· Basic research ·

Protective effect of dithiothreitol intraperitoneal injection on eye lens exposed to ultraviolet radiation in rats

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Abstract

• AIM: To study the effect of exogenously administered dithiothreitol (DTT) on eye lens of adult rats exposed to ultraviolet radiation B(UV-B) from 300nm irradiation.

• METHODS: Thirty-two male SD rats aged 6 weeks (body weight about 180g) were randomly divided into four groups of 8 rats each. The first group, the model group, was exposed daily to ultraviolet radiation from 300nm at $1\times10^3\mu W/cm^2$ for 15 minutes for one week. The second group, the experimental group, exposed in the same way as the first group, received 400μg DTT in 1mL saline 30 minutes prior to radiation, daily. The third group was also exposed to UV-B, and treated only with 400 μg DTT in 1mL saline for another week. The fourth group was kept as the control group and received a daily vehicle injection.

• RESULTS: DTT injection with radiation significantly reduced lipid peroxidation, Ca^{2+} and LDH. When DTT was injected after radiation, SOD and GSH-px enzyme activities increased significantly. Also DTT significantly reduced the turbidity of the cortical cataract.

• CONCLUSION: Our data shows that DTT has obvious inhibitory action on ultraviolet cataract.

• KEYWORDS: dithiothreitol; rat lens; ultraviolet; calciumion
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INTRODUCTION

As a serious threat to human health, cataract is one of the most common causes of blindness. Clinical studies have shown that cataract resulted from a number of factors, and ultraviolet radiation (UVR) has been specifically related to age-related cataract (ARC). With the continuous weakening of the atmospheric ozone layer, annual incidence of ARC has been increasing[1]. Epidemiologists also found positive relationship between occurrence of prevalence of cataract and elevation[2]. In Lhasa, Tibet, altitude 3658m, the rate is 41.32%; In Qamdo with an average altitude of 3147m, the incidence rate is 30.52%; Helan county of Ningxia, altitude 1111.5m, has a rate of 17.15%; and in the suburbs of Chongqing where altitude is 350m, the rate is 13.18% [3]. So it is necessary to explore new mechanisms of senile cataract and adopt early preventive interventions, particularly take preventive measures against UV in the plateau area. Therefore, finding an effective but inexpensive drug for non-surgical treatment is a desirable way to inhibit and delay the development of ultraviolet cataract.

Dithiothreitol (DTT), a commonly used reducing agent which can restore the disulfide bonds within and between the protein molecules, is usually used for the protection of protein thiol. In our preliminary studies, using our own prepared DTT eye drops could effectively inhibit the heat denaturation of βB2-crystallin, a major crystallin, delay its aggregation and help to maintain its stability[4]. In this study, a certain concentration of DTT is applied to ultraviolet rat by intraperitoneal injection, and its toxicity and efficacy is observed so as to investigate DTT inhibition of ultraviolet cataract.

MATERIALS AND METHODS

Materials Reagent dithiothreitol, imported light-packing products of the Shanghai sangon Co., Ltd., prepared with normal saline into a 25mmol/L solution (Patent of "composition for preparation of anti-cataract product". Application No: ZL200510025839. 1, Li, *et al*); anesthetic chloral hydrate, products of Xi'an Chemical Reagent, prepared with normal saline for 10% concentration; SOD, GSH-px kit, purchased from Nanjing Jiancheng Bio Co., Ltd.; Neosynephrine eye drops, product of Wuxi Ho Chi Ming credible Hill Pharmaceutical Co., Ltd.; Tropicamide, produced by Changhai Hospital; Philips UV radiation lights; TN2340 UV radiation meters, produced by Beijing Turner Co.; slit-lamp microscope (model KJ-5D) of Kang Jie, Suzhou.

Methods 6-week-old SD rats, weight about 180g. Provided by the Second Military Medical University animal experiment

Table 1 The levels of SOD, GSH, LDH and Ca^{2+} in UV-induced cortical cataract rats by intraperitoneal injection of DTT

Group (n =8)	SOD (U/mg protein)	GSH (μmol/g protein)	Ca^{2+} (mg/mg protein)	LDH (U/mg protein)
①UV radiation	530.61±52.07	1.65±0.22	0.56±0.02	34
②UV radiation+dithiothreitol	832.90±80.12	2.09±0.13	0.27±0.03	19
③UV radiation followed by dithiothreitol	905.67±89.32	3.20±0.18	0.15±0.02	10
④Control	942.80±93.80	2.00±0.37	0.21±0.02	7

center, UV radiation in rats[5,6]: 10% *chloral* hydrate 0.35mL/100g, a total of 0.6-0.7mL, intraperitoneal injection of anesthesia. As the cycloplegic agent, one drop of Phenylephrine and tropicamide eye drops was alternately used every three minutes, three times for both eyes. After the pupils dilated, the mydriatic agents in the conjunctiva were soaked up carefully with a cotton swab. Then the relative height between rat and the UV lamp was fix so that irradiance with a UV irradiance meter could be measured according to the control at 1×10^3 μW/cm^2. Now a measure of UV light from the ground is 13cm, and four rats placed below each lamp, 10cm apart, whose eye vertically faced the lamp, exposed 15 minutes for 7 days, and the cumulative radiation dose are 9kJ/m^2/ time, the same for both eyes. almost 100% of the model rats can develop the lens cortical opacity.

