• Basic Research •

Effect of epigallocatechin gallate in green tea on preventing lens opacity and αB-crystallin aggregation in rat model of diabetes

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Abstract

● **AIM:** To evaluate the effect of epigallocatechin gallate (EGCG) in preventing lens opacity and the aggregation of lens αB-crystallin in model rats of diabetes mellitus (DM).

● **METHODS:** This experimental study included Wistar rats for DM as *in vivo* models and divided into 5 groups. The treatment groups were administered EGCG by orally for 20d and were then assessed for their degree of lens opacity with binocular microscope and lens αB-crystallin expression from Western blot analyze.

● **RESULTS:** Pearson correlation test and regression analysis on EGCG exposure and final random blood sugar (RBS) obtained a significance level of $P<0.05$. EGCG exposure can significantly lower RBS with an R^2 of 0.5634 (56.34%). The same analysis on EGCG exposure and the degree of lens opacity obtained a significance level of $P<0.05$ and increased exposure to EGCG can significantly lower the degree of lens opacity with an R^2 of 0.8577 (85.77%). Correlation analysis between EGCG and the expression of lens αB-crystallin can be concluded that the higher the EGCG exposure administered, the higher the native lens αB-crystallin expression and the lower the aggregate lens αB-crystallin expression. There was also significant effect in which every 1 mg/kg body weight dose of EGCG can increase the native lens αB-crystallin expression by 0.0063 and decrease the aggregate lens αB-crystallin expression by 0.0076.

● **CONCLUSION:** The administration of EGCG at a dose of 300, 600, and 1200 mg shows a significant effect on preventing lens opacity and aggregation of αB-crystallin in diabetic rat models and this research could be a biomolecular prevention of cataract.

● **KEYWORDS:** diabetes mellitus; epigallocatechin gallate; cataract; lens αB-crystallin

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INTRODUCTION

Diabetes mellitus (DM) is a chronic systemic metabolic disease marked with hyperglycemia, associated with microvascular and macrovascular complications, which causes significant morbidity, particularly involving the kidney, eye, heart, cerebrovascular diseases, and early mortality. Its prevalence across the globe indicates that 463 million people live with diabetes. Of these people, 111 million were among the elderly population above 65 years old, which further intensify their vulnerability to the onset of complications[1-3]. In patients with DM, there is an increase in oxidative stress due to hyperglycemic conditions. Hyperglycemia can affect complications through 4 paths, which is an increased flow of glucose through the polyol pathway, the activation of protein kinase C (PKC) through *de novo* synthesis of diacylglycerol, an increased formation of glycation end-products (AGE), and increased oxidative stress. The polyol pathway has been described as a primary mediator of DM that induces oxidative stress in the lens. In this path is where the nonenzymatic glycation reaction of the crystallin protein takes place, which results in the cross-link between protein molecules, thereby allowing the addition of proteins with a higher molecular weight, that affect lens opacities and can causes cataract[4-5].

There are several types of plant extracts that can act as potent antioxidant to prevent oxidative stress in age-related diseases, such as cataract, dry eye syndrome (DES), and age-related macular degeneration (AMD). Epigallocatechin gallate (EGCG) is an active antioxidant molecule present in green tea that

can neutralize free radicals. EGCG is known for its ability to protect pancreatic β-cells, which secrete insulin from damage caused by diabetic conditions. Other studies also demonstrated a similar finding, which is the suppression of the increase in blood sugar level in diabetic rats that were administered EGCG for 4wk, compared with the controls. However, its protective effect on lenses under the condition of DM has not been much researched[6-8].

Given its potential, the administration of EGCG can hopefully trigger the prevention of the aggregation of lens αB-crystallin in rat models of DM. Furthermore, this study may subsequently serve as an early biomolecular assessment of cataract.

MATERIALS AND METHODS

Experimental Animal This study is a true experimental study involving 3-month-old male Wistar rats weighing 300–400 grams. Acclimation was done in a cage at a temperature of 25℃, standard humidity of 50%, 12-hour light-dark cycle, and they were provided food in the form of pellets and drinking water from the PDAM (local-government-owned water utilities).

