



The role of reduced glutathione on oxidative stress, reticulum endoplasmic stress and glycation in human lens epithelial cell culture

[Papel del glutatión reducido sobre el estrés oxidativo, el estrés endoplásmico del retículo y la glicación en el cultivo de células epiteliales del cristalino humano]

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Abstract

Context: Cataracts are currently the main cause of blindness worldwide. This condition caused by a low concentration of reduced glutathione (GSH) in the lens.

Aims: To evaluate the effect of glutathione (GSH) on the hyperglycemia-induced oxidative stress, reticulum endoplasmic stress, and glycation in human lens epithelial cells.

Methods: Human lens epithelial cells were cultured under high-glucose conditions. Malondialdehyde (MDA), protein carbonyl content (PCC), glucose reactive protein (GRP), and advanced glycation end product (AGE) were measured by enzyme-linked immunosorbent assay after 72 h of incubation for MDA, PCC, GRP and after 2 weeks incubation for AGE.

Results: The MDA and PCC levels increased in response to high-dose glucose administration compared to the control group. MDA and PCC levels were decreased at all GSH doses, whereas the lowest mean MDA and PCC levels were observed at GSH doses of 10 and 100 μ M, respectively. GRP levels increased after high-glucose administration as compared to the control group. Additionally, the groups co-treated with 30 and 100 μ M GSH showed reduced PCC. The AGE level was reduced at all doses of GSH compared to those in the control group.

Conclusions: The results suggest that GSH inhibits oxidative stress, reticulum endoplasmic stress, and AGE formation, which may lead to the progression of diabetic cataract. Additionally, GSH may maintain lens transparency by acting as an antiglycation and controlling the AGE formation.

Keywords: glycation; glutathione; glucose reactive protein; hyperglycemia; malondialdehyde; oxidative stress.

Resumen

Contexto: Las cataratas son actualmente la principal causa de ceguera en todo el mundo. Esta condición es causada por una baja concentración de glutatión reducido (GSH) en el cristalino.

Objetivos: Evaluar el efecto del glutatión (GSH) sobre el estrés oxidativo inducido por la hiperglucemia, el estrés endoplásmico del retículo y la glicación en las células epiteliales del cristalino humano.

Métodos: Se cultivaron células epiteliales del cristalino humano en condiciones de alto contenido de glucosa. El malondialdehído (MDA), el contenido de carbonilo proteico (PCC), la proteína reactiva a la glucosa (GRP) y el producto final de glicación avanzada (AGE) se midieron mediante un ensayo inmunoabsorbente ligado a enzimas después de 72 h de incubación para MDA, PCC, GRP y después de 2 semanas incubación para AGE.

Resultados: Los niveles de MDA y PCC aumentaron en respuesta a la administración de glucosa en dosis altas en comparación con el grupo de control. Los niveles de MDA y PCC disminuyeron en todas las dosis de GSH, mientras que los niveles medios más bajos de MDA y PCC se observaron en dosis de GSH de 10 y 100 μ M, respectivamente. Los niveles de GRP aumentaron después de la administración de glucosa alta en comparación con el grupo control. Además, los grupos tratados conjuntamente con 30 y 100 μ M de GSH mostraron una reducción del PCC. El nivel de AGE se redujo en todas las dosis de GSH en comparación con las del grupo de control.

Conclusiones: Los resultados sugieren que GSH inhibe el estrés oxidativo, el estrés endoplásmico del retículo y la formación de AGE, lo que puede conducir a la progresión de la catarata diabética. Además, GSH puede mantener la transparencia de la lente actuando como antiglicación y controlando la formación de AGE.

Palabras Clave: estrés oxidativo; glicación; glutatión; hiperglucemia; malondialdehído; proteína reactiva a la glucosa.

ARTICLE INFO

Received: September 22, 2020.

Received in revised form: November 11, 2020.

Accepted: November 11, 2020.

Available Online: November 16, 2020.



INTRODUCTION

Cataracts are currently the main cause of blindness worldwide, which occurs in more than 17 million people in Southeast Asia (Resnikoff et al., 2004). Age and diabetes mellitus are the main risk factors for cataract. The pathogenic pathways contributing to cataractogenesis under diabetic conditions are the polyol pathway (also known as the aldose reductase or sorbitol pathway), non-enzymatic glycation pathway, oxidative stress pathways, unfolded protein response, and apoptotic pathways. The polyol pathway activates oxidative stress, and hyperglycemia increases the occurrence of protein glycosylation and free radical formation. Importantly, oxidative stress can trigger the unfolded protein response which can lead to decrease of lens antioxidants such as glutathione thereby increasing the formation of reactive oxygen species (ROS) (Lee and Chung, 1999).

