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ABSTRACT:

The purpose of this research is to investigate the effect of catechin isolate from GMB4 Clone Green Tea on apoptotic cell death and oxidative stress in the lens of rats with cataract. Cataract isolate from GMB4 Clone Green Tea was induced by intraperitoneal injection of 19μmol/kg sodium selenite to ten day-old Wistar rats. The neonatal rats were randomly divided into five groups (n=5 in each group): a control group, and four cataract-induction groups, treated with either 0, 50, 100, 200mg/kg catechin Isolate from GMB4 Clone Green Tea. We performed slit-lamp bio microscopic analysis, level of GSH and GR, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay and immunohistochemistry for caspase-3. Both eyes of all rats in Group 1 did not exhibit cataract formation. In Group 2, one out of five (20%) developed grade 3 cataracts and the remaining four out of five (80%) developed grade 4 cataracts. The difference in exhibited cataract in the lens of all rats between group 2 and any eyes of groups 3 or 4 and 5 were significant (P = 0.022, 0.001, 0.001). The grade of cataract formation was decrease in group 3, 4 and 5. The mean GSH and Caspase-3 levels lenses of group II rats were significantly (P < 0.01) lower than the levels in Group I, Group III, Group IV and Group V. Apoptotic cell death and oxidative stress in the lens that increased following cataract formation in rats was suppressed by cathechin Isolate From GMB4 Clone Green Tea.

Keywords: Apoptosis Oxidative Stress Cataract Catechin Isolate Sodium selenite.

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Research

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KEYWORDS: Apoptosis, Oxidative Stress, Cataract, Catechin Isolate, Sodium selenite

INTRODUCTION:

The cataract is an opacity that develops in the crystalline lens of the eye; it varies in degree from slight to completely opaque, obstructing the passage of light. The lens epithelium covers the anterior surface of the lens. Epithelial cells near the lens equator divide and differentiated into the lens fibers. This process continues at a constant, slow rate throughout adult life, resulting in the steady growth of the lens fiber mass [1]. Damage of the lens epithelium has been a major focus in the identification of causes of cataract formation [2].

Pathogenesis of cataracts is multifactorial, with the disease developing as a result of heredity, trauma, inflammation, metabolic disorders, malnutrition and age-related changes, amongst other pathways. Some risk factors, such as oxidative damage, impaired glucose metabolism, radiation damage and toxic damage to the lens, also play an important role in the pathogenesis of cataracts. One of the most common types of cataracts is that related to age. Although the exact mechanism of age-related cataract formation is unknown, the increase in free oxygen radicals and the reduction in antioxidant enzymes in the lens have been identified as possible mechanisms. According to the theory of oxidative damage, free oxidant radicals lead to cataract formation by cross-linking and aggregation of lens proteins, the peroxidation of membrane lipids and by apoptosis of epithelial cells in the lens [3-5].

Increased amounts of oxidative substances and reduced levels of antioxidants in the lens such as glutathione were proposed to be involved in the pathogenesis of cataracts [6-8]. Researchers have uncovered the importance of increased oxidative substances and reduced levels of antioxidants in the pathogenesis of cataracts [9-11].

Glutathione is the most important antioxidant in the lens and is synthesized the lens epithelium. The reduced glutathione (GSH) exists in high concentration in the lens. GSH provides maintenance of the lens transparency by scavenging reactive oxygen species and protecting protein thiols. It has been reported that the GSH level in the lens is decreased in age-related cataract [12-15].