Extraction of Epigallocatechin Gallate EGCG was obtained from 500 grams of young green tea leaves that were dried beforehand at a temperature of 50℃ for 8h. Then, the green tea leaves were blended and steeped at a temperature of 80° for 30min, and then filtered to separate the liquid from the solid. The liquid extract was concentrated using an evaporator at a temperature of 40°C. The bioactive compound was harvested using 11% C18 silica gel to isolate it from the other substances. The product was filtered and evaporated.

Experimental Procedures

Preparing rat models for diabetes The rats were intraperitoneally injected with streptozotocin (STZ; 40 mg/kg, dissolved in a 0.1 mol/L citrate buffer, pH 4.5). Next, the rats' random blood sugar (RBS) levels were measured 3d post-STZ-injection using a glucometer. The rats with a random blood sugar level >250 mg/dL were considered to have DM. The rats were then divided into 5 groups: negative control group, positive control group, treatment 1 group, treatment 2 group, and treatment 3 group[9].

Examining the degree of lens opacity After the rats were subjected to EGCG at doses of 300, 600, and 1200 mg/kg body weight (BW), their degrees of lens opacity were assessed by 1 observer and classified as follows: clear normal lens (degree 0); peripheral vesicle (degree 1); peripheral vesicle and cortical opacity (degree 2); diffuse central opacity (degree); and mature cataract/uniform opacity (degree 4)[10].

Assessing the expression of lens αB-crystallin using Western blot The rats' lenses were dissected and washed with ice-cold saline to remove any traces of blood, and they underwent Western blot analysis using the αB-crystallin (Ser59) antibody. The right and left lenses of each research subject were homogenized in a 0.1 mol/L Tris buffer (pH 8.0) and centrifuged at 20 000 g for 20min and 4°C. The supernatant underwent electrophoresis inside 12.5% polyacrylamide gel using Tris-glycine as the running buffer. After the electrophoretic separation, the protein was transported to the immobilon nitrocellulose membrane. Immunoblotting was performed with the primary antibody (αB-crystallin antibody, diluted 1:1000) after blocking with non-fat dry milk (NFDM), then incubation was done using peroxidase tagged anti-rabbit IgG antibody. Immune complexes were detected using diaminobenzidine (0.01%) and H_2O_2. The quantification of band intensity was carried out using the gel doc system through the Quantity One software (version 4.0; Bio-Rad, Hercules, CA, USA). Images were taken from the Western blot test results was done and analyzed quantitatively for each research sample[11].

RESULTS

RBS tests were performed on all group of rats before STZ injection, after STZ injection, and at the end of the study. The initial RBS test prior to STZ injection was to prove that the rats involved in this study were rats with a normal blood sugar level (non-diabetic), which became hyperglycemic (diabetic) after the injection of STZ 40 mg/kg in the positive control group and the all-treatment groups observed 3d after the injection. At the end of this study, another RBS test was also performed to ensure that the positive control group remained diabetic and the negative control group still had a normal RBS level. This study observed that the negative control stayed normoglycemic, the positive control group and the treatment groups were hyperglycemic post-STZ-injection, and the positive control group stayed hyperglycemic until the end of this study while the treatment groups demonstrated decreased RBS levels after having been administered EGCG.

Based on the one-way ANOVA test, the significance level was 0.001 (P<0.05), which means that there was a difference in the mean final RBS level in each group. The result of Pearson correlation test of exposure to EGCG with final RBS level (after treatment) in diabetic rat models has a significance level of 0.008 (P<0.05), which means that there is a significant correlation. A strong and inverse correlation between EGCG exposure dose and final RBS level at a significance level of 5% is shown by the negative correlation value of -0.751. Therefore, it can be concluded that the higher the EGCG dose exposed, the lower the diabetic rat models' RBS level.