Some studies have shown that glutathione (GSH) activity decreases with aging and high glucose levels, and thus this condition could increase oxidative stress in lens and is considered as a main factor in cataract formation (Berthoud and Beyer, 2009; Michael and Bron, 2011; Holm et al., 2013). GSH protect against oxidative stress, free radicals, and drug detoxification in the lens and protects thiol groups on intracellular crystallin and membranes proteins from post-translational modifications that cause crosslinking (Garadi et al., 1987; Reddy, 1990; Lou and Dickerson, 1992; Mathew et al., 2006; Holm et al., 2013). Zhang et al. (2008) showed that supplementation with GSH can protect the lens against oxidative stress by increasing NADPH synthesis, which is an important process in the regeneration of glutathione disulfide (GSSG) to GSH as a pro-oxidant.

Sweeney and Truscott (1998) found that age-related cataract is typically associated with the oxidation of protein in the center of the lens. This condition is caused by a low concentration of reduced GSH in the center of the lens. GSH does not diffuse in an older normal lens but diffuses in younger lens. Thus, the presence of GSH in the lens is vital for lens clarity by maintaining the

normal hydration level and cellular membrane integrity in the cornea. GSH and related enzymes such as glutathione reductase and glucose-6-phosphate dehydrogenase also play vital roles in maintaining the reduce form of GSH (Giblin, 2000; Ganea and Harding, 2006).

We hypothesized that GSH can delay the onset of diabetic cataract. Therefore, in the present study, we examined the protective effect of GSH against oxidative damage in terms of protein oxidation (protein carbonyl content measurement), endoplasmic reticulum (ER) stress (glucose reactive protein/GRP measurement), and antiglycating agent (AGE measurement) in high-glucose treated human lens epithelial cell culture. We found that GSH protected against oxidative stress in the ER and reduced the formation of AGE. The inhibitory effect was more pronounced against glycation and oxidative stress in the ER.

MATERIAL AND METHODS

Materials

Human lens epithelial (HLE-B3) cell line was purchased from ATCC (Manassas, VA) and stock cultures were maintained in EMEM medium (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum, penicillin (50 U/mL), and streptomycin (50 U/mL) at 37°C in the presence of 5% CO₂.

Cell culture and treatments

Cells (5×10^4) were seeded into 24-well plates and incubated overnight in EMEM medium containing 10% fetal bovine serum and treated with 25 mM glucose and co-treated with three different doses of GSH (10, 30, and 100 μ M). The cells were harvested after 72 h. Cells were also harvested after 2 weeks for AGE analysis.

Malondialdehyde (MDA), Protein Carbonyl Content (PCC) and Glucose Reactive Protein (GRP 78) analysis

After 72 h exposure to high glucose levels (25 mM) and co-treated with three different doses of

GSH (10, 30, and 100 μM /P1, P2, and P3, respectively), the cells were harvested, lysed, and centrifuged at 10,000 rpm for 2-3 min (Angirekula et al., 2018). Proteins were isolated from culture cells and protein concentrations were measured using a nanodrop spectrometer. Malondialdehyde (MDA) (TBars, NWLSS), protein carbonyl content (PCC), and glucose reactive protein (GRP) [enzyme-linked immunosorbent assay (ELISA)] were measured using commercial assay kits according to the manufacturer's instruction (Life Science Specialties, LCC, Vancouver, WA, USA). Absorbance was measured at 532 nm using a spectrophotometer.

Advanced glycation end product (AGEs) analysis

In a separated experiment, the cells were harvested, and the cell lysate was collected for ELISA AGE analysis (MyBioSource, San Diego, CA, USA) using commercial assay kits according to the manufacturer's instruction. Protein concentrations were measured with a nanodrop spectrometer and AGE was determined (ELISA) by measuring the absorbance at 532 nm using spectrophotometer (Hashim and Zarina, 2011).

Statistical analysis

One-way analysis of variance or Kruskal Wallis was used to evaluate the statistical significance of

differences between groups. Data normality was tested with SaphiroWilk and Levene's test to evaluate homogeneity of the data between groups, with $p < 0.05$ was considered as significant. All data were analyzed with SPSS 18.0 for Windows (SPSS, Inc., Chicago, IL, USA).

RESULTS

Based on Fig. 1, there was an increase in MDA levels after high glucose treatment. After GSH administration, there was a decrease of the MDA levels when compared with positive controls. The lowest average MDA levels shown in P1 groups ($0.013 \pm 0.006 \mu\text{g}/\text{mL}$) followed by P3 and P2 groups accordingly.

