Preventive Effect of Eye Drops of Liposomes Containing Disulfiram and Cefmetazole on Selenite-Induced Cataract in Rat Pups

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Abstract: Disulfiram (DSF) and cefmetazole (CMZ), which possess with anti-oxidative activities were tested for anti-cataract effect in rats and were compared with pirenexine (PRX), which is a marketed product used for the treatment of cataract. These compounds were encapsulated in liposomes and the results were compared with the anti-cataract effect of suspension and solution formulations containing same contents of each other agents. Instillations of DSF- and CMZ-liposomes prevented the development of cataract in selenite-injected rat pups. On the other hand, no anti-cataract effects were observed in selenite-induced cataract rats instilled with DSF suspensions and CMZ solutions. The reduced form of glutathione (GSH) content of the lenses was decreased by approximately 60% of normal level 96 h after the sodium selenite-injection and the calcium (Ca^{2+}) content was increased. The decreased GSH and increased Ca^{2+} levels were prevented by instillation of DSF- and CMZ-liposomes. Instillation of PRX-liposomes and 0.03% PRX solution had no effect on the development of cataract. The results of this study confirmed that anti-oxidative agents such as DSF and CMZ were useful to prevent cataract development related to oxidative stress.

Key words: eye drop, cataract, antioxidant, disulfiram, cefmetazole

1 Introduction

Current hypotheses concerning the pathogenesis of senile cataracts in human patients include osmotic shock (1), genetic defects (2-4), or oxidative damage (5-8). Analyses of the lenses and aqueous humor from the eye of patients with senile cataracts has demonstrated increased hydrogen peroxide (H_2O_2) levels (9) and decreased reduced form of glutathione (GSH) levels (10) accompanied by extensive damages of protein (3,11), lipid (12) and DNA (1), suggesting the participation of reactive oxygen species (ROS). ROS scav-

engers such as vitamin C (13,14), vitamin E (15,16), or GSH (17) inhibited the onset of cataracts and protected the crystalline protein of the lens. These studies suggest the possible use of agents that suppress ROS formation for the therapeutic or prophylactic treatment of cataracts.

Disulfiram (DSF) has been widely used in the treatment of alcohol abuse (18) to inhibit the liver enzyme, aldehyde dehydrogenase (19). Diethyldithiocarbamate (DDC) is produced from DSF by the catalytic reaction of sulfhydryl groups in proteins such as albumin (20). DDC has been shown to inhibit microsomal lipid perox-

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idation in rat hepatocytes (21) and DSF and DDC have been reported to inhibit DNA breakage by hydroxyl radicals (22). Moreover, it has been reported that thiomethyltetrazole (23) also has an anti-oxidative activity and antabuse effect.

In this study we examined the anti-cataract effects of DSF, cefmetazole (CMZ, one of cephamycins that possesses a thiomethyltetrazole residue in its structure) (24), and pirenoxine (PRX) (25-27) which is already marketed for clinical use in Japan (Fig. 1). The anti-cataract effects were monitored by the ability for prevention of selenite induced cataracts in rat pups (28). We also investigated the effectiveness of liposome formulation as a carrier for these drugs.

2 Experimental

2.1 Animals

Male and female Wistar rat pups aged 13 days old were used. They were housed under standard conditions of 12 h/day fluorescent light (07:00-19:00) and 25°C room temperature and were given a commercial diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water *ad libitum* (rat pups were kept with their mother). All animal studies were conducted in accordance with the NIH

Guidelines on the Care and Use of Animals in Research.

2.2 Chemicals

DSF was kindly donated by Ouchi Shinko Chemical Industrial Co., Ltd. (Tokyo, Japan). PRX was purchased as a commercial granulated product (Catalin K, Senju Pharmaceutical Co., Osaka, Japan). CMZ was a commercial product (antibiotics for injection, Sankyo Pharmaceutical Co., Tokyo, Japan). Sodium selenite was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were obtained from Nippon Oil and Fats Co., Ltd. (Tokyo, Japan). 1-*n*-Hexa-decylpyridinium chloride monohydrate (CPC) was purchased from Kanto Chemical Co. (Tokyo, Japan). All other chemicals used were of the highest purity and were commercially available.

