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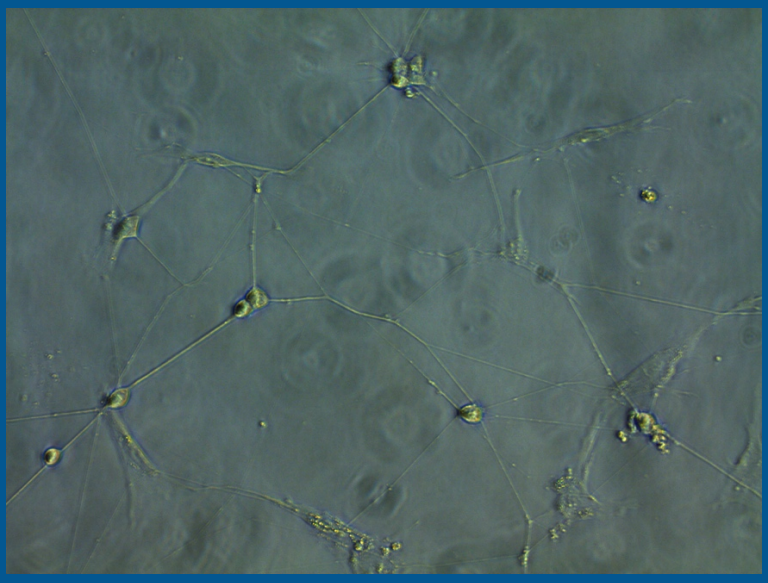

Preliminary study of anti-apoptotic effects of *Ginkgo biloba* extract on glial cells and neurons in culture

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Introduction

An estimated 84,692 deaths in 2011 were caused by Alzheimer's disease (AD) in the United States (Tejada-Vera, B., 2013). An effective drug for this disease is in demand. The development of such drug relies on our understanding of the pathology of Alzheimer's disease. One of the pathological hallmarks of Alzheimer's disease found in brain tissue is the accumulation of ß-amyloid (Kandel et al., 2013). After years of research, the underlying mechanism for the formation of ß-amyloid plaque is now believed to be initiated from the amyloid precursor protein (APP). Through a proteolysis process, the APP is cleaved into three pieces. Given the fact that APP is a transmembrane protein, one of the three pieces must contain the hydrophobic domain and ß-amyloid peptide is that piece. Thus, ß-amyloid peptides have the potential to aggregate with each other and to form insoluble plaque. Based on the original amyloid cascade hypothesis (ACH) (Hardy & Higgin, 1992), amyloid plaques have always been blamed for Alzheimer pathology. However, the current ongoing debate over the original amyloid cascade hypothesis suggests that the toxicity of the intermediate Aß oligomer that undergoes fibrillization is over that of the mature fibrils (Verma, 2015). This new perspective may help scientists resolve the conflicting reports showing the correlation between amyloid plaque and AD- symptoms (Sakano and zako, 2010).

Indeed, studies from several groups have successfully demonstrated that cells exposed to soluble Aß peptide would experience neuronal apoptosis (Florent, et al. 2006; Troy, et al., 2001; Ivins, et al., 1999). Among these studies, both Aß 1-40 fragment and Aß 25-35 fragments were commonly utilized for their neurotoxic effects. The present study utilizes Aß 25-35 fragment, which is the smallest fragment retaining the biological activity of the full-length peptide (Sublimi Saponetti M., et al., 2014). Previous studies also have supported the existence of an apoptotic event in the toxic mechanisms mediated by Aß 25-35 fragment (Clementi et al., 2005). Thus, Aß 25-35 fragment is sufficient in the present study to induce the desirable toxic effect.

Herbal medicine with phytochemical constituents is a rising field in modern pharmacology and is considered as an alternative treatment for various disorders. *Ginkgo biloba* leave extract (EGb761) had been shown to be an effective drug to Alzheimer's disease. EGb761 has been available in Europe as an herbal medicine since the early 1990s, however, produces containing EGb761 are not approved for medicinal use by the US FDA (EGb, EGb761, 2003). EGb761 is a standardized extract that contains 24% ginkgo-flavonol glycosides, 6% terpene lactones such as ginkgolides A, B, C, J and bilobalide B as the major active ingredients

(Nash&Shah., 2015). It has a broad spectrum of pharmacological activities and is able to be applied to numerous disorders occurred in cerebral, retinal, cardiac, or peripheral ischemia (Nakanishi, 2005). Most importantly, EGb761 has direct effects on apoptosis of neurons, thus is used as an effective drug for Alzheimer's disease (Behl, 2000). The interaction between EGb761 and beta-amyloid shall be investigated as a mean to reveal the mechanism of action for EGb761's neuroprotective effects. Among all the possible mechanisms of action, its antiapoptotic effect is the focus of the current paper.

Previously, EGb761 has shown its antiapoptotic effects in diverse tissue types in both animal and human studies (Serrano-Garecia, 2013). Fewer studies have been done within a cell culture model. One of the groups showed the protective effects of EGb761 against both Aß 1-40 and Aß 25-35 fragment induced apoptosis in hippocampal neurons from rats (Basrianetto, et al., 2000); and the other group showed the antiapoptotic effects of EGb761 in a bEnd.3 Endothelial cell line (Wan, et al. 2004). None studies have looked at the possible difference of EGb761's protective effects against beta-amyloid-induced toxicity among cell types. Thus, the present study tested for the possible difference in the potency of EGb761's protective effects against Aß 25-35 fragment-induced cellular apoptosis between neuronal cells and glial cells using a mixed neuronal/glial cell culture from chick embryo.