Based on Fig. 2, it can be seen that the highest PCC rate found in K^+ groups ($1.49 \pm 0.138 \text{ nmol}/\text{mL}$) and the lowest average PCC rate found in the group treated with high glucose and GSH 10 μM ($1.00 \pm 0.267 \text{ nmol}/\text{mL}$). This study proved that high glucose could promoted the increase of PCC level in the lens epithelial cells (K^+). In P1, P2, and P3 of GSH, the average of PCC level almost reached the average of control group and GSH 10 μM was the most effective to reduce PCC levels.

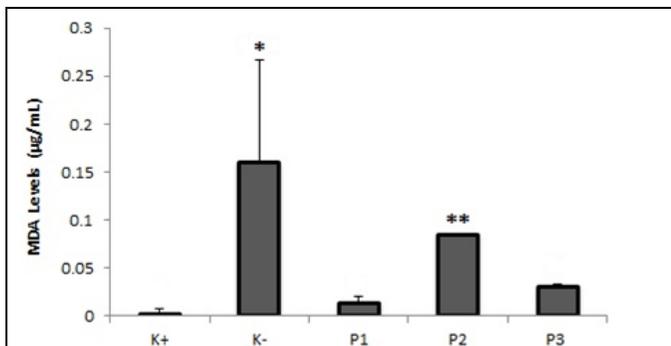


Figure 1. MDA levels in lens epithelial cell culture in each treatment group. Data were analyzed using MDA TBars-methods and tabulated into Microsoft excel.

Data are mean \pm SD values of each group with three replications ($p < 0.05$). (*) indicates a significant difference ($p < 0.05$) between the control group (K-) and K^+ . (**) indicate significant differences of $p < 0.05$ between K^+ and P2 group. Note: K-: control group; K^+ : glucose 25 mM; P1: glucose 25 mM + GSH 10 μM ; P2: glucose 25 mM + GSH 30 μM ; P3: glucose 25 mM + GSH 100 μM .

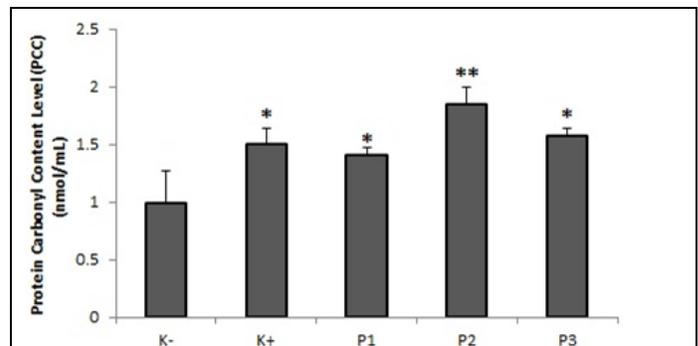


Figure 2. Administration of GSH decreasing the stress oxidative level in the protein carbonyl content (PCC) that produce by human lens epithelial cells in high glucose level.

Data were analyzed using Human ELISA PCC and tabulated into Microsoft excel. Data are mean \pm SD values of each group with three replications ($p < 0.05$). (*) indicates a significant difference ($p < 0.05$) between the control group (K-) and K^+ . (**) indicate significant differences of $p < 0.05$ between K^+ and P2 group. K-: control group; K^+ : glucose 25 mM; P1: glucose 25 mM + GSH 10 μM ; P2: glucose 25 mM + GSH 30 μM ; P3: glucose 25 mM + GSH 100 μM .

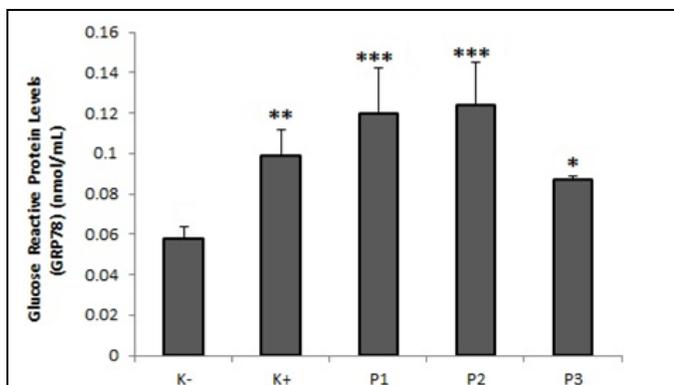


Figure 3. Administration of GSH decreasing the reticulum endoplasmic stress oxidative level (Glucose Reactive Protein/GRP78/BiP) that produced by human lens epithelial cells in high glucose level.