Apoptosis, also known as programmed cell death, is a form of cell death that serves to eliminate dying cells in proliferating or differentiating cell populations. Thus, apoptosis plays a crucial role in normal development and tissue homeostasis. Previous studies have shown that apoptosis of lens epithelial cells plays an important role in the development of several types of cataracts. These studies have suggested that apoptosis of lens epithelial cells appears as a common cellular mechanism mediating stress-induced non-congenital cataractogenesis [16].
Apoptosis can be detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay, a measure of DNA fragmentation in tissue sections, and by observation of a DNA ladder, a measure of fragmentation in DNA extracted from cells or tissues. In human cataract research, TUNEL-positive cells indicate apoptotic cell death in the lens epithelium. Another important characteristic of apoptosis is caspase activation. Caspase-3 is one of the most widely studied caspases, and it is a key executor of apoptosis [17].
Cataract is a major health problem and the major cause of blindness throughout the world [18]. Currently, the only available treatment for the disease is the surgical extraction of the cataractous lens followed by replacement with a synthetic implant. Although such a surgical replacement of the natural lens with an artificial lens is significantly effective in restoring vision to most patients, it is not free of complications. Attempts to reverse cataract formation, or at least significantly retard the onset of the disease would be of great value [19, 20, 21].
The functional roles of catechin have been well documented, but its effect on the lens epithelium following cataract formation remains poorly understood [22]. Accordingly, research is needed to prove the effect of GMB4 clone green tea catechin isolates that can protect lens epithelial cells against oxidative stress and apoptosis so as to delay the onset of cataracts. The aim of the study was to evaluate the effects of Catechin Isolate from GMB4 Clone Green Tea on sodium-selenite induced cataract formation and activities of the enzymes glutathione (GSH), and Caspase-3.

MATERIAL AND METHODS:

This study was performed in Biosains Laboratory of Brawijaya University. Twenty five Wistar-albino rat pups were housed with their mother in special wire-bottom cages and in standard conditions (12-hour daylight-dark cycle, ventilated, constant room temperature). It has been considered that solid-bottom cages are more adequate for the housing of the rat pups. The rat pups, were divided into five groups (four experimental and one control), each consisting of five pups. Group 1 received only subcutaneous saline injection and was the control group. In Group 2, sodium-selenite (19 nmol/g body weight, Sigma Chem. Co., St Louis, USA) was injected subcutaneously on postpartum Day 10. In Group 3, subcutaneous sodium-selenite (19 nmol/g body weight) was injected on postpartum Day 10 and injection of isolate catechin (50 mg/kg body weight) , starting one day before sodium-selenite injection (on postpartum Day 9) and was continued for 5 days (till postpartum Day 13). The procedures performed on Group 3 rats were also performed on Group 4 and Group 5, the difference being the dosage of isolate catechin. Group 4 had used 100 mg/kg body weight of isolate catechin and Group 5 was 200 mg/kg body weight.
On postpartum Day 17 all rats were anesthetized with intraperitoneal ketamine injection (80 mg/kg) and xylazine (15 mg/kg). The rat pups were taken out and the pupils were dilated with tropicamide 0.5% every 30min for two hours. All lenses were evaluated and were morphologically staged for cataract development and staging was performed by slit-lamp bio microscopy on a scale of 0 to 4; Grade 0 was a normal clear lens, Grade 1 was a sub capsular opacity, Grade 2 was a nuclear cataract, Grade 3 was a strong nuclear cataract with an opacity in the perinuclear area, and Grade 4 was a mature dense opacity involving the entire lens [23]. Lens photos ×25 magnifications were taken using a camera attached to slit-lamp (Topcon, Tokyo, Japan) (Figure 1). The lens was then taken immediately after euthanasia, the eyes were enucleated. Frozen lens samples were weighed and homogenized in ice cold phosphate buffered saline solution (0.01 mol/L and pH 7.4). Homogenization procedures were carried out using Bullet Blend tissue Homogenizer (Next Advanced Inc, Averill Park, NY, USA), according to the manufacturer's instructions at 4 ℃. These homogenates were centrifuged at 10 000 g for 30min at 4 ℃, and supernatants were obtained. Supernatants were used for the measurement of the levels of GSH and caspase-3.
The GSH measurements were carried out using a GSH kit (ImmuchromGmbH, Hessen, Germany) with high-performance liquid chromatography. During the reaction of derivatisation glutathione is converted into a fluorescent probe. The precipitation step removes high molecular substances. After centrifugation, the fluorescent probe is cooled (2℃ -8℃) and 20 µL samples are injected into the HPLC system. Measurements were carried out on the HPLC system with a fluorescence detector at 385 nm (excitation) and 515 nm (emission). Results were expressed as micromoles per liter. To visualize caspase-3 expression, we performed caspase-3 immunohistochemistry using a previously described method [24]. Sections were drawn from each lens and incubated overnight with mouse anti-caspase-3 antibody (1:500; Santa Cruz Biotech) and then for another 1 h with biotinylated mouse secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Bound secondary antibodies were then amplified with a Vector Elite ABC Kit® (1:100; Vector Laboratories). The antibody-biotin-avidin-peroxidase complexes were visualized using 0.03% DAB, and the sections were finally mounted onto gelatin-coated slides. The slides were air dried overnight at room temperature, and coverslips were mounted using Permount®.