Material and Method

Material and equipment

Ginkgo biloba extract (EGb761) was obtained by dissolving one commercial Tebonin® EGb761 tablet purchased from Amazon (B0096R3POA). Aß 25-35 oligomer (lot number A4559) was purchased from Sigma (USA). The purchased Aß 25-35 oligomer were white solids. Trypan blue powder (Allied Chemicals No.508) were taken from the Wheaton College chemical compound stock. Peripheral neuron cells were prepared from dorsal root ganglion of chick embryo at ten-day old. Material and reagent required for dissection of chick embryo were summarized in previous protocol (Morris, 2015). Dense cell culture with mixed glial cell and neuronal cells were used.

The images were acquired by SPOT® Ideal Camera (model: 27.2-3.1 MP color; serial number 257279) on a Nikon Eclipse TS 100 inverted microscope. Image analysis was done by ImageJ (version 1.51m9) and Photoshop (2017.1.1 Release). Statistical analysis was done by R-Studio (version 1.0.136).

Preparation of EGb761 stock solution

Each Tebonin® EGb761 tablet contains 120mg standard EGb761 extract and thus, an assumption was made that the described dissolving method achieved the full solubility of the EGb761 in the solvent of choice. The solvent of choice was deionized water because EGb761 shows moderate solubility in water and the usage of water eliminated the possible influence from reagent carrier (Beek and Montoro, 2009). The dissolving process went like following: One tablet was grounded into powders with mortar and pestle. The obtained powders were dissolved in 10-ml of deionized water in a falcon tube. The dissolving process was facilitated by incubating the tube in a 37^{O}C water bath periodically and with vigorous shaking. The dissolving process continued until no more powders could dissolve. The tube was then centrifuged for one minute with Sorvall® H-1000B Rotor in order to separate the undissolved tablet coat and inert ingredients from the solution. The revolutions per minute (RPM) was set at 2200g to create a

relative centrifugal force (RCF) of 1000g. The clear supernatant was then filtrated with a 0.22µm filter to sterile the solution. The final EGb761 stock solution has a concentration of 12mg/ml under the assumption that a full solubility of EGb761 was achieved.

Preparation of Aß 25-35 oligomer stock solution

The Aß 25-35 oligomers were in white solid forms. The peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2.5mM and stored at -20^{O}C. This concentration has been shown to lead to the predominance of the soluble monomeric form of the peptides (Pike, et al., 1995). 377µl of DMSO was added to 1mg of peptide solids directly within the product bottle. The addition of DMSO was followed by stirring to make sure a complete dissolving. The stock solution preparation process was accomplished by Cassandra Kennie, Nathaniel Awkerman, Ryan McKeon and Lena O'Flynn.

Preparation of Trypan blue stock solution

Trypan blue powder with dark blue color was used. The protocol for trypan blue stock solution preparation was based on product information from HiMedia Laboratory (product sheet from the original company was not found). For Cell culture application, trypan blue is generally used in HBSS at 0.4% concentration. In order to achieve this final concentration in 2ml HBSS, a 10X stock solution was made. For the stock solution, the solvent of choice was deionized water because trypan blue power is soluble in water. The stock solution was stored at room temperature away from bright light. Experimentally, a small amount of trypan blue powders was measured out and put into a Falcon tube. The corresponding volume of deionized water required to achieve 4% concentration was calculated and then added. The Falcon tube was shaken vigorously with periodic 37^{O}C water bath incubation to facilitate the dissolving process. The solution was considered fully dissolved when there was no observable powder at the bottom of the tube. Then, the solution was sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns.

Cell culture

Dissection and culture of chick embryo neurons and glia were performed as described by Morris (2015). The cell cultures were plated at day0 on a coverslip that had previously been coated with poly-D-lysine and laminin. The poly-D-lysine coating functioned as an adhesion medium and the laminin was a contact signal for neuronal cells. Cell cultures were grown in F-Plus-medium supplemented with NGF. Cells were maintained in growth medium at 37^{O}C in a humidified atmosphere until the day of the experiment.

Experiment treatment

On the day of the experiment, the growth medium was removed in exchange for growth medium supplemented with three different treatment: Control 1 was supplemented with 12.5µM Aß 25-35 oligomers and 100µg/ml deionized water. Control 1 (Aß treatment) would show us the natural neurotoxic effects of Aß 25-35 peptides. Control 2 (Control treatment) was supplemented with 12.5µM DMSO and 100µg/ml ginkgo solution. Control 2 would show the normal cell growth in the absence of Aß 25-35 peptides. Experiment treatment (Aß +Ginkgo treatment) was supplemented with 12.5µM Aß 25-35 fragments and 100µg/ml Ginkgo solution. This experiment treatment would tell us the influence of Ginkgo on the neurotoxic effects of Aß 25-35 peptides. The final volume of growth medium in each petri dish after the exchange was always 2ml. The

chosen applied concentration of Aß 25-35 peptides has been previously shown to successfully induce the desired apoptotic effects (Bastianetto et al., 2000). The chosen applied concentration of the EGb761 solution was the highest of previously tested concentrations in other cell types (Bastianetto et al., 2000). The highest concentration was adapted to ensure the potency of EGb761. All reagents were prepared fresh on the day of the experiment by diluting the stock solution. After the exchange of growth medium, cells were incubated at 37^{o}C in exposure to one of the three treatments for 48 hours. The previous study has shown that Aß 25-35 peptides bring about a larger toxic effect on cell viability after 48hours of incubation (Clementi, et al., 2005).

