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L-Theanine Protects against Methylglyoxal-Induced Oxidative Stress and Tight Junction Disruption in Human Cerebral Microvascular Endothelial Cells

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Abstract

The brain's blood microvessels restrict the exchange of substances between the blood and brain tissue through the blood-brain barrier (BBB). Methyl-glyoxal (MG), a byproduct of glucose metabolism, contributes to the formation of advanced glycation end products (AGEs) and disrupts the BBB, which is associated with neurodegenerative diseases. L-Theanine (TA), an amino acid found in green tea with antioxidant properties, may protect the BBB. This study aimed to determine whether MG increases reactive oxygen species (ROS) and permeability by reducing tight junction proteins in human cerebral microvascular endothelial cells (hCMEC/d3), and whether TA pretreatment can counteract these effects. Our findings demonstrated that MG treatment led to increased BBB permeability, decreased transendothelial electrical resistance (TEER) values to 39% of control levels, reduced expression of Claudin-5 to 53% and Occludin to 69% of control levels, and elevated intracellular ROS levels. TA pretreatment restored barrier integrity, preserved tight junction protein expression, and decreased ROS accumulation to levels comparable to control levels. These findings suggest that TA effectively prevents MG-induced BBB dysfunction by reducing oxidative stress and maintaining tight junction proteins, showing promise as a protective agent for the BBB in conditions associated with elevated MG and AGEs.

Keywords

L-Theanine, Methylglyoxal, Oxidative Stress, Tight Junction, Blood-Brain Barrier

1. Introduction

The brain's blood microvessels safeguard neurons by restricting the exchange of substances between the blood and brain tissue through the blood-brain barrier (BBB). The BBB effectively blocks molecules larger than 500 Da from crossing and selectively regulates the entry of smaller molecules [1]. Disruption of the BBB is associated with neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease [2]. Structurally, cerebral blood vessels comprise three layers: the intima (endothelial cells), media (smooth muscle), and adventitia (connective tissue). Endothelial cells are interconnected by tight junction proteins (TJs, including Claudin-5, Occludin, and ZO-1) and adherens junction proteins (AJs, such as VE-cadherin), which collectively limit paracellular transport into the brain tissue [3].

In hyperglycemic conditions, advanced glycation end products (AGEs) form through non-enzymatic reactions (glycation) between sugar aldehyde groups and protein amino groups, generating dicarbonyl compounds such as methylglyoxal (MG), glyoxal, and 3-deoxyglucosone in the process [4]. MG, in particular, is produced abundantly in vascular endothelial cells [5]. Previous studies have shown that MG and AGEs induce oxidative stress, inflammation, apoptosis, and activate receptors for AGE (RAGE) signaling pathways, all contributing to cellular dysfunction [6] [7].

L-Theanine (γ-glutamylethylamide, TA), an amino acid found in green tea at approximately 8 mg per cup, is metabolized into glutamic acid in the body, promoting the synthesis of glutathione (GSH) [8] [9]. TA also enhances the activity of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT) [10] [11]. Additionally, TA has demonstrated neuroprotective effects by promoting the synthesis of acetylcholine and brain-derived neurotrophic factor (BDNF) [12]. Clinical studies have shown that regular green tea consumption is associated with reduced cognitive impairment and improved working memory [13]-[15]. However, the effects of MG on cerebral vascular endothelial cells and the potential BBB-protective effects of TA are not fully understood.

This study aimed to investigate whether MG increases intracellular reactive oxygen species (ROS) and enhances BBB permeability by reducing the expression of tight junction proteins in a human cerebral microvascular endothelial cell line (hCMEC/d3) BBB model. Additionally, we examined whether TA pretreatment could mitigate these MG-induced effects, evaluating TA's potential to prevent MG-induced endothelial cell dysfunction.

2. Materials and Methods

2.1. Materials

Antibodies used in this study including anti-Claudin-5 (Cat# 34-1600; RRID: AB_2533157) and anti-occludin (Cat# 71-1500; RRID: AB_2533977), Goat anti-Rabbit IgG (H + L), Alexa Fluor 488 (Cat# A-11008; RRID: AB_143165), were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The human

cerebral microvascular endothelial cell line hCMEC/d3 (Cat# SCC066) was acquired from Merck Millipore (Darmstadt, Germany). Chemical compounds and drugs used in the experiments were H2DCFDA (Cat# D399) from Thermo Fisher Scientific, Hoechst 33,342 (Cat# H342) from Dojindo Laboratories (Kumamoto, Japan), L-Theanine (Cat# T0954) from Tokyo Kasei Kogyo Co. (Tokyo, Japan), N-Acetyl-L-cysteine (Cat# 017-05131) and L-Glucamine (Cat# 070-00502) from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), and Methylglyoxal (Cat# 155558) from MP Biomedicals (Illkirch, France).