Data are presented as mean ± standard deviation and differences between groups were analyzed using one-way ANOVA with SPSS 17.0 Statistical Package. The post-hoc test was used if the ANOVA was significant. P< 0.01 was considered as statistically significant.
A 0.45 µm nylon filter (Pall life Sciences, Mumbai, India) was used. All other chemicals and reagents used were analytical grade unless otherwise indicated.

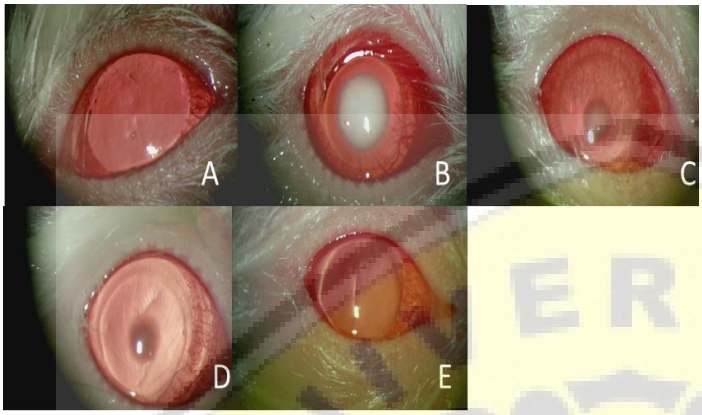

Fig.1: The Slit-lamp Pictures of Representative Lenticular Opacities
(A) clear lens (grade 0) in control group, (B) grade IV in group of only sodium-selenite, (C) grade III in sodium-selenite with isolate catechin 50 mg/kg body weight group, (D) grade II in sodium-selenite with isolate catechin 100 mg/kg body weight, (E) grade I in sodium-selenite with isolate catechin 200 mg/kg body weight group.

The comparison of the right eye and left eye with paired samples correlations method were not significant (p= 0,749) and paired samples test (p=1,00).

Table 1: The Opacity Grading of the Lens in All of the Groups

Experimental Groups	Number of Wistar Rats	Number of pups with different degrees of lenticular opacification					Number of pups in which lenticular opacification occurred
		0	+	++	+++	++++	
Group 1 (Normal)	5	5	-	-	-	-	0
Group II (Sodium Selenite only)	5	-	-	-	1	4	5 (100%)
Group III (Sodium Selenite + catechin 50 mg/kg) body weight group	5	-	1	2	2	-	5 (100%)
Group 1V (Sodium Selenite + catechin 100 mg/kg) body weight group	5	2	1	1	1	-	3 (60%)
Group V (Sodium Selenite + catechin 200 mg/kg) body weight group	5	4	1	-	-	-	1 (20%)