Also, in this study, it was found that EGCG exposure has a significant and negative effect on the final RBS of diabetic rat models. In other words, an increase in exposure to EGCG can significantly lower RBS in diabetic rat models. The magnitude of the effect of EGCG exposure on the decrease of the final

RBS level can be seen from the coefficient of determination (R squared), where the *R* squared was 0.5634 (56.34%). After having assessed the degree of cataract opacity, the results shows that the positive control group has the highest degree of lens opacity than the other groups, and the treatment 3 group, which received a dose of EGCG of 1200 mg/kg BW, has the lowest degree of lens opacity compared with the other two treatment groups (Figure 1).

In the one-way ANOVA test, the significance level obtained was <0.001 (P<0.05), which can be concluded that there is a difference in the degree of lens opacity in each group. In the Pearson correlation test between EGCG exposure and lens opacity in diabetic rat models, the significance level obtained was <0.001 (P<0.05), which means that there is a significant correlation. A strong and inverse correlation between the dose of EGCG exposure and the degree of lens opacity at a significance level of 5% is marked with a correlation value of -0.921 (92.1%). As a consequence, it can be concluded that the higher the EGCG exposure dose administered, the higher the ability to lower lens opacity in diabetic rat models (Figure 2).

In this study, EGCG significantly and negatively affects lens opacity in diabetic rat models. In other words, an increase in EGCG exposure can significantly decrease the degree of lens opacity in diabetic rat models. The magnitude of the effect of EGCG exposure on the decrease of lens opacity can be seen from the coefficient of determination (R^2), where the R^2 was 0.8577 (85.77%; Figure 3).

After testing the expression of lens αB-crystallin using the Western blot method, the highest decrease in native lens expression of αB-crystallin was detected in the positive control group, which consists of rats with DM that did not receive EGCG treatment. Meanwhile, higher expressions of native lens αB-crystallin were found in the treatment groups compared with the positive control group. In other words, the higher the EGCG dose administered, the higher also the expression of native lens αB-crystallin.

The expression of aggregate lens αB-crystallin shows a contrasting condition when compared with that of native lens αB-crystallin in which the highest expression of aggregate αB-crystallin belongs to the positive control group, while the lowest expression is in the negative control group. Moreover, in the treatment groups exposed to EGCG, it can be seen that the higher the EGCG, the lower the aggregate lens αB-crystallin expression (Figure 4).

The one-way ANOVA of mean expression of native lens αB-crystallin obtained a significance level of 0.000, which is lower than α (0.05). Thus, it can be concluded that there is a difference in the mean expression of lens αB-crystallin in each group. Based on the result of the correlation analysis, it can be concluded that the higher the EGCG exposure, the higher the native lens αB-crystallin expression and the lower the aggregate lens αB-crystallin expression with a significance level of 0.000 (Figure 5).

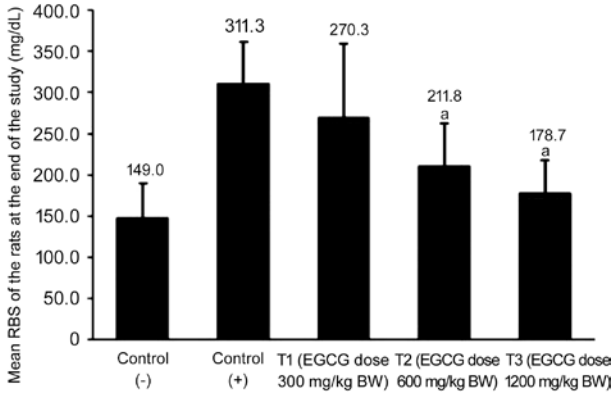

Figure 1 The mean RBS of the rats at the end of the study The highest mean RBS level can be found in the positive control group, and the higher the EGCG dose given, the more the RBS level decreases. [a]Significant difference compared with the (+) control group. RBS: Random blood sugar; BW: Body weight; EGCG: Epigallocatechin gallate.