2.3 Determination of DSF, CMZ and PRX in Liposomes

The amount of DSF in the liposomes was determined by an HPLC method previously described (29). Fifty μL of the sample solution was added to 100 μL of the internal standard solution (5 μg of indomethacin/mL of methanol). Two μL of the mixture was injected into an HPLC instrument, Shimadzu LC-10AD, equipped with a column oven, CTO-6A. The chromatography was performed under the following conditions: a Wakosil-II 3C18 AG (4.6 $\times$ 150 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) column, mobile phase of 60% acetonitrile containing 0.1% trifluoroacetic acid, flow-rate of 1.0 mL/min, column temperature of 35°C and a 215 nm wavelength for detection.

The amount of CMZ in the liposomes was determined by an HPLC method. Fifty μL of the sample solution was added to 100 μL of the internal standard solution (30 μg of L-tryptophan/mL of methanol). Two μL of the mixture was injected into an HPLC instrument, Shimadzu LC-10AD, equipped with a column oven, CTO-6A. The chromatography was performed under the following conditions: a Capcell Pack C18 (4.6 $\times$ 150 mm, Shiseido Co., Ltd., Tokyo, Japan) column, mobile phase of 50 mM sodium citrate buffer (pH 5.0) containing 8% acetonitrile, flow-rate of 1.0 mL/min, column temperature of 35°C and a 254 nm wavelength for detection.

The amount of PRX in the liposomes was determined

Fig. 1 Structures of the Compounds Tested for Anti-Aataract Activity. The part of the structure within the dotted outline represents a residue of thiomethyltetrazole.

by an HPLC method described in Japanese Pharmacopoeia (30). Fifty μL of the sample solution was added to 100 μL of the internal standard solution (5 μg of methyl-*p*-hydroxybenzoate/mL of methanol). Two μL of the mixture was injected into an HPLC instrument, Shimadzu LC-10AD, equipped with a column oven, CTO-6A. The chromatography was performed under the following conditions: a Capcell Pack C18 (4.6×150 mm, Shiseido Co., Ltd., Japan) column, mobile phase of 10 mM sodium phosphate buffer (pH 6.5) containing 25% acetonitrile, 0.15% tetra-*n*-butylammonium chloride and 3.5% tetrahydrofuran, flow-rate of 1.0 mL/min, column temperature of 35°C and a 230 nm wavelength for detection.

2.4 Preparation of Liposomes Containing DSF, CMZ and PRX and DSF Suspensions

Liposomes containing DSF (DSF-liposomes) were prepared by reverse phase solvent evaporation with a minor modification (31). DSF (10 mg), DPPC (201 mg), DMPC (750 mg) and CPC (49 mg) were dissolved in 20 mL of dichloromethane. The molar ratio of DPPC, DMPC and CPC was 2:8:1 and the total weight was 1.0 g. This solution was used as the hydrophobic phase. The hydrophobic phase was mixed with 10 mL of phosphate buffered saline (pH 7.4) containing 0.04% methyl and *n*-propyl *p*-hydroxybenzoate and was sonicated under a stream of nitrogen using a bath-type sonicator (Branson Ultrasonics Division Emerson-Japan, Yokohama, Kanagawa, Japan). The w/o emulsion was aspirated for 30 min at 37°C to remove the dichloromethane. The liposomes were passed through an Excluder® (Lipex Biomembranes Inc., Vancouver, Bc., Canada) with polycarbonate membranes (pore sizes of 1.0, 0.6, 0.2 and 0.1 μm , Coster Scientific Corp., Cambridge, Ma., USA), which was repeated 10 times for each membrane. The particle sizes of the liposomes were determined using a laser diffraction scattering particle analyzer, Coulter N4S (Coulter Corp., U.S.A.). The particle size of the liposomes was approximately 80 nm and the DSF content was 0.98 ± 0.08 mg/mL.

Liposomes containing CMZ (CMZ-liposomes) or PRX (PRX-liposomes) were prepared by the same method that was used for DSF-liposomes except CMZ and PRX were dissolved in a buffer solution and a different molar ratio of phospholipids was used. DPPC

(774 mg), DMPC (179 mg) and CPC (47 mg) were dissolved in 20 mL of dichloromethane which was used as the lipophilic phase. The molar ratio of DPPC, DMPC and CPC was 8:2:1 and the total weight was 1.0 g. The lipophilic phase was mixed with 10 mL of CMZ solution (1000 mg/mL) or PRX (0.3 mg/mL) dissolved in buffer and was sonicated under a stream of nitrogen. The emulsion was aspirated for 30 min at 37°C to remove dichloromethane. The liposomes were passed through an Excluder® with polycarbonate membranes (pore sizes of 1.0, 0.6, 0.2 and 0.1 μm) in the same manner as described above. To separate the liposomes from the unencapsulated CMZ or PRX, the liposomes were chromatographed on a column of Superose 6 (2.5×30.0 cm, Pharmacia LKB Biotechnology AB, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 145 mM NaCl and 1 mM EDTA. The white turbid fractions were collected and used as CMZ- or PRX-liposome preparations. The particle size of the liposomes was approximately 100 nm and the CMZ and PRX contents were 32.3 ± 8.7 mg/mL and 0.24 ± 0.02 mg/mL, respectively.