Lenses in both eyes of all control rats (Group 1) remained clear [Fig. 1A]. Subcutaneous injection of Na2SeO3 (19 µmol/kg) on postpartum day 10 was sufficient to induce cataract formation, which was visible by the time the rat pups opened their eyes. Inspection of the rat pups' eyes with a slit lamp microscope confirmed that all animals injected only with Na2SeO3 developed cataracts: one out of five (20%) developed grade 3 cataracts (Fig. 1C) and the remaining four out of five (80%) developed grade 4 cataracts (Fig. 1B). In comparison, Na2SeO3 with Catechin 50mg/kg injections showed that the severity of cataract formation decreased; two rats out of 5 (40%) developed grade 3 cataracts (Fig. 1C), two rats out of 5 (40%) developed grade 2 cataracts (Fig. 1D) and one out of five (20%) developed grade 1 cataract (Fig. 1E) while grade 4 cataract (Fig. 1B) was not founded. Na2SeO3 with Catechin 100mg/kg injections decreased the severity of cataract formation; one rat out of 5 (20%) developed grade 3 cataracts, one rat out of 5 (20%) developed grade 2 cataracts while three out of five (60%) did not develop any cataracts (grade 0). Na2SeO3 with Catechin 200mg/kg injections decreased the severity of cataract formation; only one rat out of five (20%) developed grade 1 while four out of five (80%) did not develop cataract (grade 0).These results indicated that Catechin especially 200 mg/kg BB dosage, was successful in preventing cataract formation. The grading of the lens in all of the groups is tabulated in Table 1, and the slit-lamp pictures of representative lenticular opacities observed for each group are shown in Fig. 1. No toxic effects to the cornea or conjunctiva of the

eye. This difference was statistically significant. The comparison between group 2 with group 1, 4, and 5 were significant (p= 0.000, 0.000, 0.000) while group 3 was not significant (p= 0,022). The comparison between group 3 with group 1 and 5 were significant (p=0.001, 0.003) while group 2 and 4 were not significant (p= 0.022, 0.253).

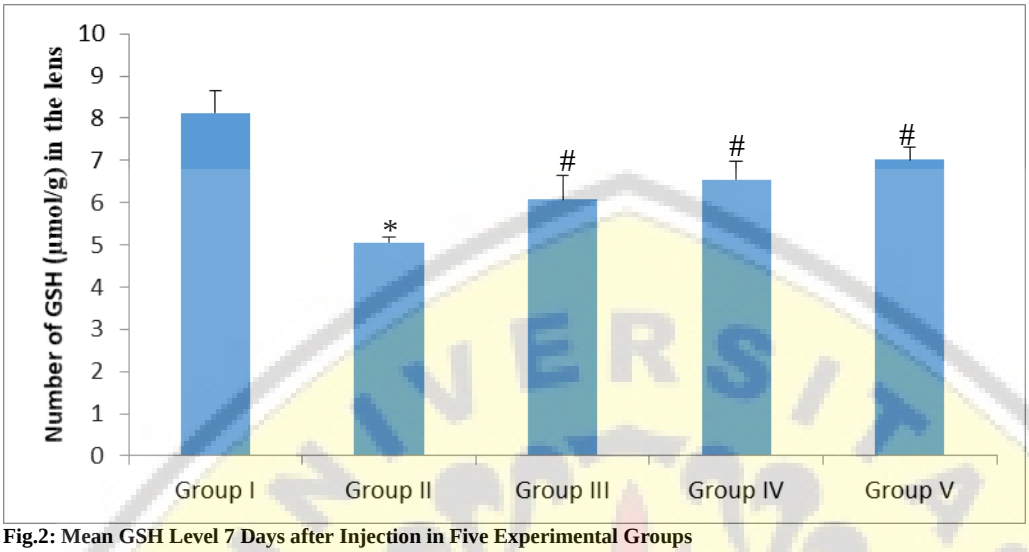

Fig.2: Mean GSH Level 7 Days after Injection in Five Experimental Groups
The scale bar represents 50 mm number of GSH expression in each group. (1) group 1, (2) group 2, (3) group 3, (4) group 4, (5) group 5.
*p < 0.01 - compared to the control group (group 1); #p < 0.01 - compared to the cataract induced group (group 2).

The mean GSH levels lenses (5.06 ± 0.13) of group II rats were significantly (P < 0.001) lower than the levels in Group I lens (8.36 ± 0.51)], Group III lens (6.08±0.56)], Group IV (6.54±0.45), and Group V (7.02±0,29) (Figure 2). Significant differences were also observed in levels of GSH in lenses (P < 0.001) between group III and group I. The lens GSH level decreased gradually with increase in the stages of lens opacity (that is, with increasing opacification) in group II and group III.
GSH levels in lenses from the Na2SeO3 group were found to be significantly (p < 0.01) lower than those of the lenses from the control and Catechin groups. Treatment with Catechin in the Catechin + Na2SeO3 group (Fig. 2) significantly (p < 0.01) increased GSH levels.