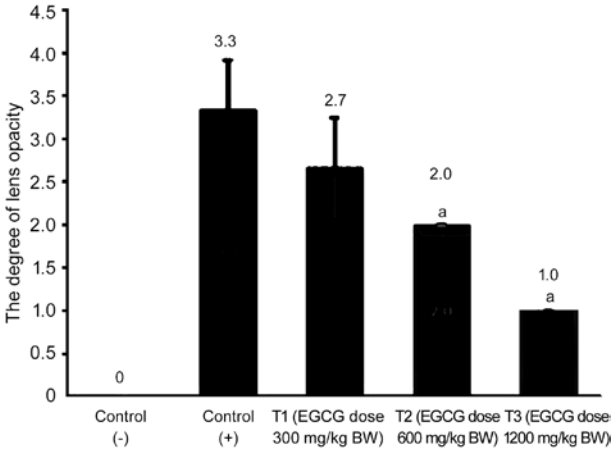

Figure 2 The degree of lens opacity The positive control group has the highest degree of lens opacity compared with the other groups, and the treatment 3 group has the lowest degree of cataract opacity compared with the treatment 1 and treatment 2 groups. [a]Significant difference compared with the (+) control group; BW: Body weight; EGCG: Epigallocatechin gallate.

This study produced a simple linear regression equation between EGCG exposure (X) and the expression of native lens αB-crystallin Y=-0.97+0.00063X and the expression of aggregate lens αB-crystallin Y=12.65−0.0076X. Therefore, this study concludes that for each increase in the EGCG exposure dose by 1 mg/kg BW, the expression of native lens αB-crystallin increases by 0.00063× and the expression of aggregate lens αB- crystallin decreases by 0.00076×.

DISCUSSION

This study found that the treatment groups that were administered EGCG demonstrated a statistically significant decrease in final RBS level compared with the positive control group, and the higher the EGCG dose administered, the higher

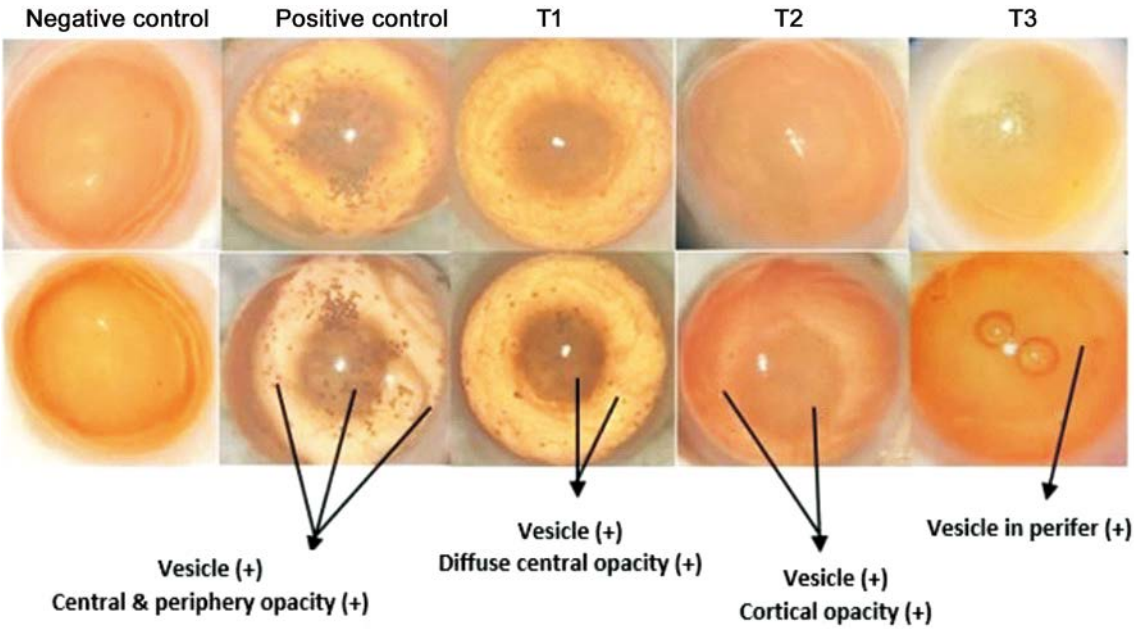

Figure 3 Degree of lens opacity The negative control group shows degree 0, the positive control group shows degrees 2–4, the T1 group shows degrees 2–3, the T2 group shows degrees 1–2, and the T3 group shows degrees 0–1.