2.1. Cell Culture

Human brain endothelial cells (hCMEC/d3), derived from microvessels in the human temporal lobe, were cultured using the EGM-2MV kit (Lonza, Bend, OR, USA). This kit includes Endothelial Basal Medium (EBM-2) supplemented with gentamicin/amphotericin-B (GA), human epidermal growth factor (hEGF), ascorbic acid, vascular endothelial growth factor (VEGF), R3-insulin-like growth factor-1 (R3-IGF-1), hydrocortisone, human fibroblast growth factor-beta (hFGF-β), and 5% (v/v) fetal bovine serum. Cells were maintained at 37˚C in a humidified incubator with 5% CO_2.

2.2. Permeability-Study

hCMEC/d3 cells (1.0×10^5 cells/well) were seeded onto Transwell inserts (Cat#3460, Corning Life Sciences, MA, USA) and cultured for 72 h. Following this, the cells were treated with MG, TA, NAC, and GLU for 4 h. Transepithelial electrical resistance (TEER) values were then measured using a Millicell ERS-2 meter (Millipore, Bedford, MA). The TEER value of an empty filter was subtracted from each measurement, and the resulting TEER values (in Ohms) were multiplied by the filter surface area (1.12 cm^2) as follows: TEER ($\Omega \times cm^2$) = (cell monolayer Ω − empty Transwell filter Ω) × surface area (cm^2).

After measuring TEER, the culture medium was replaced with Hanks' balanced salt solution (HBSS, pH 7.4). Fluorescein-Na was added to the apical side at a final concentration of 1 μg/mL, and samples were collected from the basal side at 15, 30, and 60 min. Fluorescence was measured using an EnSpire 2300 fluorescence microplate reader (PerkinElmer, Turku, Finland) with 475 nm excitation and 512 nm emission. The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \left(1/A \times C_0\right) dC/dt$$

Where dC/dt represents the rate of change in fluorescein concentration on the basal side over time, C_0 is the initial fluorescein concentration in the apical compartment at time zero, and A is the surface area of the filters (1.12 cm^2).

2.3. Immunofluorescence Staining

Cells were seeded on 8-well chamber slides (Cat#SCS-N08, Matsunami Glass Industry, Osaka, Japan) and cultured for 72 h. The cells were then treated with MG,

TA, NAC, and GLU for 4 h. Following treatment, cells were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 15 min at room temperature. Permeabilization and blocking were then conducted by incubating the cells in 0.1% Triton X-100 and 2% bovine serum albumin (BSA) in PBS for 30 min at room temperature. After blocking, cells were incubated overnight at 4˚C with the primary antibody. The following day, cells were washed with PBS-T and incubated with a fluorophore-conjugated secondary antibody for 1 h at room temperature in the dark. Coverslips were then mounted onto glass slides using ProLong Gold Antifade Mountant with DAPI (Cat#P36935, Thermo Fisher Scientific) and stored in the dark at 4˚C. Fluorescence images were captured using an LSM700 confocal microscope (Carl Zeiss, Jena, Germany).

2.4. Measurement of DCF Fluorescence

The fluorogenic dye H_2DCFDA was used to measure ROS production. Once inside the cells, H_2DCFDA is deacetylated by cellular esterases into a non-fluorescent form, which is then oxidized by ROS to form the fluorescent compound 2', 7'-dichlorofluorescein (DCF) [16]. Cells were seeded in a glass-bottom dish (Cat#627870, Greiner Bio-One, Kremsmünster, Austria) and cultured for 48 h. H_2DCFDA was added to the culture medium at a final concentration of 200 μM, followed by incubation for an additional 24 h. The cell nuclei were counterstained with Hoechst 33,342. Afterward, the cells were treated with the specified reagents. Fluorescence confocal microscopy was used to capture images, and fluorescence intensity was quantified using ImageJ software (version 1.53 t).

2.5. Statistical Analysis

All results are presented as mean ± standard error (S.E.). Data were analyzed using EZR (R version 4.2.2, EZR version 1.61) [17]. One-way ANOVA followed by Tukey's post-hoc test was used for multiple group comparisons. A P-value of < 0.05 was considered statistically significant.

3. Results

3.1. Effects of MG and TA on Permeability

To examine the effects of methylglyoxal (MG) and L-Theanine (TA) on the permeability of the BBB model formed by hCMEC/d3 cells, we assessed cell permeability after MG and TA treatments. Cells were seeded in the upper chamber of Transwell inserts, pretreated with TA for 24 h, and then exposed to MG for 4 h. Fluorescein-Na was added to the upper chamber, and permeability was measured over time by recording fluorescence intensity to calculate P_{app}. Results showed that MG increased P_{app} values in a dose-dependent manner (**Figure 1(A)**). However, this MG-induced increase in P_{app} was prevented by TA pretreatment.