Trypan blue Exclusion test of Cell Viability

Trypan blue exclusion was utilized to do the cell viability assay. Trypan blue is a dye that can only be taken by apoptotic cells whose plasma membrane is no longer intact. Experimentally, after 48 hours of incubation of one corresponding experimental treatment, the cell culture had its growth medium replaced by 2ml of HBSS containing 20µl of freshly dissolved 10x stock solution of trypan blue to achieve the desired concentration of 0.4%. Followed by a 5-10 incubation at room temptation, the cell cultures were washed with 2 portions of 2-ml of HBSS to dilution the applied trypan blue solution. Cells were then observed with 10X lenses on a Nikon Eclipse TS 100 inverted microscope in incubation of HBSS.

Images were acquired by SPOT® Ideal Camera (model: 27.2-3.1 MP color; serial number 257279). To gather a representative sample of images of cells in a dish, seven non-overlapping, highly focused images were collected from random positions on each coverslip. These seven images typically included 70-80 cells. Each of the seven images was analyzed as described below.

Quantification of cell viability

The extent of cell injury was quantified by assessing the extent of cellular uptake of the trypan blue dye. This quantification was done by measuring the blue staining intensity with ImageJ. Images were first contrast-enhanced within Photoshop by adjusting the black level threshold to the dimmest detected pixels. Then, the contrast-enhanced image was opened in ImageJ. The image was first converted to the grayscale (image>type>8-bite), and then the background coloration was removed (Process>Subtract Background> Rolling ball radius:50pixels > OK). Finally, the image was to be converted to the inverted color model (Edit>Invert). This had the benefit of increasing the readability of the image.

For each analyzed image, following qualities were recorded. First, the number of neuronal cells and glial cells respectively were recorded. For the purpose of this study, a neuronal cell was identified as one with clear phase halo and a near spherical cell body. A glial cell was identified as one with polygon shape. Possible glial cells that happen to have elongated cell bodies that resembled axons were excluded. Any object without defined cellular characteristic was excluded. Cells on the image margin that showed partial structures were excluded. Overlaid cells were excluded as the results would be biased when measuring the staining intensity. For all identified neuronal cells and glial cells, staining intensity was further measured. Staining intensity was measured within ImageJ by using polygon tool to outline around the cell body of a neuronal cell or a glial cell and set it as the region of interest (ROI). The best judgment was made about where the edges of the cell body were. The mean gray value for the ROI was then measured and recorded (Analyze>measure). The mean gray value was measured and recorded for every identified cell.

In total, 44 neuronal cells and 46 glial cells in 6 images from the control treatment were analyzed. 25 neuronal cells and 43 glial cells in 7 images from Aß treatment were analyzed. For Aß + ginkgo treatment, 40 neuronal cells and 51 glial cells in 7 images were analyzed.

Assessment of apoptotic cell

Due to the fact that staining cells varied in their extent of blue color, the apoptotic cells were identified by setting a threshold intensity. Cells with staining intensity over the threshold intensity were countered as apoptotic cells. For the purpose of the present study, the threshold intensity for one cell type was defined as the upper quartile of the ordered dataset of all the staining intensities obtained before for one cell type. The upper quantile was obtained by using statistics software R-studio (Version 1.0.136). The obtained threshold intensity for a neuronal cell to be considered as apoptotic was 87.84 and that of a glial cell was 48.65. The number of apoptotic cells and the percentage of apoptotic cells were counted and calculated with Microsoft® Excel for Mac (Version 15.40) by sorting the dataset on value (Data>Sort).

In total, staining intensities of analyzed 140 glial cells in 20 images from three treatments together were used to obtain the threshold staining intensity. Similarly, staining intensities of analyzed 109 neuronal cells were used to obtain the threshold staining intensity.

Statistical analysis

A Welch Two Sample t-test was performed to test the significance of the difference in the mean staining intensity between Aß treatment and Aß with ginkgo treatment with a significance level at 0.95. Thus, a p-value smaller than 0.05 was considered as statistically significant. All the statistical tests were done with software R-studio (Version1.0.136).

Results

Aß 25-35 oligomer induce cellular death by forming plaque

Cell cultures' growing conditions after 24-hours of exposure to the corresponding treatment were observed under 20X lenses of Nikon Eclipsed TS100 inverted microscope (images not included). From preliminary observation, there were small plaques formed by soluble Aß 25-35 oligomer in presence of living cells. Cell culture that was exposed to Aß 25-35 (12.5µM) had possible cell body fragments present in the petri dish. But, axons looked healthy and no obvious blebbing were observed. Control cell culture that was exposed to EGb761 (100µg/ml) showed healthy axon growing and no obvious blebbing. Cell culture exposed to Aß 25-35 (12.5 µM) along with EGb761 (100Mµg/ml) also showed healthy axons and no blebbing were detected. These results implied that neurotoxic effects of Aß 25-35 had not achieved the maximum extent. No quantification but only preliminary observations were made after 24-hours of incubation.