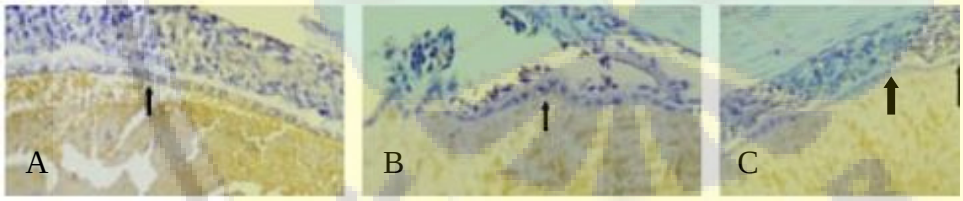

Fig.3: Caspase-3 (A) Group 1 Results, (B) Group 2, (C) Group 5

Effect of catechin on caspase-3-expression in the lens epithelium induced by cataracts. Photomicrographs of caspase-3-positive cells in the lens epithelium. (A) control group, (B) cataract-induction group, (C) cataract-induction and 200 mg/kg catechin group. The sections were stained for caspase-3 immunoreactivity (brown).

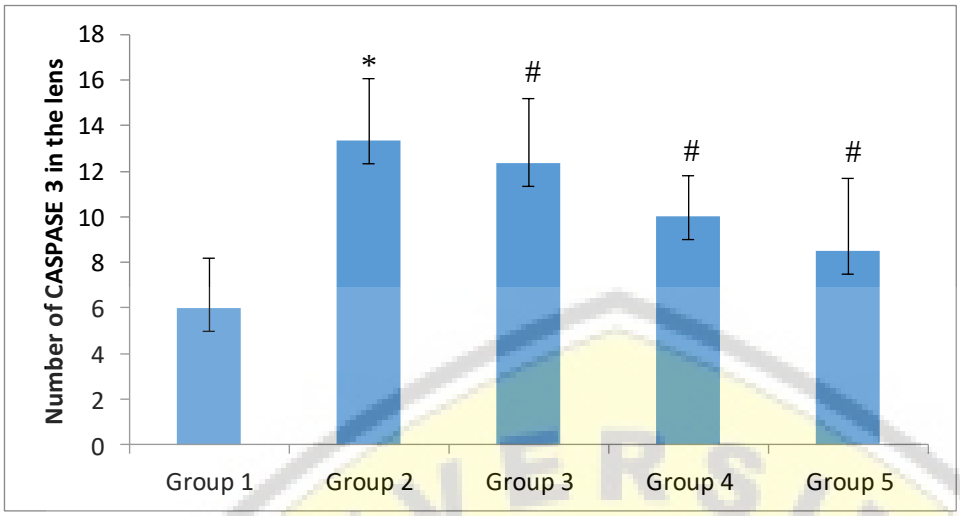

Fig.4: Effect of Catechin on Caspase-3-Expression in the Lens Epithelium Induced by Cataracts
The scale bar represents 50 mm number of caspase-3-positive cells in each group. (1) group 1, (2) group 2, (3) group 3, (4) group 4, (5) group 5. *p < 0.01 - compared to the control group (group 1); #p < 0.01 - compared to the cataract induced group (group 2).

DISCUSSION:

Cataract is the leading cause of blindness worldwide, and surgical replacement of the opacified lens with an artificial lens is currently the only way to remedy vision loss. Although cataract surgery is considered to be very successful in terms of visual outcome, the cost, need for trained personnel, and postsurgical complications limit the worldwide availability and accessibility to this procedure. Hence, development of alternatives to surgical intervention is warranted [23].