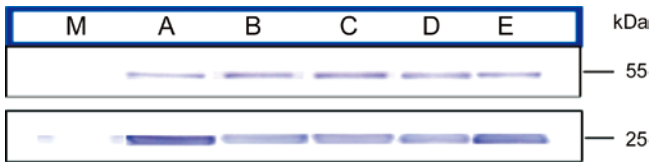

Figure 4 The expression of lens αB-crystallin using the Western blot M: Standard protein marker; A: Negative control; B: Positive control; C: T1 group; D: T2 group; E: T3 group. The band with a molecular weight of ±25 kDa represents native αB-crystallin native and that of ±55 kDa represents aggregate αB-crystallin.

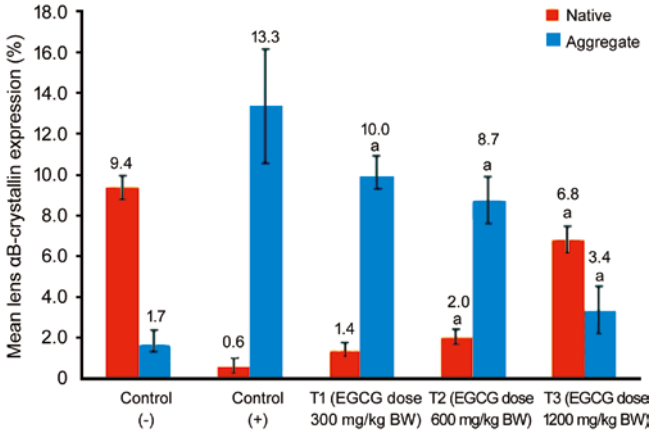

Figure 5 Mean lens αB-crystallin expression The blue-colored bars denote native αB-crystallin expression; the orange-colored bars denote aggregate αB-crystallin expression. The higher the EGCG dose in diabetic rat models, the higher the native αB-crystallin expression but the lower the aggregate αB-crystallin expression. [a]Significant difference compared with the (+) control group; BW: Body weight; EGCG: Epigallocatechin gallate.

the decrease in the final RBS. This seems to be a secondary finding in this study, which reveals that there is a statistically significant correlation and effect of EGCG dose and diabetic rat models' final RBS. In one study it was stated that there was an emphasis on increasing levels of RBS in diabetic rats' model after given EGCG for 3wk. EGCG is known to protect insulin-secreting pancreatic β cells from damage due to diabetes. Another study on EGCG suggested that a similar role as insulin was demonstrated in the HepG2 cell, which was able to decrease the phosphorylation of Ser307 from the insulin receptor substrate-1 (IRS-1) through the activation of the AMPK pathway. In the endothelial cells of the retina in a hyperglycemic condition, 40 mmol/L of EGCG may provide a protective effect by regulating inflammatory cytokines and reducing the expression from the phosphorylation of p38 mitogen-activated protein kinase (MAPK), extracellular regulated kinase (ERK), and vascular endothelial growth factor (VEGF). This shows that EGCG-induced glycemic control may provide a protective effect of STZ-induced diabetic conditions[10-13].

In the inhibition pathway of gluconeogenesis, EGCG is also considered to have the same effect as insulin, which can thereby reduce the expression of gluconeogenesis enzymes. In addition, EGCG can also increase tyrosine phosphorylation from insulin receptors and phosphoinositide 3-kinase with similar functions to insulin[13].

STZ-induced rats have a higher glucose level, this exact hyperglycemic condition is what causes the change in membrane permeability, therefore increasing Ca^{2+}, Na^{+}, Mg^{2+}, and decreasing K^{+}. This results in the overactivation of protease, insolubility, and aggregation of proteins, which, in turn, affects lens opacity. The results of this study show that the negative control group (normal rats) showed clear

lenses. In the positive control group, there was lens opacity with a range of degrees 2–4 by the end of week 4 (at the end of this study). On the other hand, the treatment 3 group that received an EGCG dose of 1200 mg/kg BW showed the lowest degree of cataract opacity compared with the treatment 1 and treatment 2 groups. There was a significant difference between the positive control group and the treatment groups. Moreover, there was also a significant correlation and effect of EGCG dose on the degree of cataract opacity in diabetic rat models. This illustrates that the higher the administration of EGCG, the milder the lens opacity that occurs[14-16].