After 48-hour incubation, the Aß 25-35 oligomer formed large plaque such that they could be seen without a microscope. Trypan blue exclusion assay results showed the presence of apoptotic glial cells (Figure1) and neuronal cells (Figure2) whose staining intensity exceeded the threshold staining intensity. There were both apoptotic cells detected in cell culture that was having Aß treatment and cell culture that was having Aß with EGb761 treatment.

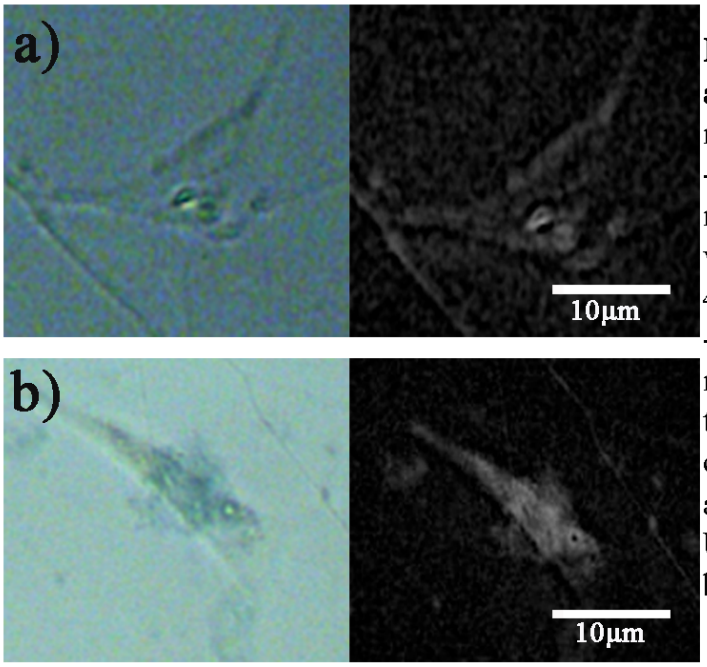


Figure1 A representative healthy glial cell and a representative apoptotic glial cell.a) A representative healthy glial cell that has a polyg -on shape and has no trypan-bule staining.The mean staining intensity for this cell is 21.596 which is smaller than the threshold intensity of 48.650 for a glial cell. b) A representative apop -totic glial cell with trypan blue staining. The mean intensity for his cell was 50.781, exceeding the same threshold intensity of 48.650. Both cells were shown as a phase microscopy image along with image under the inverted color mode. Under inverted colore mode, the part with trypan blue staining shows the highest brightness.

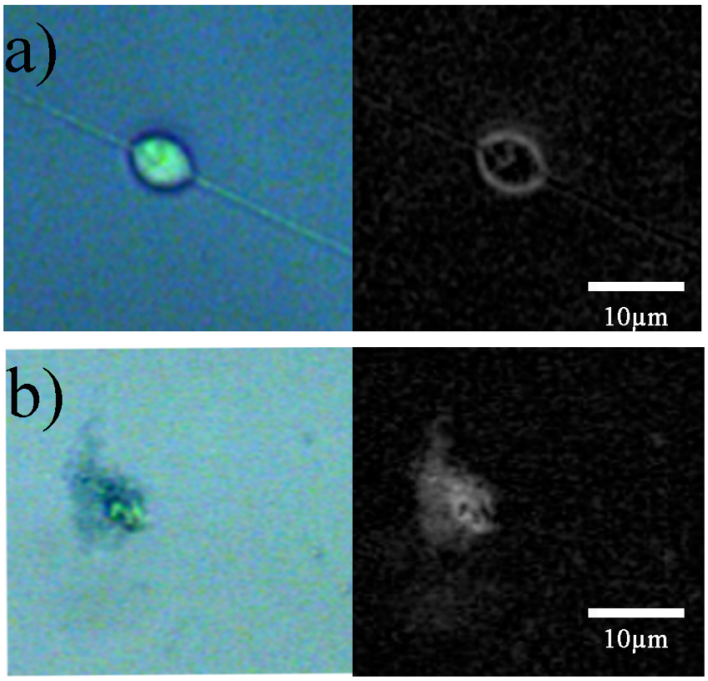


Figure 2 A representative healthy neruonal cell and a representative apoptotic neuronal cell. a) A representative healthy neuronal cell with phase halo and spherical cell body. It has no trypan blue staining. The cell has a mean intensity of 30.907 which is smaller than the threshold intensity of 87.840. b) A representative apoptotic neuronal cell with trypan blue staining. The apoptotic cell has intensity of 91.626 which is bigger than the same threshold intensity of 87.840. Both cells were shown in a phase microscopy image and a image under inverted color mode. Under inverted color model,the part with trypan blue staining shows the highest brightness.

EGb761's neuroprotective effects differ between glial cells and neuronal cells

By quantifying the percentage of apoptotic glial cells and apoptotic neuronal cell respectively under three experimental treatments, we were able to compare the difference in EGb761's neuroprotective effects between glial cells and neuronal cells. Figure 3 shows the percentage of apoptotic glial cells under three treatments. With the presence of EGb761, the percentage of apoptotic cells wad decreased by about 2 folds. Virtually no apoptotic glial cells were detected in control cell culture that was exposed to EGb761 only. Figure 4, on the other hand, shows the percentage of apoptotic neuronal cells under three treatments. The presence of EGb761 also brought about a small reduction in the percentage of apoptotic neuronal cells. The reduction fold was much smaller than that of the glial cells. There were also no apoptotic neuronal cells being detected in cell culture that exposed to EGb761 only.