Data were analyzed using Human ELISA PCC and tabulated into Microsoft excel. Data are mean \pm SD values of each group with three replications ($p < 0.05$). (*) indicates a significant difference ($p < 0.05$) between the control group (K-) and P3 group. (**) indicate significant differences of $p < 0.05$ between K- and K+ group. (***) indicate significant differences of $p < 0.05$ between K+ and P1 and P2 group. K-: control group; K+: glucose 25 mM; P1: glucose 25 mM + GSH 10 μ M; P2: glucose 25 mM + GSH 30 μ M; P3: glucose 25 mM + GSH 100 μ M.

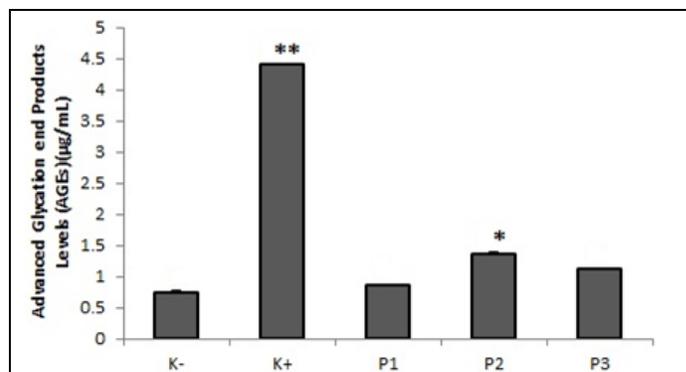


Figure 4. Administration of GSH decreasing the AGEs level that produced by human lens epithelial cells in high glucose level.

Data were analyzed using MDA TBars-methods and tabulated into Microsoft excel. Data are mean \pm SD values of each group with three replications ($p < 0.05$). (*) indicate significant differences of $p < 0.05$ between K- and P2 group. (**) indicate significant differences of $p < 0.05$ between K- and K+ group. K-: control group; K+: glucose 25 mM; P1: glucose 25 mM + GSH 10 μ M; P2: glucose 25 mM + GSH 30 μ M; P3: glucose 25 mM + GSH 100 μ M.

This next step was to evaluate the endoplasmic reticulum stress marker (GRP78/BiP) induced by high glucose. We observed that the average of GRP level in Fig. 3 increased after exposed with high glucose (K+) comparing with the control group (K-) (0.580 ± 0.28 vs. 0.990 ± 0.14 nmol/mL). After P2 and P3 of GSH administrations, the average of GRP level was decreased below the average of control group. This study proved that administration of GSH could decrease GRP78/BiP levels.

Based on Fig. 4, the highest AGE concentration rate found in the K+ groups (4.41 ± 0.003 μ g/mL) and the lowest AGEs average was found in the group treated with high glucose and GSH 10 μ M (0.64 ± 0.028 μ g/mL). This study proved that high glucose could promote the increase of AGEs level significantly in the lens epithelial cells (K+). Administration of 10, 30 and 100 μ M GSH were able to decrease the AGEs levels.

DISCUSSION

Chronic hyperglycemia may affect GSH levels in lens epithelial cells. Chronically increased glucose levels in diabetes mellitus play an important

role in the occurrence of cataracts, which may also be associated with decreased GSH levels in the lens. This study revealed elevated levels of MDA in high glucose conditions, which is indicated by an increase in MDA levels in the positive control that corresponds to the theory above that hyperglycemia can cause oxidative stress.

GSH functions as an antioxidant in the detoxification of products formed from lipid oxidation, triggered by ROS such as malonyldialdehyde and 4-hydroxy-2-nonenal as well as other ROS products that interact with cellular components. Thiyl radicals formed from this reaction can join with other molecules and form GSSG. GSH could also conjugate with many electrophilic components when electrophiles become reactive through the action of glutathione S-transferase (Strange et al., 2000; Aquilano et al., 2014).

Carbonylation involves the addition of aldehydes or ketones to specific amino acid residues through non-enzymatic reactions. This carbonylation is the most common oxidative modification of proteins. Carbonyl proteins may affect metabolic stability, and modified proteins play an important

role in the pathophysiology of disorders associated with oxidative stress. Carboxylation affects the proteins through two main mechanisms, namely oxidative (direct) and non-oxidative (indirect). The oxidative mechanism uses an ionic catalyst that directly reacts with hydrogen peroxide or lipid hydroperoxide with Lys, Arg, and Pro and produces 2-aminoadipic semialdehyde and γ -glutamyl semialdehyde (Bizzozero, 2009). Non-enzymatic carbonylation of proteins occurs through a reaction at a Cys, His or Lys residue. ROS are produced by lipid oxidation (HNE, MDA, acrolein) and carbohydrates (methylglyoxal, methylglyoxal) in the central area of nucleophiles (Zheng et al., 2018).