DSF suspensions were prepared by a solvent evaporation method. One hundred mg of DSF was dissolved in 30 mL of dichloromethane and this solution was filtered through a membrane filter (0.22 μm of pore size). The DSF solution was mixed with 50 mL of phosphate buffered saline (pH 7.4) containing 0.04% methyl and *n*-propyl *p*-hydroxybenzoate and was sonicated for 10 min. The emulsion was aspirated for 1 h at room temperature to remove the organic solvent. The DSF suspension (2 mg/mL) was diluted with an equal volume of the same buffer containing 0.5% hydroxypropylmethylcellulose 2906 (Shin-Etsu Chemical Co., Tokyo, Japan). The particle size of DSF in the suspension was 18.6 ± 2.6 μm , and the final content of DSF in the suspensions was 1.02 ± 0.06 mg/mL.

Solutions of 3.0% CMZ and 0.03% PRX were prepared by dissolving each solid agent in 10 mM sodium phosphate buffer (pH 7.4) containing 145 mM NaCl.

2.5 Selenite-Induced Cataracts of Rats and Treatment by DSF-, CMZ- and PRX-liposomes

For the induction of cataracts, rat pups aged 13 days were given a single subcutaneous injection of sodium selenite dissolved in 0.9% NaCl solution (19 $\mu\text{mol/kg}$ body weight) (28). The eyelids of rat pups were opened

gently and carefully with blunt tweezers. Five μL of liposomes containing DSF (0.98 mg/mL), CMZ (32.3 mg/mL) or PRX (0.24 mg/mL) were instilled into the eyes. The eyes were kept open for approximately one minute to prevent the liposomes from overflowing. The treatment for both eyes was completed 1.5 h before the sodium selenite injection and was repeated 4 times per day (at times of 06:30, 12:00, 18:00, 23:00) for one week after the selenite injection. Both eyes of the control group rats were instilled with agent free liposomes as described above. Rat pups were killed by decapitation and bled out at selected time points after sodium selenite injection and the lenses were removed from the eye. All animals were alive during the monitoring of lens opacification.

2·6 Determination of Reduced Form of Glutathione (GSH), Sodium (Na^+), Potassium (K^+) and Calcium (Ca^{2+}) Ions in Lenses of Selenite-Induced Cataract Rats

The GSH and Na^+ , K^+ , and Ca^{2+} ions in the lenses were measured as described below.

GSH levels were assayed by the HPLC method of Tsunasawa with minor modifications (32). The lenses were homogenized in 0.5 mL of purified water using a Teflon tissue homogenizer. GSH in the homogenate was determined by the following HPLC method. Fifty μL of the lens homogenate were added into 100 μL of acetonitrile containing 10 μg of sodium pantothenate (internal standard) and centrifuged at 12,000 rpm for 10 min. The supernatant (10 μL) was injected into a Supersphere 100 RP-18 (4 μm , column size: 4.0×250 mm) column (Kanto Chemical Co., Inc., Tokyo, Japan), using a Shimadzu LC-10AD system equipped with a column oven CTO-6A (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of 5% methanol containing 0.1% trifluoroacetic acid. The flow rate was 1.0 mL/min, the column temperature was 35°C , and the wave length for detection was 215 nm.

Na^+ , K^+ and Ca^{2+} contents of the lenses were assayed by the method of Bunce *et al.* with minor modification (33). The lenses were dried in a vacuum at 100°C for 8 h and then digested in 60% nitric acid (100 μL /lens) at 80°C . One mL of purified water was added to the digests and the tubes were centrifuged at $300 \times g$ for 10 min. Two tenth mL of the supernatant was mixed with 2 mL of purified water for the Na^+ and K^+ assays. One

tenth mL of 100 $\mu\text{g}/\text{mL}$ lanthanum chloride was added to the supernatant for the determination of the Ca^{2+} content. The Na^+ , K^+ and Ca^{2+} levels in the lenses were determined by atomic absorption spectroscopy, Shimadzu AA-6400 (Shimadzu Corp., Kyoto, Japan).