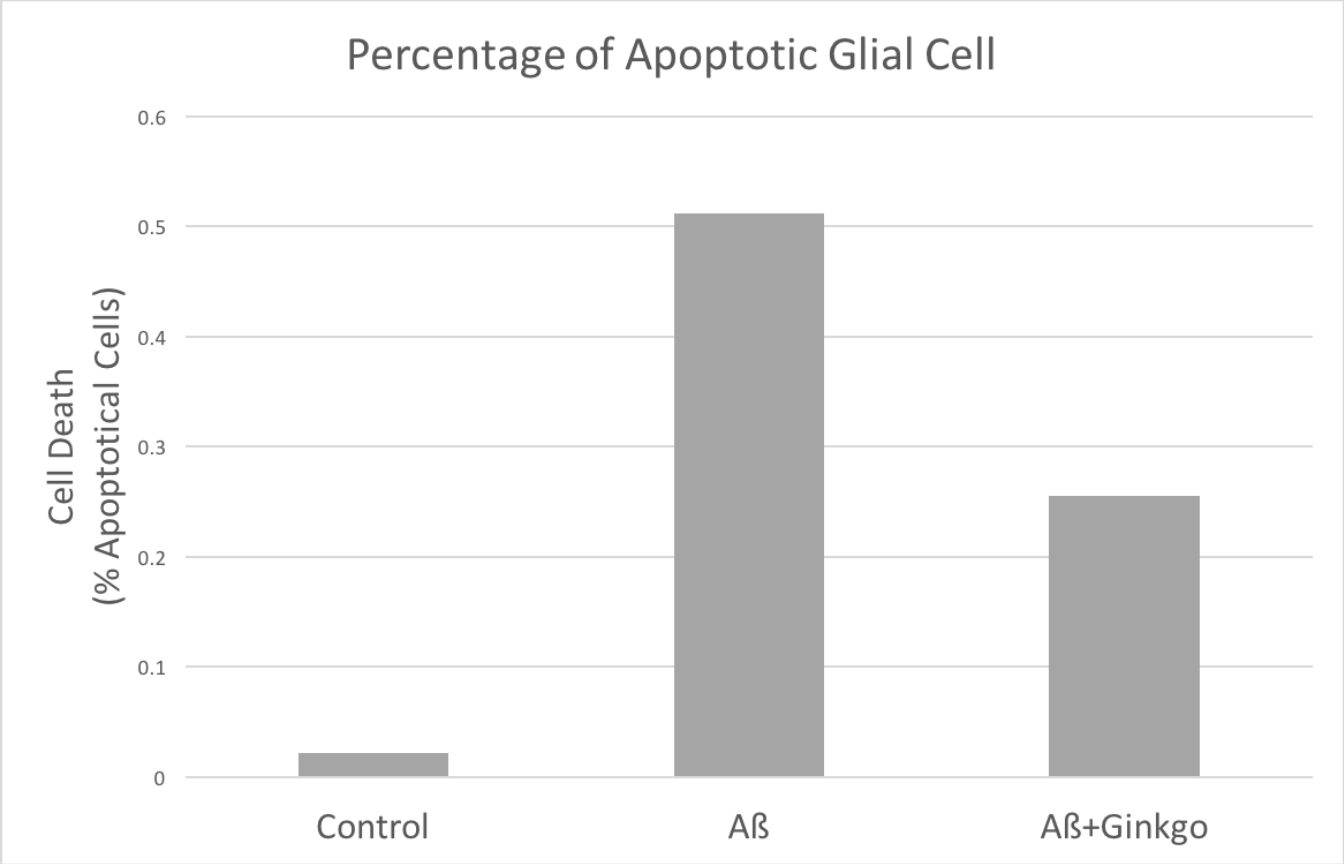


Figure 3 Percentage of apoptotic Glial cells under three treatments after 48-hour of incubation. The percentage of apoptotic glial cells increased by about 5-fold in exposure to Aß 25-35 (12.5μM) for 48 hours. The percentage of apoptotic glial cells were reduced by 1-fold in cell culture in exposure to both Aß 25-35 (12.5μm) and EGb761 (100μg/ml) after 48-hour of incubation. Only one apoptotic glial cells were detected in cell culture after exposing to EGb761 (100μg/ml) for 48 hours.