We further evaluated transendothelial electrical resistance (TEER), a measure of intercellular tightness. MG treatment led to a dose-dependent reduction in TEER **Figure 1(B)**). In contrast, cells pretreated with TA before MG exposure

maintained TEER levels comparable to untreated controls, indicating preserved barrier integrity. However, when TA was administered after MG treatment, it failed to prevent the MG-induced increase in P_{app} and decrease in TEER (**Figure 1(C, D)**), suggesting that TA may act protectively when applied prior to MG exposure.

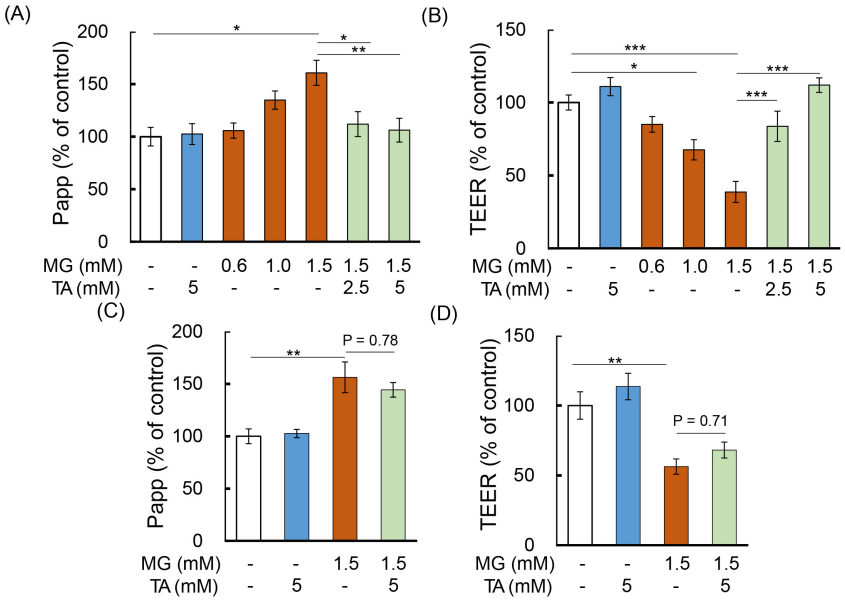


Figure 1. Effect of MG and TA on BBB permeability in hCMEC/d3 cells. (A, B) hCMEC/d3 cells were cultured for 48 h, pretreated with TA (2.5 - 5 mM) for 24 h, and then exposed to MG (0.6 - 1.5 mM) for 4 h (n = 6). TEER was measured, followed by replacing the culture medium with HBSS. Fluorescein-Na (10 μg/mL) was added to the upper chamber and incubated for 1 h. HBSS was collected from the lower compartment at 15, 30, and 60 min after fluorescein-Na addition. The apparent permeability coefficient (P_{app}) was calculated and displayed in the bar graph. (C, D) hCMEC/d3 cells were cultured for 72 h, treated with MG for 4 h, and subsequently treated with TA for 24 h (n = 5). TEER measurement and other procedures were performed as described in (A, B). Data are presented as mean ± S.E.; *P < 0.05, **P < 0.01, ***P < 0.001.

3.2. Effects of MG and TA on Tight Junction Proteins

To assess the effects of MG and TA on tight junction proteins, we analyzed the expression of Claudin-5 and Occludin in hCMEC/d3 cells treated with MG and TA using immunofluorescence staining. After 4 h of MG exposure, Claudin-5 protein levels were significantly reduced (**Figure 2(A, B)**). Pretreatment with TA mitigated the MG-induced decrease in Claudin-5 expression. It is possible that TA is converted into L-glutamic acid (GLU) once taken up by the cells [9]. Thus, we also examined Claudin-5 expression in cells pretreated with GLU before MG exposure. Although GLU treatment slightly alleviated the MG-induced reduction in Claudin-5, it was less effective than TA (**Figure 2(A, B)**).

Similarly, we observed a decrease in Occludin expression following MG treatment. This MG-induced reduction in Occludin expression was also suppressed by TA pretreatment (**Figure 2(C, D)**).

3.3. Effects of MG and TA on ROS Levels

Given that MG and AGEs are known to induce ROS production, we examined ROS levels in hCMEC/d3 cells treated with MG and TA. Menadione (MEN) served as a positive control for ROS generation [18], while N-acetyl-L-cysteine (NAC) was used as a positive control antioxidant [19]. ROS levels were monitored over time using confocal microscopy to observe the fluorescence intensity of DCF, which fluoresces in the presence of ROS. We observed a time-dependent increase in DCF fluorescence intensity following MG addition, reaching levels comparable to MEN within 4 h (**Figure 3(A, B)**). Pretreatment with TA effectively reduced ROS levels to values similar to those achieved by NAC (**Figure 3(C)**). These findings indicate that TA's ability to suppress ROS generation may be beneficial in protecting the BBB.