Oxidative stress is an imbalance between the rate of oxidant production and degradation [25]. Substantial supporting evidence suggests that reactive oxygen species (ROS) and oxidative damage are involved in the development of cataracts [26, 27]. Cataract formation has a multifactorial etiology. Oxidative stress, resulting in the depletion of antioxidant defense systems in the lens, is considered to be a major factor in the formation of cataracts. Lens transparency is dependent on the preservation of a favorable redox balance, which is, in part, maintained by its high GSH content [28, 29]. De-creased levels of GSH in the lens can lead to free radical accumulation, resulting in lipid peroxidation and de-creased antioxidant enzyme activity [30, 31], all of which lead to cataract development. Therefore, an alternative method to prevent or treat cataracts would be the use of antioxidant. Based on this, we have investigated the effects of antioxidant, Catechin in selenite-induced cataracts. Results from morphological observation indicate that Catechin was able to prevent the formation of cataracts in the Catechin + Na2SeO3 group (Table 1).

As discussed earlier, GSH is the most important anti-oxidant in the lens; it is the first line of defense against oxidative stress [32]. Our results show a significant decrease in GSH levels in the lenses of the Na2SeO3 group (Fig. 2) when compared to those of the control group indicating oxidative stress. Treatment with Catechin significantly increased the GSH levels in the Catechin-treated group. This suggests that Catechin was able to prevent oxidative stress by restoring GSH levels [33].

However, significant improvement in the GSH was observed in the Catechin + Na2SeO3 group (Fig. 2). Furthermore, changes in the levels of GSH was seen to affect the activity of GR. This enzyme regenerates GSH from its oxidized form and is imperative to GSH homeostasis. Increased activity of GR in the lenses of the Na2SeO3 group could be attributed to the activation of the lens antioxidant defense network against a change in the redox status. Furthermore, Catechin treatment increased the levels of GSH and restored GR activity.

Several animal species experience spontaneously occur- ring cataract of known inheritance and offer valuable model for studying human cataract [34]. Various chemicals are known to contribute to the development of cataract in animals. Among these chemicals, catechin, a direct-acting alkylating agent that does not require metabolic activation, is known as a cataractogenic agent in rats [35]. In addition, young animals are reported to be more susceptible to catechin than are adult animals. Therefore, in this study, a cataract model was constructed using a single intraperitoneal injection of catechin in rats at postnatal day 10.

Division of the lens epithelial cells is confined to the periphery of the lens. These cells move toward the equator and then differentiate into lens fibers. Apoptosis of lens epithelial cells can occur during this differentiation process [36, 37]. It is well known that apoptotic death of lens epithelial cells induces lens opacification. Lens epithelial cells play a vital role in the metabolic homeostasis and maintenance of transparency in the lens [38], and damage to lens epithelial cells potently contributes to cataractogenesis. Moreover, apoptosis of lens epithelial cells has been reported to be the earliest event in the experimental formation of cataracts, such as those inducted by hydrogen peroxide and catechin [39]. In human studies, caspase-3 is up-regulated and activated in the early stages of apoptosis following cataractogenesis [40].

We found that the numbers of caspase 3-positive cells in the lens epithelium were significantly higher following cataract induction (Fig.3). Opacification in the eye- ball was also greater following cataract induction. These findings indicate that catechin injection-induced cataracts increased apoptosis in the lens epithelium.

We observed that catechin significantly suppressed both cataract-induced increases in DNA fragmentation and caspase-3 expression in the lens epithelium in dose-dependent manners. In addition, catechin alleviated the degree of opacity induced by cataract formation.

In summary, our data indicate that oxidative stress and apoptosis plays a role in cataract formation, particularly in glutathione and caspase-3 maintenance and suppression of apoptotic cell death in the lens epithelium. The data support our hypothesis that Catechin especially with 200mg/kg body weight protects the lens by increase number of GSH and decrease number of caspase-3.
Our present and future studies may eventually help prevent cataract formation in high-risk populations and treat early-stage cataracts without need for surgical intervention. Catechin could potentially be used to delay cataractogenesis through the suppression of apoptotic cell death and oxidative stress in the lens epithelium.

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CONFLICT OF INTEREST:
The authors declare no conflict of interest.

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