Totally 32 rats were randomly divided into four groups: model group (n = 8), modeling, without any treatment, experimental group (n = 8), injected the DTT solution intraperitoneally 1mL, 30 minutes before UV radiation, for 7 days; experimental group two (n = 8), injected the DTT solution intraperitoneally 1mL, 30 minutes before UV radiation, for 14 days (injected only after 7 days radiation), the control group (n = 8), no UV radiation, normal rats, a daily intraperitoneal injection of 1mL saline.

These rats were sacrificed after anesthesia. Lens were extracted intracapsularly carefully with micro-scissors, rinsed with phosphate buffered saline (PBS). The whole lenses were homogenized in 900μL crystallines extract (PH7.4 Tris-HCI buffer own preparationed). The resulting suspension was centrifuged at 12000rpm, 4℃ for 20 minutes. The supernatant was retained to detect indicators. Antioxidant enzymes level was examined using SOD, GSH test kit ; the remaining was send to biochemical inspection for Ca^{2+}, Na, LDH content.

Statistical Analysis All quantitative data are analyzed using mean ± standard deviation. The significance of differences between means was verified by analysis of variance (ANOVA). Statistical significance was declared if a P value was <0.05.

RESULTS

Role of UV Radiation on Lens After UV irradiation, antioxidant enzymes SOD, GSH levels in the lens were lower than those of control groups. Ca^{2+}, Na, and LDH increased significantly (Table 1). This demonstrated that UV radiation had a significant effect of increasing lipid peroxidation.

Intervention of Dithiothreitol Lipid peroxidation was clearly reduced and the level of peroxidase increased after dithiothreitol intraperitoneal injection of ultraviolet cortical cataract rat. And Ca^{2+} content, LDH activity were significantly decreased at the same time. Similar results were detected in the groups 2 weeks after intraperitoneal injection DTT (Table 1). No rats died with this DTT dose.

Characterization of Lens Structure and Development Compared with control groups (Figure 1A), slit-lamp micrographs demonstrated that development of lens opacities in the posterior portion of the cortex could be observed after radiation (Figure 1B). Cortical cataracts mitigate when DTT was used together with irradiation. And the turbidity showed little difference between group 2 and 3 (Figure 1C, D).

DISCUSSION

UV-B between 320-290nm wavelength directly affecting on the skin, cornea and lens and other tissues can cause free radical damage [7]. Lens exposed to UV radiation has been confirmed in previous studies as an important factor in causing cataract in vertebrates [8]. It has been studied that UV-B radiation produces oxidative damage in the lens enzymes and antioxidants [9]. Nakamura *et al*[10] has found that UV-B light increased the scattering of soluble protein in lens, and calcium-activated inhibitors and antioxidants GSH can reduce light scattering. Lou *et al* pointed out that the size of GSH pool is diminished in the age-related cataract and the lens under oxidative stress. UV radiation-induced lens is damaged due to imbalance between oxidative damage and antioxidant. The lens directly exposed to UV radiation can cause a significant SOD reduction. And antioxidant enzymes GSH can also be used as an indicator of lens injury to some extent[6]. In this study, the change of the antioxidant enzymes SOD and GSH on lens in each group were consistent with previous research.

The mechanism of UV radiation damaging the lens can be summarized as follows. Lens molecular absorbs ultraviolet photon which can produce free radicals, resulting in oxidative damage on the lens. About the pathogenesis of cataract, Spector *et al*[11] proposed that the formation of oxidative damage is the start of cataract factors, which can harm DNA, protein and lipid. Oxidative damage or UV photosensitization can affect DNA, and lead to the rupture of DNA single-stranded, double-stranded, forming pyrimidine dimers, which can change lens protein transcription and translation, resulting in rearrangement of the structure and arrangement of protein[12].