2·7 Image Analysis

Lens opacification was observed with a Scheimpflug camera and recorded at selected time points from 0 to 7 days. The pupils of the rat pups were dilated with 1% pivallephrine (Santen Pharmaceutical Co., Ltd., Osaka, Japan) without anesthesia 5 min before taking slit photographs using a photoslit lamp microscope and an anterior eye segment analysis system (EAS-1000, Nidek, Gamagori, Aichi, Japan) at selected time points from 24 to 96 h. The lens images were obtained using an EAS-1000 equipped with a CCD camera. Flash level was 100 Watt-seconds and the slit length was 4.4 mm. The area of the opacity, in pixels, was analyzed using an EAS-1000 (34,35). The outline of the slit lens image was determined by selecting 4 points on the image. Then the transparent area within the lens outline and threshold level were set automatically by the computer software. There are approximately 100 threshold levels both for a normal lens and a lens with a cataract. The total area of opacity of the lenses, expressed as pixels, was calculated by the following equation:

Pixels within opacity = pixels within outline – pixels within transparent area

2·8 Statistical Analysis

All values were expressed as the means $\pm$ SEM in a total of 3 to 8 experiments. Significance of the mean differences in each experiment was analyzed by Student's *t*-test, and a *p* value of <0.05 was considered significant.

3 Results

Figure 2 shows the Scheimpflug camera images of eyes from rats 96 h after selenite injection. The lenses had significant opacity in the nuclear portion of the lenses (positive control). On the other hand, the lenses from selenite-injected rats treated with DSF-liposomes were similar in transparency to those from control rats. The lenses of selenite injected rats treated with CMZ-liposomes were either clear or had a trace of opacity in

Fig. 2 Slit Images of Eyes from Selenite-Injected Rat Pups Instilled with DSF-Liposomes (DSF-Lipo), CMZ-Liposomes (CMZ-Lipo), PRX-Liposomes (PRX-Lipo), 0.1% DSF Suspension (DSF-Susp), 3.0% CMZ Solution (CMZ-Soln), and 0.03% PRX Solution (PRX-Soln). Thirteen-day old rats were instilled with 5 μ L of the formulations into both eyes at 1.5 h before sodium selenite-injection (19 μ mol/kg) and 4 times per day after sodium selenite-injection. The instillations were continued for one week. The slit images were taken at 96 h after the sodium selenite-injection. Control shows rat pup without selenite injection.

the nuclear portion of the lens. The slit images of lenses were digitized and the results are presented in **Fig. 3**. The lens opacities from selenite-injected rats treated with DSF- or CMZ-liposomes were less than 60% of those levels in the selenite treated positive control group. No anti-cataract effect was observed using 0.1% DSF suspension, 3.0% CMZ solution, 0.03% PRX solution or PRX-liposomes.

The effect of DSF- and CMZ-liposomes on the GSH, Ca^{2+} contents and the Na^+/K^+ ratio in lenses of selenite-treated rats is shown in **Fig. 4a, b, c**, respectively. Significant preservation of the GSH content by pre- and post-treatment with DSF- or CMZ-liposomes was observed. However, instillation of the other preparations had no effect on lens GSH content. The Ca^{2+} content in the selenite-treated positive control lenses was

Fig. 3 The Effect of Instillation of DSF-Liposomes, CMZ-Liposomes, PRX-Liposomes, 0.1% DSF Suspension, 3.0% CMZ Solution and 0.03% PRX Solution on the Opacification of Lenses in Rat Pups 96 h after Sodium Selenite-Injection. The experiment was carried out under same conditions described in **Fig. 2**. Each value represents the mean $\pm$ SEM of 8-12 rats. * $p < 0.01$ versus Selenite group.

elevated to approximately 5 times the level observed in the non-treated lenses. The instillation of DSF- and CMZ-liposomes (1.05 ± 0.41 and 1.31 ± 0.36 mM, respectively) restored the Ca^{2+} content to normal level (0.82 ± 0.32 mM). There was no effect on the lens Ca^{2+} content in the other treated groups. In particular, two formulations of PRX had no effect on GSH and Ca^{2+} levels. There were no differences between the Na^+/K^+ ratios in lenses with and without anti-cataract agents, and the ratios were almost the same as that observed in the normal rat lens.