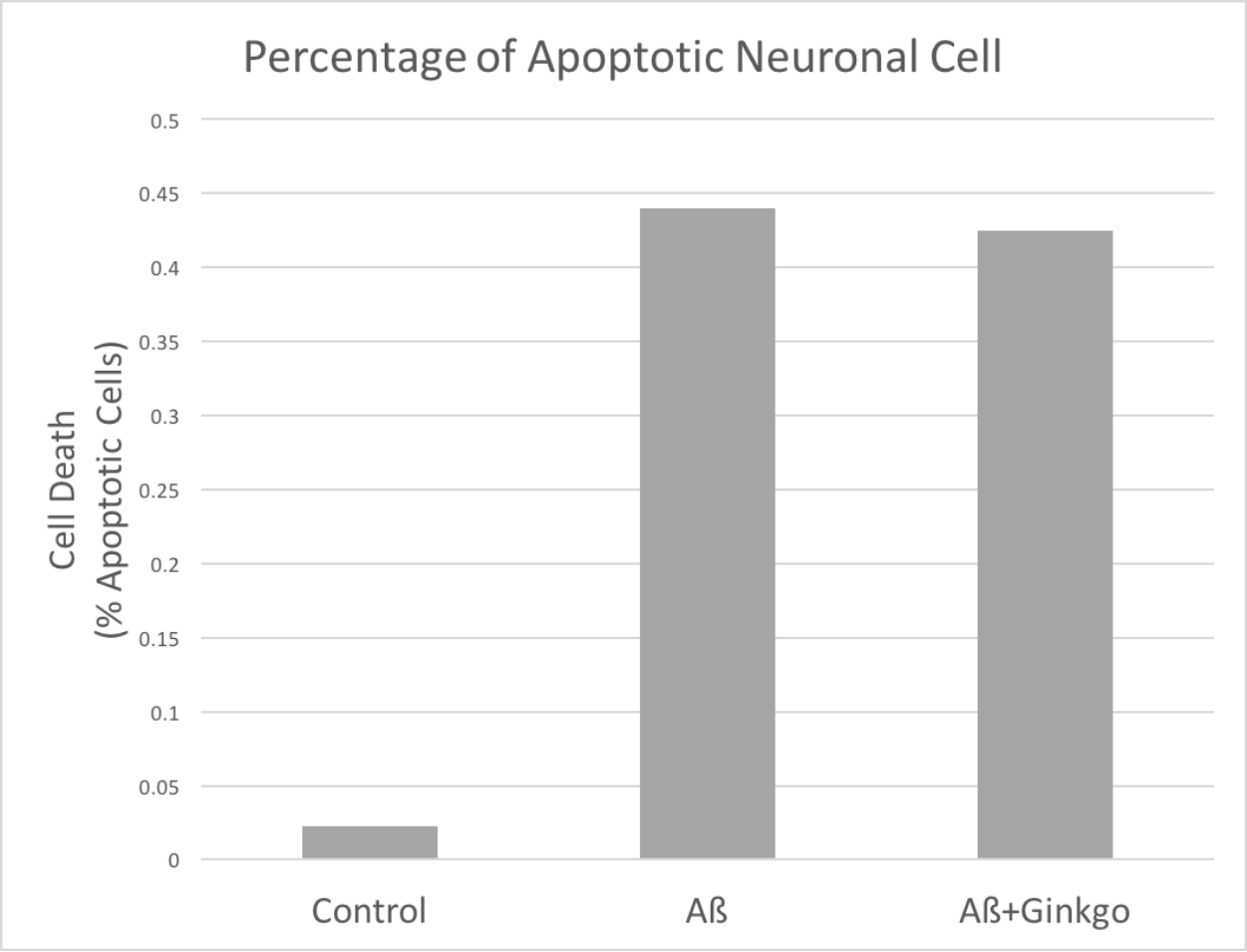
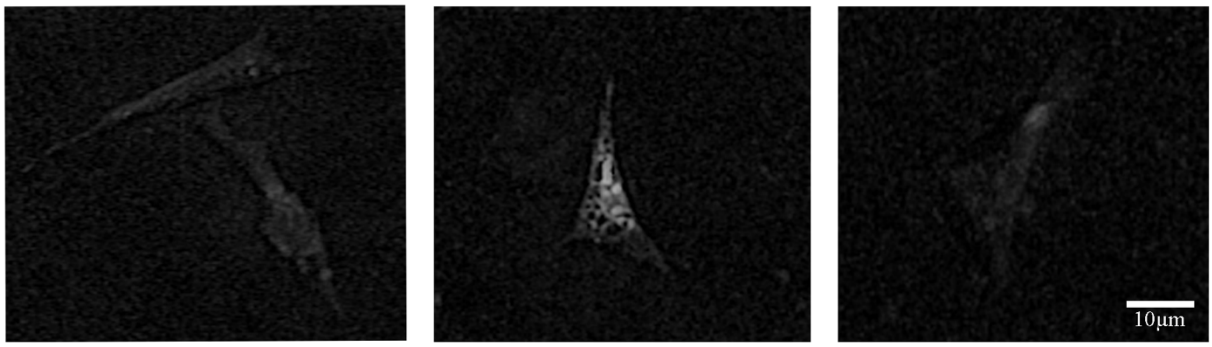


Figure 4 Percentage of apoptotic Neuronal cells under three treatments after 48-hour incubation. The percentage of apoptotic neuronal cells increased by more than 4 folds in exposure to Aß 25-35 (12.5μM) for 48 hours. The percentage of apoptotic glial cells were reduced by less than 1 percent in cell culture in exposure to both Aß 25-35 (12.5μm) and EGb761 (100μg/ml) after 48-hour of incubation. Only one apoptotic neuronal cell was detected in cell culture after exposing to EGb761 (100μg/ml) for 48 hours.