A decreased GSH/GSSG ratio is known to open the mitochondrial permeability transition pore by oxidation of dithiol in a protein complex (Zheng et al., 2018). If GSH decreased to normal levels, permeability transition will occur followed by mitochondrial membrane potential collapses. This causes an increase in superoxide production through the action of the redox cycle of ubiquinone in complex III (Chen et al., 2003) or through electron transport from succinate to NADH in complex I (Lambert and Brand, 2004). Loss of cytochrome C from mitochondria also increases the production of O_2 free radicals by decreasing the redox pathway of complex IV. Zheng et al. (2018) evaluated the mechanism of protein carbonylation in rat brains and found a correlation between GSH reduction and protein carbonylation. Addition of GSH in our study reduced PCC levels and maintained the GSH/GSSG ratio to inhibit the oxidation reaction of superoxide production and other free radicals.

GRP78/Bip is an ER stress marker. ER stress can be caused by several mechanisms, including errors in continuously occurring protein folding processes. GSH significantly reduced GRP78/BiP levels at a dose of 30 μ M GSH. Thus, GSH can improve oxidative stress both on the cell membrane and in the ER. GSH in the ER may reduce non-native disulfide bonds formed in proteins by oxidative stress processes and enzymes that catalyze oxidation (Jansens et al., 2002).

Two studies have shown that GSH plays an important role in the isomerization of non-native disulfide bonds. Both studies revealed an increase in non-native disulfide bond formation when total GSH levels decrease were decreased (Winter et al., 2002; Chakravarthi and Bulleid, 2004). Increased oxidation is also accompanied by an increased formation of non-native disulfide bonds. A decrease or loss of cytosolic GSH levels causes protein disulphide isomerase (PDI) to become more oxidized and interferes with the isomerization of non-native disulfide bonds, which require a long time for isomerization. This reinforces the hypothesis that GSH is involved in disulfide bond isomerization both directly and indirectly.

GSH is also the main reducing agent responsible for maintaining oxidoreductase enzyme in the ER to remain in a reduced form. This enzyme is required to reduce or isomerize the non-native disulfide bonds through 1) glutathionylation of cysteine residues and 2) GSH molecules which attack glutathionylated proteins to release GSSG and reduce protein substrates. The CXXC motif in PDI found in the oxidoreductase ER also functions to maintain efficient reduction or isomerization processes. Polypeptide binding domains in PDI also increase the isomerization of disulphide bonds (Winter et al., 2002). Administration of GSH in our study reduced GRP 78 levels; based on previous studies, addition of GSH maintains protein folding in ER to prevent oxidative stress.

AGEs accumulate in the intracellular and extracellular environment of the eye structure and are associated with diabetic cataracts (Emre et al., 2008). Prolonged hyperglycemia can cause the formation of AGEs, which are irreversible glycation products. This study demonstrated that AGE levels are increased in epithelial cells exposed to high glucose compared to negative controls. GSH addition significantly reduces the level of AGE. In this case, GSH may be involved in the Maillard reaction as an anti-glycation agent. GSH also functions as a cofactor in methylglyoxal elimination (AGEs) by using two glyoxalase, glyoxalase I and glyoxalase II (Lushchak, 2012).

CONCLUSIONS

GSH can protect the lens from cataractogenesis that may occur due to increased generation of oxidative stress, reticulum endoplasmic stress and AGE formation in the human lens epithelial cells under hyperglycemic conditions.

CONFLICT OF INTEREST

The authors declare no conflicts of interests.

ACKNOWLEDGMENTS

The researcher delivers the gratitude for the grant support provided by Indonesia Ministry of Technology, Research and Education. This work was supported by Indonesia Ministry of Technology, Research and Education (Grant No. 338.177/UN10.C10/PN/2018).

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AUTHOR CONTRIBUTION:

Contribution	Handayani N	Permatasari N	Sujuti H	Rudijanto A
Concepts or ideas	x			
Design	x			
Definition of intellectual content	x			
Literature search		x		
Experimental studies		x		
Data acquisition			x	
Data analysis			x	
Statistical analysis			x	
Manuscript preparation	x			
Manuscript editing				x
Manuscript review	x	x	x	x

Citation Format: Handayani N, Permatasari N, Sujuti H, Rudijanto A (2021) The role of reduced glutathione on oxidative stress, reticulum endoplasmic stress and glycation in human lens epithelial cell culture. J Pharm Pharmacogn Res 9(2): 175-181.