4 Discussion

Selenite-induced cataracts in rat pups is a rapid and convenient animal model for the development of cataracts and is widely used for the initial screening for potential anti-cataract agents (28). Although the mechanism of selenite-induced lens opacification is still unclear, selenite has been reported to generate ROS including the superoxide anion and the hydroxyl radical (36). Accumulation of H_2O_2 and decreased GSH are observed in both the selenite-induced cataracts and human cataracts (37). Devamanoharan *et al.* reported that treatment with vitamin C had a preventive effect on selenite-induced cataracts in rat pups (14). However, in their study vitamin C and selenite were both administered intraperitoneally and the vitamin C may have

Fig. 4 The Effect of Instillation of DSF-Liposomes, CMZ-Liposomes, PRX-Liposomes, 0.1% DSF Suspension, 3.0% CMZ Solution and 0.03% PRX Solution on GSH (a), Ca²⁺ (b) and Na⁺/K⁺ Ratio (c) of Lenses in Rat Pups 96 h after Sodium Selenite-Injection. Each value represents the mean $\pm$ SEM of 8-12 rats. * p < 0.01 versus Selenite group; ** p < 0.05 versus Selenite group.

reacted locally with the selenite. Therefore, in this study we investigated the preventive effect of DSF and CMZ on selenite-induced cataract development in rat pups by the instillation of liposomes containing these agents into the eyes of the rat pups.

DSF is a lipophilic agent and is encapsulated in the lipid phase of liposomes. The recovery of DSF encapsulated in liposomes was approximately 100% in this study. On the other hand, CMZ and PRX are hydrophilic agents and were encapsulated in the inner water phase of liposomes. The recoveries of CMZ and PRX encapsulated in liposomes were approximately 3 and 80% respectively after removing those agents in

outer water phase of liposomes by a column chromatography. The higher recovery of PRX than CMZ may be owe to adsorption ability of PRX to the lipid phase of liposome. The similar result was reported for a doxorubicin encapsulated in liposomes (38).

DSF is absorbed rapidly from the gastrointestinal tract but no or only a small amount of the DSF appears in blood because it is rapidly reduced to DDC, an active metabolite of DSF, by glutathione reductase in erythrocytes and by the non-enzymatic reduction associated with the albumin sulfhydryl groups (19,22). Our previous experiments had demonstrated that after instillation of DSF-liposomes into rabbit eyes, only DDC was detected in the aqueous humor (29), suggesting that a liposome formulation was effective for the transcorneal delivery of DDC. The similar result was obtained for CMZ-liposomes. Cephamycin antibiotics such as CMZ are degraded non-enzymatically in a neutral or acidic solution to thiomethyltetrazole, that possesses reductive and/or antioxidative activity (23). Therefore, the metabolite may act as an anti-cataract agent.

There have been several reports that PRX had antioxidative activities in *in vitro* experiments (39,40). It was proposed that PRX may have specifically scavenged the superoxide anion. The effects of DSF and CMZ on the development of selenite-induced cataracts in rats were compared with PRX because it is the most widely available preparation in Japan for the treatment of senile cataracts (25-27). However, some investigators have reported that PRX was not effective for the treatment of human cataracts (41,42). In this study we used PRX-liposomes and a PRX solution at 5 and 6 times the concentration used clinically. The anti-cataract effect by PRX was not observed (Fig. 4).

Control of the level of Ca²⁺, Na⁺ and K⁺ ions is essential for lens function. Na⁺,K⁺-ATPase and Ca²⁺-ATPase in the epithelial cells of the lens membrane maintain the levels of these ions in the lens. Increased levels of Ca²⁺ and Na⁺ have been reported in many cataractous lenses (43). In contrast, Shearer *et al.* reported that there was an elevation of Ca²⁺ but no increase of the Na⁺ content in the lens of selenite-injected rat pups (44). Similar results were observed in our study (Fig. 4b, c). Instillations of DSF- and CMZ-liposomes suppressed the increased Ca²⁺ content in lenses of selenite injected rat pups (Fig. 4b). These results suggest that the restoration of the Ca²⁺ content in the lens to near normal levels may be the result of protection of Ca²⁺-ATPase activity

by DSF and CMZ (45).

5 Conclusion

We demonstrated that DSF and CMZ have anti-cataract effects in the selenite-induced cataract in rat pups. The liposome formulations of DSF and CMZ were more effective than suspensions and solutions in treating the selenite-induced rat cataracts. We plan to investigate methods to stabilize the DSF- and CMZ-liposomes and to potentially develop more effective formulations such as emulsions and solid dispersions for increased transcorneal penetration by these compounds.

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