The distributions of trypan blue staining intensity for neuronal cells and glial cells respectively were further investigated to quantify the results statistically. The staining intensity was measured to quantify the extent of cell viability. A healthy cell would have a relatively lower staining intensity, and an apoptotic cell would have a relatively higher staining intensity. The distribution of staining intensity thus showed an overview of the cell viability within one petri dish under one corresponding treatment. The distribution was shown in the form of a boxplot. Each boxplot was plotted from data obtained from all analyzed neuronal cells or glial cells within one petri dish (one corresponding treatment). Data points at the bottom of the

boxplot were from healthy cells and data points at the top region of the boxplot were from apoptotic cells. Side-by-side boxplots were shown to do a comparison among three treatments.

Figure 5 shows the distribution of trypan blue staining intensity for glial cells under three treatments. In cell culture being exposed to Aß 25-35 along with the presence of EGb761, since both healthy cells and apoptotic cells were present, the boxplot showed a large range. In cell culture being exposed to Aß 25-35 only, the neurotoxic effects of Aß 25-35 led to a large proportion of apoptotic cells. Thus, this boxplot appeared higher than the other two boxplots. In the control treatment where cell culture was exposed to EGb761 only, the cell culture had only one apoptotic cell. Therefore, the boxplot of control treatment looked much lower than the other two boxplots. The one apoptotic cell in the control treatment appeared as an outlier in the boxplot. Because the staining intensity of this apoptotic cell was much larger than the rest of the data set, it was considered as an outlier statically. The mean staining intensities of all the analyzed glial cells for the three treatments were represented by the line within the box (n=46 for control treatment; n=43 for Aß treatment; and n=51 for Aß with Ginkgo treatment). The mean intensity for the Aß with ginkgo treatment was lower than that of the Aß treatment. A Welch two-sample t-test was performed to test whether this reduction in mean staining intensity was significant or not with a significant level at 95%. The p-value from this test was smaller than 0.001, meaning that the reduction in the mean staining intensity in the Aß with ginkgo treatment was significant compared to that of the Aß treatment. Figures of glial cells in the inverted mode under three treatments were also presented to show the difference in staining intensity visually.



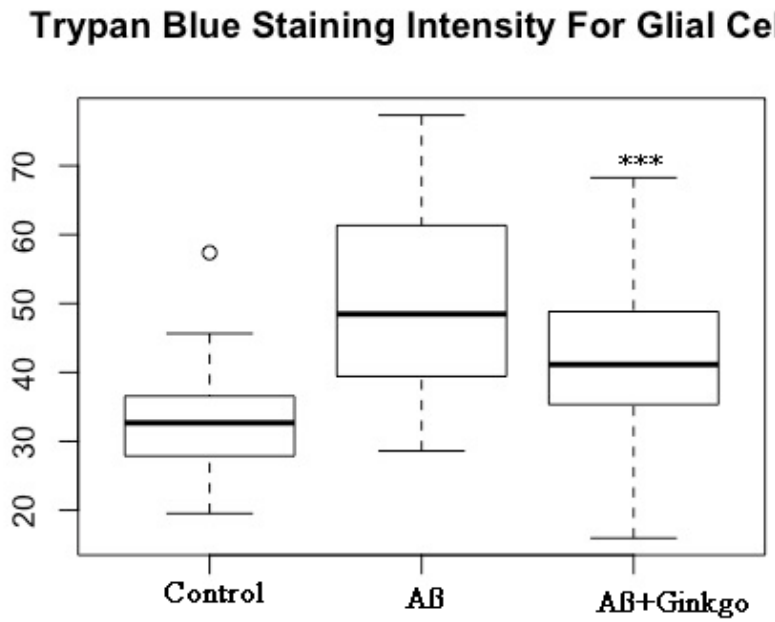
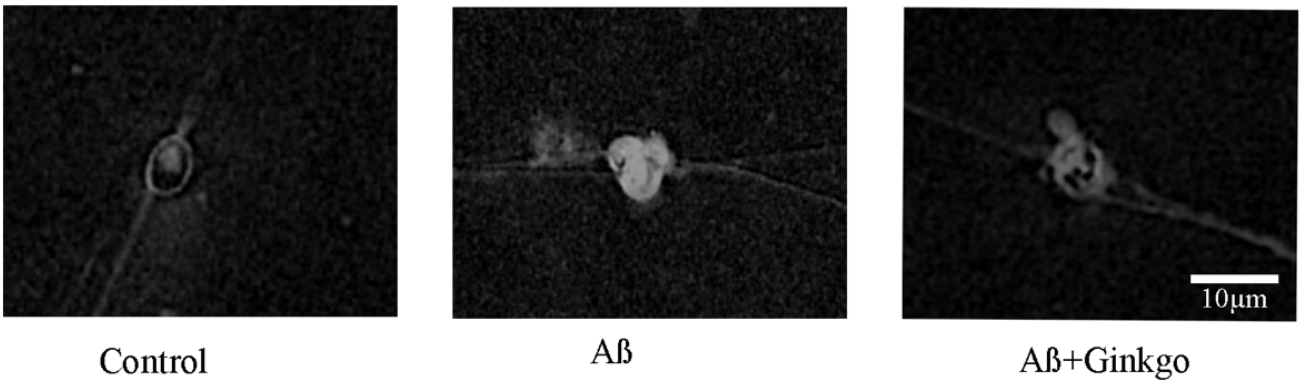