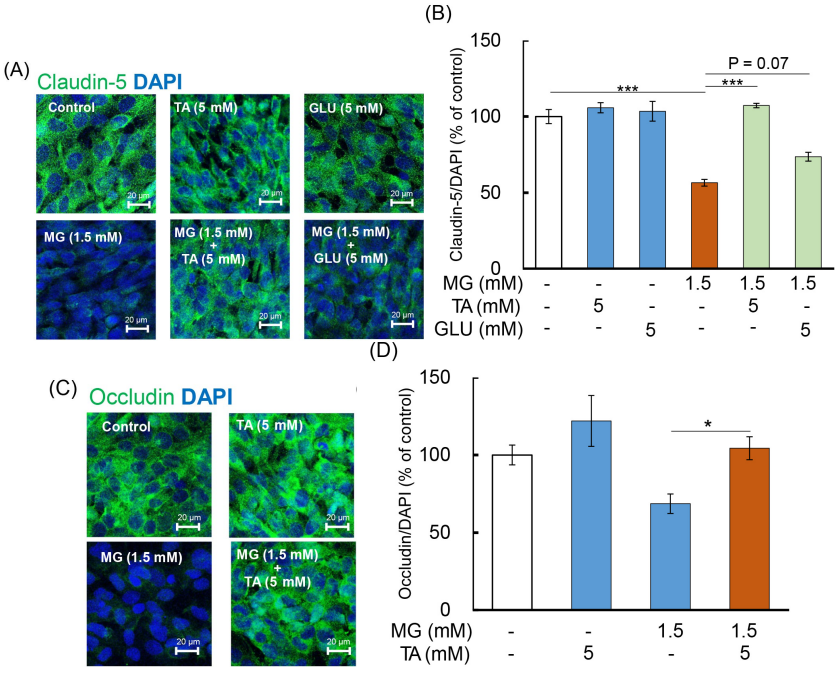
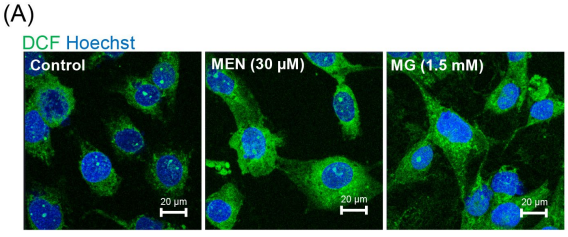


Figure 2. MG and TA on tight junction protein levels in hCMEC/d3 cells. hCMEC/d3 cells were seeded in an 8-well chamber slide, cultured for 48 h, pretreated with TA or GLU (5 mM) for 24 h, and then treated with MG (1.5 mM) for 4 h. Cells were fixed with 4% paraformaldehyde and immunostained with an-ti-Claudin-5 (A) or anti-Occludin (C) antibodies. Nuclei were counterstained with DAPI, and cells were imaged using fluorescence confocal microscopy. Fluorescence intensity was quantified and shown in bar graphs (B) and (D) for Claudin-5 and Occludin, respectively. Data are presented as mean ± S.E.; *P < 0.05, ***P < 0.001, n = 3.



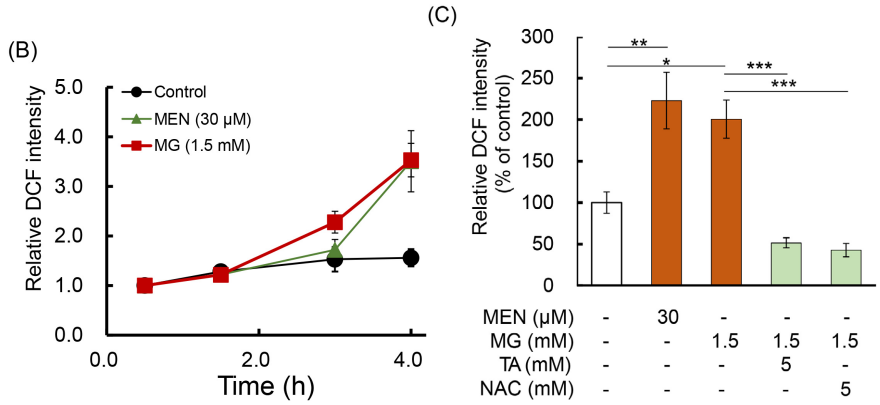


Figure 3. Effect of MG and TA on ROS levels in hCMEC/d3 cells.hCMEC/d3 cells were seeded in a glass-bottom dish and incubated for 48 h. H_2DCFDA (200 μM), TA (5 mM), and NAC (5 mM) were added and incubated for 24 h, after which MG (1.5 mM) or MEN (30 μM) was added and incubated for 0.5, 1.5, 3.0, and 4.0 h. Nuclei were counterstained with Hoechst 33,342, and cells were imaged using fluorescence confocal microscopy. (A) Representative images were captured after 4 h of treatment. (B) Time-dependent effects of MG and MEN on DCF fluorescence intensity. (C) Fluorescence intensity of cells after 4 h of treatment. Data are presented as mean ± S.E.; *P < 0.05, **P < 0.01, ***P < 0.001. n = 3 – 7.