According to our research hypothesis, EGCG is a compound primarily contained in green tea and has a potent antioxidant effect. Therefore, it plays a vital role in preventing oxidative stress and the aggregation of lens protein in cataracts. EGCG's activities as an antioxidant include combating free radicals, binding transition metals, and increasing the expression of antioxidant enzymes. Another study involved the human lens epithelial cells treated with H_2O_2 to trigger oxidative stress, which showed the dysfunction of mitochondria and the increase in mitochondrial cytochrome C release into the cytoplasm. However, EGCG administration can suppress the increase in reactive oxygen species and prevent the dysfunction of mitochondria and the release of mitochondrial cytochrome C. A previous study also suggested that H_2O_2 was able to induce the activation of caspase-9 and caspase-3 in the epithelial cells of the lens, and EGCG may play a role in the protection of lens epithelial cells from apoptosis by blocking the expression of caspase-9 and caspase-3[17-20].

The role of α-crystallin as a molecular chaperone of the lens acts to prevent the formation of aggregates, which are involved in the occurrence of cataracts. In the lens, α-crystallin is a polydisperse molecule that consists of unit A and unit B with a ratio of 3:1. Different from αA-crystallin, a study pointed out that αB-crystallin is included in the small heat shock protein group. Thus, it can be assumed that αB-crystallin will be more actively expressed in various systemic diseases, such as DM, where the human body system will produce stress[21-23]. In this study, there was a decrease in native lens αB-crystallin expression along with an increase in aggregate lens αB-crystallin expression in the positive control group, which was the diabetic rat model group that did not receive an EGCG treatment. These expressions showed statistically significant differences compared with those of the negative control group. The mechanism of lens cell protection against environmental and/or metabolic stresses was demonstrated with the increase in α-crystallin expression. The increase in α-crystallin, which functions as a chaperone, that is not in balance with the aggregation of lens proteins due to oxidative stress will result in the phosphorylation of α-crystallin, which triggers changes in the structure of the chaperone and, therefore, is unable to impede protein aggregation. In this study, such a condition was shown by the decrease in native lens αB-crystallin expression and the increase in aggregate lens αB-crystallin expression, which has an impact on the degree of lens opacity[23-26].

Another finding was also recorded in this study. As the EGCG dose administered increased, the native lens αB-crystallin expression increased but the aggregate lens αB-crystallin expression decreased. This condition implies that the administration of EGCG as an antioxidant is able to suppress oxidative stress that causes protein aggregation in the lens. Therefore, a higher expression of native lens αB-crystallin compared with the positive control group can be explained by the fact that there is a higher concentration of expression in its original form, *i.e.*, not oxidized. In addition, αB-crystallin also helps to provide protection through its chaperone activities that prevent protein aggregation, thereby hindering lens opacity. This study demonstrated a lower expression of aggregate αB-crystallin and a lower lens opacity compared with the positive control group. A similar result was also produced, where curcumin, which is considered to be a potent antioxidant, was able to reduce the expression of aggregate lens αA-crystallin and αB-crystallin after having been induced with selenium for 24h. Early in the formation of oxidative stress due to ROS, an increase in α-crystallin is triggered, which is a mechanism of adaptation of the lens cells to stress. However, when there is an imbalance between the increase in α-crystallin that acts as a chaperone and the protein aggregation in the lens that takes place due to oxidative stress, then the damage to the lens will continue to accumulate, which results in unavoidable lens opacity[27-30].

A limitation of this study is that we only conducted assessments at the end of this study (*i.e.*, not periodically each week). In other words, we were unable to monitor the onset or the progressivity of cataracts.

In conclusion, this study showed a significant effect of EGCG administration on preventing lens opacity and aggregation of αB-crystallin in diabetic rat models. As the EGCG dose that is given increases, the degree of lens opacity becomes milder, the expression of native lens αB-crystallin becomes higher, and the expression of aggregate lens αB-crystallin becomes lower.

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