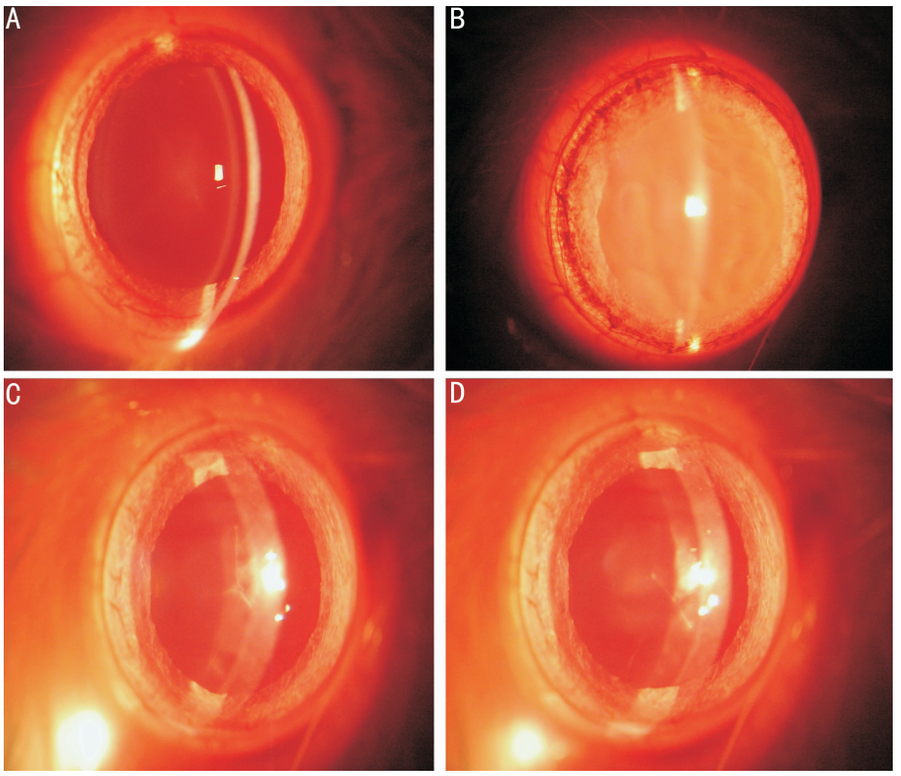

Figure 1 Lens slit-lamp biomicroscopy examination of different groups Rats were examined with slit-lamp after dilation of the pupils. A: No cataract was observed in control group rats; B: The whole lens cortex becomes opaque after radiation in model group; C: Cortex cataract began to mitigate at the end of using DTT with irradiation one week, which looks like "fishbone" pattern in the Y-sutures; D: And opacity level of continuing using DTT for another week is similar with just a week DTT.

Peroxidation and oxidase inhibition may upset cell membrane function and cell metabolism, ultimately lead to cell swelling[13]. All these disrupted the order and spatial location of lens cells, causing an increase in light scattering.

However, little has been known about the function of dithiothreitol in the lens. We speculated that dithiothreitol may play a role as a regulator of rhythmic activity in the lens protein activity. It is a common reducer that can restore disulfide bonds of the intra-protein molecules and inter-protein molecules, acting as a protein thiol protective agent. Our group has found that DTT effectively inhibits one of the major crystallins βB2 of heat denaturation, also delaying the non-specific protein aggregation and maintaining protein stability[4]. Using DTT eye drops on rat selenium cataract and ultraviolet cataract has exhibited good inhibiting effects. Therefore, this study will apply certain concentration of DTT by means of intraperitoneal injection on ultraviolet rats cataract and investigate the mechanism of DTT and proper concentration.

Our experiment results show DTT while radiation can reduce lipid peroxidation, increases antioxidant enzyme activity and reduce oxidative damage. Oxidation mechanism plays an important role in lens malformation, so any defects in antioxidant defense system may lead to and accelerate development of cataracts. As we know, the lens nucleus become dark yellow and even brown when cataract grows. Van Heyningen first reported the lens contain fluorescent pigments material in 1971 (glucose side glycosides)[14]. They are kynurenine (Kyn) evolving from the oxidation products of tryptophan in lens fiber cells, which mainly include 3-hydroxy-kynurenine, 3-hydroxy-kynurenine glucoside and glucose side 4-(2-amino-3-hydroxyphenyl)-4- OLD-glucose side oxygen acid glycosides. These substances increase crystallins aggregation, pigmentation and non-tryptophan fluorescence substances when subjected to oxidation damage. Lens opacity closely related to the changes above-mentioned. Kynurenine combining glutathione and nucleophilic amino acids (such as cystein) may become 3 - hydroxy-kynurenine glutathione adduct of glucose side glycosides. Gamer *et al*[15] found that bovine lens proteins can produce colored (365nm wavelength) and fluorescence (excitation 380nm, emission 450-490nm) protein adducts modified by kynurenine. These adducts gathered to aggravate light scattering, lower lens transparency. Oxidative damage heightens free radicals that not only result in lipid peroxidation but also accumulate the 3-hydroxy-kynurenine concentrations in lens and then cataract ensued. Recent research study how DTT can protect lens opacity by directly inhibiting lipid peroxidation and delaying non-specific protein aggregation[16], while indirectly increasing the amount of the endogenous antioxidant GSH.