4. Discussion

This study demonstrated that MG induces ROS production in the brain microvascular endothelial cell line hCMEC/d3, decreases the expression of tight junction proteins Claudin-5 and Occludin, and consequently increases permeability (**Figure 4**). Furthermore, we found that TA pretreatment mitigates these MG-induced effects, preserving BBB function.

MG treatment of hCMEC/d3 cells led to reduced expression of Claudin-5 and Occludin, compromising BBB structural integrity (**Figure 2**). MG is known to induce oxidative stress and apoptosis, impairing cellular function [20]. It also glycates DNA, leading to genetic mutations [21] [22]. Additionally, MG-induced AGE formation activates RAGE-mediated signaling, increasing vascular permeability through mDia1 [23]. These findings suggest that MG disrupts the BBB, a critical barrier protecting the central nervous system.

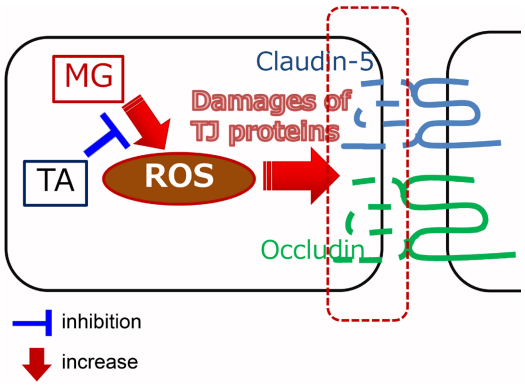


Figure 4. Protective effects of L-Theanine against methylglyoxal-induced oxidative stress and tight junction disruption in hCMEC/d3 cells.

Our results also showed that MG increased ROS production in hCMEC/d3 cells (**Figure 3**), suggesting ROS as a key mediator of MG-induced endothelial damage. Brain endothelial cells express drug efflux transporters such as P-glycoprotein (P-gp) and contain 4 - 5 times more mitochondria than peripheral vascular endothelial cells to meet high ATP demands, which makes them more vulnerable to ROS [24]. MG and AGE stimulation of RAGE has been shown to activate NADPH oxidase, raising cytoplasmic ROS levels [25], and contributing to mitochondrial superoxide production [26]. Liu et al. reported that MG-induced mitochondrial ROS contributes to cytotoxicity in rat pancreatic beta cells [27]. Excessive ROS can cause mitochondrial damage and lead to the degradation of tight junction proteins via autophagy or mitophagy [28]. Therefore, reducing ROS generation in brain endothelial cells is essential for BBB protection.

This study showed that TA exhibits antioxidant properties in brain endothelial cells, preventing the MG-induced reduction in tight junction protein levels and increased permeability. However, adding TA after MG treatment did not restore BBB integrity (**Figure 1(C, D)**), suggesting that MG-induced damage may be irreversible or that TA acts preventively. TA has previously been shown to protect against amyloid-beta (Aβ)-induced neurotoxicity by inhibiting oxidative stress and the activation of ERK/p38 MAPK and NF-κB pathways [29]. In a D-galactose-induced brain injury model, TA administration significantly reduced AGEs and Aβ1-42 concentrations in brain tissue [12]. Intracellularly, MG is conjugated with glutathione and degraded by glyoxalase enzymes [30]. In diabetes, glutathione levels [31] and glyoxalase activity [32] decrease, suggesting that TA, as a glutathione precursor, may elevate intracellular glutathione, exert antioxidant effects, and reduce MG-induced AGE formation.

While TA is converted into GLU intracellularly, adding GLU alone had minimal effect on restoring MG-induced Claudin-5 reduction (**Figure 2(A, B)**). GLU is an excitatory neurotransmitter, and high extracellular GLU levels may impair barrier function by activating N-methyl-D-aspartate (NMDA) receptors in endothelial cells [33]. Since TA is transported into the brain via LAT1 and LAT2 amino acid transporters [34] [35], it holds promise as a compound with BBB-protective potential. Additionally, a recent report introduced an efficient biosynthesis method for L-theanine, potentially enabling its large-scale production and therapeutic application [36].

In summary, this study in a BBB model using hCMEC/d3 cells revealed that MG enhances permeability by inducing ROS production and reducing tight junction protein levels and that TA can prevent MG-induced BBB impairment. Although cells in this study were acutely treated with high MG concentrations, BBB damage in vivo likely accumulates due to chronically elevated MG and AGE levels. Thus, exploring the effects of long-term MG exposure would be meaningful. Furthermore, hCMEC/d3 cells form stronger tight junctions under shear stress conditions mimicking blood flow [37]. Given that diabetes reduces blood flow due to increased blood viscosity [38], employing a BBB model that simulates blood flow may provide insights closer to physiological conditions.

