein thiol involved in the antioxidant cellular defence. It is a tripeptide composed of cysteine, glutamic acid and glycine, and its active group is represented by the thiol (-SH) of the cysteine residue [23]. In cells, total glutathione can be free or bound to proteins [21]. Free glutathione is present mainly in its reduced form, which can be converted to the oxidised form during oxidative stress, and can be reverted to the reduced form by the action of the enzyme glutathione reductase. The redox status depends on the relative amounts of the reduced and oxidized forms of glutathione (GSH/GSSG) and appears to be a critical determinant in the cell. In normal conditions, the glutathione redox couple is present in mammalian cells in concentrations between 1 and 10mM, with the reduced GSH predominating over the oxidised form. In the resting cell, the ratio exceeds 100, whereas in various models of oxidative stress, this ratio was reported to decrease to values between 10 and 1 [24]. Furthermore, glutathione can be bound to the proteins, leading to the formation of glutathionylated proteins. Glutathione not only protects cell membranes from oxidative damage, but also helps to maintain the sulphydryl groups of many proteins in the reduced form, a requirement for their normal function. Irreversible cell damage supervenes when the cell is no longer able to maintain its GSH content[25].

Carnosine, through its distinctive combination of antioxidant and anti-glycating properties, is able to attenuate cellular oxidative stress and can inhibit the intracellular formation of reactive oxygen species and reactive nitrogen species. By controlling oxidative stress and chelating metal ions, is able to reduce harmful sequelae such as DNA damage [26].

Furthermore, in Maĭchuk's study, trials of the drug, carnosine eye drops, on mice, rats, rabbits, and dogs showed it to be well tolerated at both total and local levels [27]. In animals the eye drops did not affect the diameter of the pupil, nor did they increase the intraocular pressure. In our previous and present studies, eye irritation was not noticed, either [15]. Thus, this dose of carnosine used as eye drops is safe.

In the present study, the thiol contents of the carnosine-treated group were increased in the corneas compared with the untreated group, suggesting that carnosine attenuated oxidative stress through its distinctive combination of antioxidant and anti-glycating properties.

Aspirin could protect lens proteins against a variety of chemicals relevant to cataract formation [28]. This protective action appears to be brought about by acetylation of vulnerable groups of lens proteins[29], and then, acetylation of a single lysine in human crystallin was identified [30]. The decrease in glutathione and the related enzymes, and the increase in glycation were related to the progression of lens opacification. In our present study, aspirin can not raised the thiols contents in the cornea, possibly because there were not the single lysine to acetylation. And it is need to be a further study.

Cornea is very important as a protective barrier for the eye. Because the corneal surface comes in direct contact with various kinds of environmental stress, multiple defence systems are required to reduce chemical and oxidative stress, to confer mechanical strength, and provide an immunologic surveillance system[9]. GSH plays an important role in maintaining normal hydration level, in protecting cellular membrane integrity, and degrading xenobiotic agents in cornea. When the cornea is under oxidative stress, a rapid turnover of endothelial GSH via glutathione reductase and the hexose monophosphate shunt is required[9]. However, unlike the rat lens, the synthesis of glutathione in rat cornea forms a minor portion of the L-cysteine metabolic products [10]. The presence of an imbalance in the redox system under such circumstances has been proved by the decreased GSH level in galactose-fed guinea pigs, as well as a reduced level and cellular uptake of GSH by cornea with age[12].

In our present study, the thiol contents in corneas of the untreated group was obviously lower than that of the normal control group, indicating that the diabetes would change the thiol levels in corneas. Meanwhile, compared with the normal control group, the levels of thiols were increased in the carnosine-treated group, whereas, in the other groups they were decreased. And the thiols contents in the carnosine-treated group were higher than that in the untreated and the aspirin-treated groups. It suggested that carnosine protected the redox state of the cornea in the diabetic rats.

In conclusion, the present results indicate that diabetes decreased the levels of thiols (from GSH and proteins) in cornea. The carnosine eye drops in our study may function to protect the cornea to resist the glycation and oxidative damages that diabetes caused. The combination eye drops also have a certain protection for the diabetic cornea.

Acknowledgements: We are grateful to the Welcome Trust for financial support for Prof. John J. Harding and Prof. H. Yan (International Research Development Award, No. 070667); and to Shaanxi Provence, China for a grant to support this research.