It has been confirmed that high concentrations of glutathione in the lens could protect mercaptan and enzymes existing in

the structural proteins to ensure personal biological functions. GSH in lens can be biosynthesized and regenerated to maintain the level 4-6mm[17]. While GSH decreased significantly in lens which is aging or under oxdative stress. Our study show that DTT raised GSH activity, removed free radicals, OH^- (the most damaging free radicals) and the metabolites such as cyclic 3- hydroxy-kynurenine. Research has demonstrated that DTT contributed to the repair of oxidative damage on crystallins and enzymes at year 2003[18]. Speculate intraperitoneal injection of DTT works on lens through the blood circulation to aqueous humor. Its oxidation resistance may be because one is highly lipophilic[19], and the other is fully hydrophilic[20]. DTT is more easily accessible to various parts of cells than other antioxidants. Our experiments has demonstrated that DTT can protect membrane lipid of ultraviolet rats cataract, reduce oxidation and may have some effect through restraining the oxidation on membrane unsaturated fatty acids.

The balance of Ca^{2+} in the lens is the key element in cataractogenesis. The level of Ca^{2+} in the lens is about 2-10μmol/L, compared to 30μmol/L in extracellular. This great disparity of concentration gap relies on Ca^{2+}-ATP enzyme. Ca^{2+} imbalance may seriously influence the lens physiological function by repressing glucose metabolism, causing macromolecular protein aggregation and activating destructive proteases activation and so on. Ca^{2+} levels rise in lens after ultraviolet radiation, which is consistent with observations of UV-B short-term radiation[21]. It can induce calcium proteolytic system activation and then cause lens opacity [22]. We know calcium proteolytic plays an important role in lens metabolism and cell cytoskeleton regulation. Transparent protein solution will be turbid around high concentration Ca^{2+} environment[23]. Ca^{2+} inflow in the early period of cataractogenesis and high cell osmotic pressure cause cell membrane dysfunction, with gradual development of tiny opacities[24]. We found that lens epithelial cells showed excessive apoptosis before opacity in Hydrogen, calciumion, ultraviolet irradiation-induced cataract lens *in vitro*. Evidence shows that Ca^{2+} takes effect on apoptosis too. Elevated cytoplasmic calcium levels can activate DNA endonucleases, further up-regulate apoptosis related gene expression, which will cause lens epithelial cells over-apoptosis[25]. Threshold dose radiation (300nm), irradiating 15 minutes a day for a week, was prone to cause procedural cell death, and then dead cells removed from epithelium by phagocytosis[26].

Intraperitoneal injection of DTT can reduce the level of Ca^{2+} in the lens, the possible mechanism of which is considered to be related to the fact that reduced lipid oxidation inhibits Ca^{2+} into the cell and/or increases membrane stability. A transient cytoplasmic calcium rise in response to ATP is well documented in lens epithelium[27]. And DTT could stop Ca^{2+} from inositol triphosphate mobilization, or prevent Ca^{2+} inflow through voltage-gated channel. Ca^{2+} released from the storage pool acting as antioxidants under oxidative stress causes greater damage than the lipid peroxidation[28]. So the most effective way to protect cells against oxidative damage is the combination of antioxidants and Ca^{2+} inhibitor[29]. While DTT exhibits such properties, our results also show that DTT can protect lens from opacity, playing its role through directly inhibiting lipid peroxidation, reducing cytosolic Ca^{2+} concentration, and indirectly increasing the endogenous antioxidant.

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二硫苏糖醇对紫外线诱导大鼠白内障的保护作用

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摘要

目的:探讨外源性给予二硫苏糖醇(DTT)对于紫外线诱导SD大鼠白内障的保护作用。

方法:32只6周龄SD大鼠(约180g)分为4组:第一组为模型组,每天辐射300nm紫外线B(ultraviolet B)15min,辐射强度$1\times10^3\mu W/cm^2$,连续辐射7d;第二组为实验组,辐射UVB联合每天腹腔注射DTT溶液1mL(腹腔注射于辐射30min前);第三组实验组同第二组外,7d辐射结束后继续用DTT 1wk;第四组为空白对照组,不辐射UV-B,每天腹腔注射等量的生理盐水。

结果:腹腔注射DTT可以明显减少紫外线辐射引起的脂质过氧化,提高过氧化物酶(SOD)、还原型谷胱甘肽(GSH)含量,并且可以降低晶状体中钙离子(Ca^{2+})和乳酸脱氢酶(LDH)含量。

结论:二硫苏糖醇对紫外线诱导白内障SD大鼠具有良好的抑制作用。

关键词:二硫苏糖醇;大鼠晶状体;紫外线;钙离子