5. Conclusion

TA shows promise as a preventative agent against MG-induced BBB dysfunction, likely through its antioxidant effects and preservation of tight junction proteins. This study enhances our understanding of the mechanisms behind BBB impairment in conditions involving MG and AGEs, such as diabetes and Alzheimer's disease, and supports the development of new preventive and therapeutic strategies.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

[1] Kaya, M. and Ahishali, B. (2020) Basic Physiology of the Blood-Brain Barrier in Health and Disease: A Brief Overview. *Tissue Barriers*, **9**, Article ID: 1840913. https://doi.org/10.1080/21688370.2020.1840913

[2] Sweeney, M.D., Sagare, A.P. and Zlokovic, B.V. (2018) Blood-Brain Barrier Breakdown in Alzheimer Disease and Other Neurodegenerative Disorders. *Nature Reviews Neurology*, **14**, 133-150. https://doi.org/10.1038/nrneurol.2017.188

[3] Tajes, M., Ramos-Fernández, E., Weng-Jiang, X., Bosch-Morató, M., Guivernau, B., Eraso-Pichot, A., *et al.* (2014) The Blood-Brain Barrier: Structure, Function and Therapeutic Approaches to Cross It. *Molecular Membrane Biology*, **31**, 152-167. https://doi.org/10.3109/09687688.2014.937468

[4] Lai, S.W.T., Lopez Gonzalez, E.D.J., Zoukari, T., Ki, P. and Shuck, S.C. (2022) Methylglyoxal and Its Adducts: Induction, Repair, and Association with Disease. *Chemical Research in Toxicology*, **35**, 1720-1746. https://doi.org/10.1021/acs.chemrestox.2c00160

[5] Schalkwijk, C.G., van Bezu, J., van der Schors, R.C., Uchida, K., Stehouwer, C.D.A. and van Hinsbergh, V.W.M. (2006) Heat-Shock Protein 27 Is a Major Methylglyoxal-Modified Protein in Endothelial Cells. *FEBS Letters*, **580**, 1565-1570. https://doi.org/10.1016/j.febslet.2006.01.086

[6] Ott, C., Jacobs, K., Haucke, E., Navarrete Santos, A., Grune, T. and Simm, A. (2014) Role of Advanced Glycation End Products in Cellular Signaling. *Redox Biology*, **2**, 411-429. https://doi.org/10.1016/j.redox.2013.12.016

[7] Kalapos, M.P. (2008) The Tandem of Free Radicals and Methylglyoxal. *Chemico-Biological Interactions*, **171**, 251-271. https://doi.org/10.1016/j.cbi.2007.11.009

[8] Keenan, E.K., Finnie, M.D.A., Jones, P.S., Rogers, P.J. and Priestley, C.M. (2011) How Much Theanine in a Cup of Tea? Effects of Tea Type and Method of Preparation. *Food Chemistry*, **125**, 588-594. https://doi.org/10.1016/j.foodchem.2010.08.071

[9] Sugiyama, T. and Sadzuka, Y. (2004) Theanine, a Specific Glutamate Derivative in Green Tea, Reduces the Adverse Reactions of Doxorubicin by Changing the Glutathione Level. *Cancer Letters*, **212**, 177-184.

https://doi.org/10.1016/j.canlet.2004.03.040

[10] Deng, Y., Xiao, W., Chen, L., Liu, Q., Liu, Z. and Gong, Z. (2016) In Vivo Antioxidative Effects of L-Theanine in the Presence or Absence of Escherichia Coli-Induced Oxidative Stress. *Journal of Functional Foods*, **24**, 527-536. https://doi.org/10.1016/j.jff.2016.04.029

[11] Li, Z., Chen, L., Huang, Z., Jia, G., Zhao, H., Liu, G., *et al.* (2024) Supplementation with L-Theanine Promotes Intestinal Antioxidant Ability via Nrf2 Signaling Pathway in Weaning Piglets and H_2O_2-Induced IPEC-J2 Cells. *Journal of Functional Foods*, **121**, Article ID: 106433. https://doi.org/10.1016/j.jff.2024.106433

[12] Zeng, L., Lin, L., Chen, L., Xiao, W. and Gong, Z. (2021) L-theanine Ameliorates D-galactose-Induced Brain Damage in Rats via Inhibiting AGE Formation and Regulating Sirtuin1 and BDNF Signaling Pathways. *Oxidative Medicine and Cellular Longevity*, **2021**, Article ID: 8850112. https://doi.org/10.1155/2021/8850112