REFERENCES

1 Kowluru RA, Kennedy A. Therapeutic potential of antioxidants and diabetic retinopathy. *Expert Opin Investig Drugs* 2001;10(9):1665-1676

2 Kowluru RA, Kern TS, Engerman RL. Abnormalities of retinal metabolism in diabetes or experimental galactosemia: IV. Antioxidant defense system. *Free Radic Biol Med* 1997;22(4):587-592

3 Kyselova Z, Stefek M, Bauer V. Pharmacological prevention of diabetic cataract. *J Diabetes Complications* 2004;18(2):129-140

4 Harding JJ. Cataract: biochemistry, epidemiology, and pharmacology. London: Chapman and Hall; 1991

5 Spector A. Review: Oxidative stress and disease. *J Ocul Pharmacol Ther* 2000; 16:193-201

6 Wiernsperger NF. Oxidative stress as a therapeutic target in diabetes: revisiting the controversy. *Diabetes Metab* 2003;29(6):579-585

7 Harding JJ, Blakytny R, Ganea E. Glutathione in disease. *Biochem Soc Trans* 1996;24(1):1-4

8 Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr* 2004;134(3):489-492

9 Ganea E, Harding JJ. Glutathione-related enzymes and the eye. *Curr Eye Res* 2006;31(1):1-11

10 Li J, Rathbun WB, Murray DL. Cysteine and glutathione metabolism in organ-cultured rat corneas. *Curr Eye Res* 1990;9(9):883-891

11 Stitt AW. Advanced glycation: an important pathological event in diabetic and age related ocular disease. *Br J Ophthalmol* 2001;85(6):746-753

12 Kannan R, Mackic JB, Zlokovic BV. Corneal transport of circulating glutathione in normal and galactosemic guinea pigs. *Cornea* 1999;18(3):321-327

13 Reddy VP, Garrett MR, Perry G, Smith MA. Carnosine: a versatile antioxidant and antiglycating agent. *Sci Aging Knowledge Environ* 2005;2005(18):12

14 Yan H, Harding JJ. Carnosine inhibits modifications and decreased molecular chaperone activity of lens α-crystallin induced by ribose and fructose 6-phosphate. *Mol Vis* 2006;12:205-214

15 Yan H, Gou Y, Zhang J, Ding ZH, Ha WJ, Harding JJ. Inhibition of cataracts in diabetic rats by carnosine, aminoguanidine and aspirin drops. *Mol Vis* 2008;14: 2282-2291

16 Cotlier E. Senile cataract: evidence for acceleration by diabetes and deceleration by salicylates. *Can J Ophthalmol* 1961;16:133-138

17 Shi Q, Yan H, Li MY, Harding JJ. Effect of a combination of carnosine and aspirin eye drops on streptozotocin-induced diabetic cataract in rats. *Mol Vis*

2009;15:2129-2138

18 Harding JJ, Egerton M, Harding RS. Protection against cataract by aspirin, paracetamol and ibuprofen. *Acta Ophthalmol* 1989;67(5):518-524

19 van Heyningen R, Harding JJ. Do aspirin-like analgesics protect against cataract? A case-control study. *Lancet* 1986;1(8490):1111-1113

20 Bradford MM. A rapid and sensitive method for the quantotation of mincrogram quantities of proterin utilizing the prinviple of protein-dye binding. *Anal Biochem* 1976;72:248-254

21 Harding JJ. Free and protein bound glutathione in normal and cataractous human lenses. *Biochem J* 1970;117(5):957-960

22 Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* 1 985;113:548-552

23 Meister A, Larsson A. Glutathione synthetase deficiency and other disorders of the g-glutamyl cycle. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The metabolic basis of inherited disease. 6th ed. New York: McGraw-Hill; 1989: 855-868

24 Chai YC, Ashraf SS, Rokutan K, Johnston RB Jr, Thomas JA. S-thiolation of individual human neutrophil proteins including actin by stimulation of the respiratory burst: evidence against a role for glutathione disulfide. *Arch Biochem Biophys* 1994;310(1):273-281

25 Reed DJ, Fariss MW. Glutathione depletion and susceptibility. *Pharmacol Rev* 1984;36(2 Suppl):25S-33S

26 Boldyrev AA. Problems and perspectives in studying the biological role of carnosine. *Biochemistry* 2000;65(7):751-756

27 Ma chuk IuF, Formaziuk VE, Sergienko VI. Development of carnosine eyedrops and assessing their efficacy in corneal diseases. *Vestn Oftalmol* 1997; 113(6):27-31

28 Huby R, Harding JJ. Non-enzymic glycosylation (glycation) of lens proteins by galactose and protection by aspirin and reduced glutathione. *Exp Eye Res* 1988;47 (1):53-59

29 Qin W, Smith JB, Smith DL. Reaction of aspirin with cysteinyl residues of lens γ-crystallins: a mechanism for the proposed anti-cataract effect of aspirin. *Biochimica Biophysica Acta* 1993;1181(2):103-110

30 Lin PP, Barry RC, Smith DJ, Smith JB. *In vivo* acetylation identified at lysine 70 of human lens α A-crystallins. *Pro Sci* 1998;7(6):1451-1457