[13] Noguchi-Shinohara, M., Yuki, S., Dohmoto, C., Ikeda, Y., Samuraki, M., Iwasa, K., *et al.* (2014) Consumption of Green Tea, but Not Black Tea or Coffee, Is Associated with Reduced Risk of Cognitive Decline. *PLOS ONE*, **9**, e96013. https://doi.org/10.1371/journal.pone.0096013

[14] Kuriyama, S., Hozawa, A., Ohmori, K., Shimazu, T., Matsui, T., Ebihara, S., *et al.* (2006) Green Tea Consumption and Cognitive Function: A Cross-Sectional Study from the Tsurugaya Project. *The American Journal of Clinical Nutrition*, **83**, 355-361. https://doi.org/10.1093/ajcn/83.2.355

[15] Baba, Y., Inagaki, S., Nakagawa, S., Kaneko, T., Kobayashi, M. and Takihara, T. (2021) Effects of L-Theanine on Cognitive Function in Middle-Aged and Older Subjects: A Randomized Placebo-Controlled Study. *Journal of Medicinal Food*, **24**, 333-341. https://doi.org/10.1089/jmf.2020.4803

[16] Gomes, A., Fernandes, E. and Lima, J.L.F.C. (2005) Fluorescence Probes Used for Detection of Reactive Oxygen Species. *Journal of Biochemical and Biophysical Methods*, **65**, 45-80. https://doi.org/10.1016/j.jbbm.2005.10.003

[17] Kanda, Y. (2012) Investigation of the Freely Available Easy-To-Use Software 'EZR' for Medical Statistics. *Bone Marrow Transplantation*, **48**, 452-458. https://doi.org/10.1038/bmt.2012.244

[18] Chiou, T., Zhang, J., Ferrans, V.J. and Tzeng, W. (1997) Cardiac and Renal Toxicity of Menadione in Rat. *Toxicology*, **124**, 193-202. https://doi.org/10.1016/s0300-483x(97)00162-5

[19] Tenório, M.C.D.S., Graciliano, N.G., Moura, F.A., Oliveira, A.C.M.D. and Goulart, M.O.F. (2021) N-Acetylcysteine (NAC): Impacts on Human Health. *Antioxidants*, **10**, Article 967. https://doi.org/10.3390/antiox10060967

[20] Fukunaga, M., Miyata, S., Liu, B.F., Miyazaki, H., Hirota, Y., Higo, S., *et al.* (2004) Methylglyoxal Induces Apoptosis through Activation of P38 MAPK in Rat Schwann Cells. *Biochemical and Biophysical Research Communications*, **320**, 689-695. https://doi.org/10.1016/j.bbrc.2004.06.011

[21] Hou, S., Nori, P., Fang, J. and Vaca, C.E. (1995) Methylglyoxal Induces *HPRT* Mutation and DNA Adducts in Human T-Lymphocytes *in Vitro*. *Environmental and Molecular Mutagenesis*, **26**, 286-291. https://doi.org/10.1002/em.2850260404

[22] Pischetsrieder, M., Seidel, W., Münch, G. and Schinzel, R. (1999) N2-(1-Carboxyethyl)Deoxyguanosine, a Nonenzymatic Glycation Adduct of DNA, Induces Single-Strand Breaks and Increases Mutation Frequencies. *Biochemical and Biophysical Research Communications*, **264**, 544-549. https://doi.org/10.1006/bbrc.1999.1528

[23] Zhou, X., Weng, J., Xu, J., Xu, Q., Wang, W., Zhang, W., *et al.* (2018) Mdia1 Is Crucial

for Advanced Glycation End Product-Induced Endothelial Hyperpermeability. *Cellular Physiology and Biochemistry*, **45**, 1717-1730. https://doi.org/10.1159/000487780

[24] Oldendorf, W.H., Cornford, M.E. and Brown, W.J. (1977) The Large Apparent Work Capability of the Blood-Brain Barrier: A Study of the Mitochondrial Content of Capillary Endothelial Cells in Brain and Other Tissues of the Rat. *Annals of Neurology*, **1**, 409-417. https://doi.org/10.1002/ana.410010502

[25] Wautier, M., Chappey, O., Corda, S., Stern, D.M., Schmidt, A.M. and Wautier, J. (2001) Activation of NADPH Oxidase by AGE Links Oxidant Stress to Altered Gene Expression via Rage. *American Journal of Physiology-Endocrinology and Metabolism*, **280**, E685-E694. https://doi.org/10.1152/ajpendo.2001.280.5.e685

[26] Coughlan, M.T., Thorburn, D.R., Penfold, S.A., Laskowski, A., Harcourt, B.E., Sourris, K.C., *et al.* (2009) Rage-induced Cytosolic ROS Promote Mitochondrial Superoxide Generation in Diabetes. *Journal of the American Society of Nephrology*, **20**, 742-752. https://doi.org/10.1681/asn.2008050514

[27] Liu, C., Cao, B., Zhang, Q., Zhang, Y., Chen, X., Kong, X., *et al.* (2020) Inhibition of Thioredoxin 2 by Intracellular Methylglyoxal Accumulation Leads to Mitochondrial Dysfunction and Apoptosis in INS-1 Cells. *Endocrine*, **68**, 103-115. https://doi.org/10.1007/s12020-020-02191-x

[28] Kim, D., Kim, K., Kim, J., Kim, E. and Bae, O. (2020) Methylglyoxal-Induced Dysfunction in Brain Endothelial Cells via the Suppression of Akt/HIF-1α Pathway and Activation of Mitophagy Associated with Increased Reactive Oxygen Species. *Antioxidants*, **9**, Article 820. https://doi.org/10.3390/antiox9090820

[29] Kim, T.I., Lee, Y.K., Park, S.G., Choi, I.S., Ban, J.O., Park, H.K., *et al.* (2009) L-theanine, an Amino Acid in Green Tea, Attenuates β-Amyloid-Induced Cognitive Dysfunction and Neurotoxicity: Reduction in Oxidative Damage and Inactivation of ERK/p38 Kinase and NF-κB Pathways. *Free Radical Biology and Medicine*, **47**, 1601-1610. https://doi.org/10.1016/j.freeradbiomed.2009.09.008

[30] Aragonès, G., Rowan, S., Francisco, S.G., Whitcomb, E.A., Yang, W., Perini-Villanueva, G., *et al.* (2021) The Glyoxalase System in Age-Related Diseases: Nutritional Intervention as Anti-Ageing Strategy. *Cells*, **10**, Article 1852. https://doi.org/10.3390/cells10081852

[31] Whiting, P.H., Kalansooriya, A., Holbrook, I., Haddad, F. and Jennings, P.E. (2008) The Relationship between Chronic Glycaemic Control and Oxidative Stress in Type 2 Diabetes Mellitus. *British Journal of Biomedical Science*, **65**, 71-74. https://doi.org/10.1080/09674845.2008.11732800

[32] Morgenstern, J., Katz, S., Krebs-Haupenthal, J., Chen, J., Saadatmand, A., Cortizo, F.G., *et al.* (2020) Phosphorylation of T107 by Camkiiδ Regulates the Detoxification Efficiency and Proteomic Integrity of Glyoxalase 1. *Cell Reports*, **32**, Article ID: 108160. https://doi.org/10.1016/j.celrep.2020.108160

[33] Sharp, C.D., Hines, I., Houghton, J., Warren, A., Jackson, T.H., Jawahar, A., *et al.* (2003) Glutamate Causes a Loss in Human Cerebral Endothelial Barrier Integrity through Activation of NMDA Receptor. *American Journal of Physiology-Heart and Circulatory Physiology*, **285**, H2592-H2598. https://doi.org/10.1152/ajpheart.00520.2003

[34] Yamamoto, S., Kimura, T., Tachiki, T., Anzai, N., Sakurai, T. and Ushimaru, M. (2012) The Involvement of L-Type Amino Acid Transporters in Theanine Transport. *Bioscience, Biotechnology, and Biochemistry*, **76**, 2230-2235. https://doi.org/10.1271/bbb.120519

[35] Yokogoshi, H., Kobayashi, M., Mochizuki, M. and Terashima, T. (1998) Effect of Theanine, r-Glutamylethylamide, on Brain Monoamines and Striatal Dopamine Release in Conscious Rats. *Neurochemical Research*, **23**, 667-673. https://doi.org/10.1023/a:1022490806093

[36] Phumsombat, P., Sano, C., Ikezoe, H., Hayashi, J., Itoh, T., Hibi, T., *et al.* (2020) Efficient Production of L-Theanine Using Immobilized Recombinant *Escherichia coli* Cells Expressing a Modified *γ*-Glutamyltranspeptidase Gene from *Pseudomonas nitroreducens*. *Advances in Biological Chemistry*, **10**, 157-171. https://doi.org/10.4236/abc.2020.106012

[37] Griep, L.M., Wolbers, F., de Wagenaar, B., ter Braak, P.M., Weksler, B.B., Romero, I.A., *et al.* (2012) BBB on CHIP: Microfluidic Platform to Mechanically and Biochemically Modulate Blood-Brain Barrier Function. *Biomedical Microdevices*, **15**, 145-150. https://doi.org/10.1007/s10544-012-9699-7

[38] Lowe, G.D.O., Lowe, J.M., Drummond, M.M., Reith, S., Belch, J.J.F., Kesson, C.M., *et al.* (1980) Blood Viscosity in Young Male Diabetics with and without Retinopathy. *Diabetologia*, **18**, 359-363. https://doi.org/10.1007/bf00276814