Figure 5 Trypan Blue Staining Intensity Distribution for Glial Cell. Cell cultures were incubated with or without EGb761 (100µg/ml) while in exposure to Aß (12.5µM) for 48 hours. Cells were then assayed by trypan blue staining and viewed under phase microscopy. Images of glial cells under three treatments under inverted color mode show the difference in staining intensity. Glial cells in exposure to Aß without the presence of ginkgo showed the highest intensity, while, this high intensity was reduced in the presence of Ginkgo. The distribution of staining intensity was then plotted with R-studio. The boxplot shows the reduction of the mean staining intensity of all the analyzed glial cells that were having Aß with ginkgo treatment. A t-test was performed to show the significance of this difference between Aß group and Aß with ginkgo group. In Control group, a total of 46 glial cells were included in the boxplot; in Aß group, a total of 43 glial cells were included in the boxplot, and in Aß with ginkgo group, a total of 51 glial cells were included in the boxplot. (***$p<0.001$ in comparison with Aß group)

The distribution of trypan blue staining intensity for neuronal cells was shown in figure 6. For neuronal cells, the boxplot for Aß treatment and the boxplot for Aß with ginkgo treatment had the similar range, meaning that the cell viabilities under these two treatments were similar. Interestingly, the boxplot for Aß treatment did not show a relatively higher position compare to two other boxplots, like the case in glial cells. This may imply that Aß did not show as much strong toxic effects on neuronal cells as on glial cells. Like that of the glial cells, the boxplot for control group looked much lower than the two other boxplots, meaning that the cells under control treatment were healthier than the cells under other two treatments. The control boxplot showed one outlier which came from the only apoptotic cell detected in the control petri dish. The Aß treatment boxplot had two outliers that were form two neuronal cells with intense blue staining. The mean staining intensity of all the analyzed neuronal cells under three treatments were presented by the line within the box as well (n=44 for control treatment; n=25 for Aß treatment; and n=40 for Aß with ginkgo treatment). From the boxplot, there was a small reduction in the mean when comparing between Aß treatment and Aß with ginkgo treatment. A two-sample t-test was then performed to test for the significance of this reduction. The p-value from this t-test was large, therefore, there was no sufficient evidence to claim that there was a reduction in the mean staining intensity in Aß with ginkgo treatment when compared with that of the Aß treatment. Figures of neuronal cells under inverted color mode under three treatments were also presented to show the difference in staining intensity among three treatments. Visually, the staining intensity of neuronal cells with Aß with ginkgo treatment was reduced, but, based on the results of the statistical test, this reduction was not able to be considered as significant.



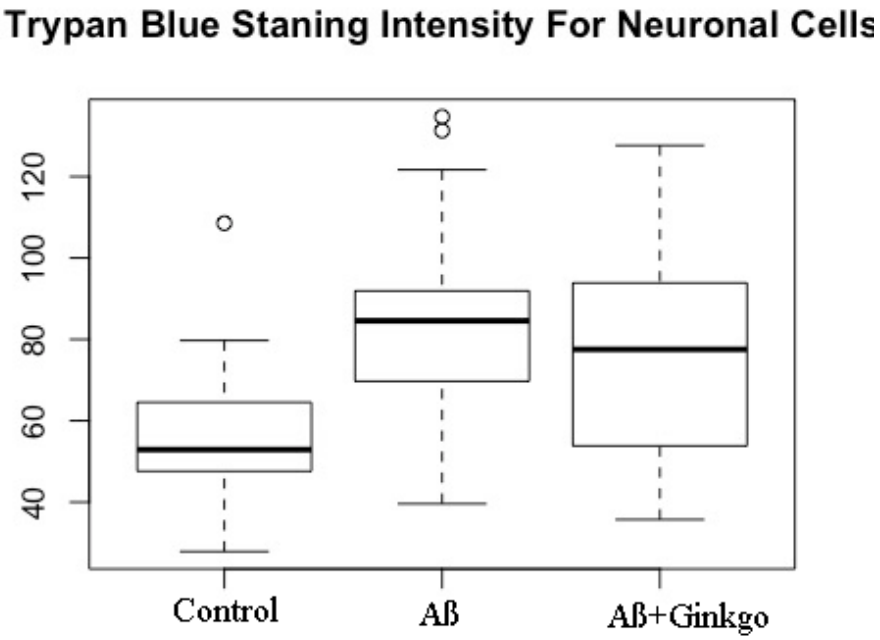


Figure 6 Trypan Blue Staining Intensity Distribution for Neuronal Cell. Cell cultures were incubated with or without EGb761 (100µg/ml) while being exposed to Aß (12.5µM) for 48 hours. Cells were then assayed by trypan blue staining and viewed under phase microscope. Pictures in the inverted color mode show a reduction in intensity in the neuronal cells when in incubation of Aß in the presence of EGb761. Side-by-side Boxplot of the distribution of staining intensity under three treatments was plotted with R-studio. A two-sample t-test was performed to test for the significance of the reduction on mean staining intensity between Aß treatment and Aß with ginkgo treatment. T-test results failed to reject the null-hypothesis and there was not enough evidence to claim that there was a significant reduction in staining intensity in the present of ginkgo. In Control group, a total of 44 neuronal cells were counted; in Aß group, a total of 25 neuronal cells were counted, and in Aß with ginkgo group, a total of 40 neuronal cells were countered.

Discussion and Conclusion

The hypothesis was supported by the present results that the neuroprotective effects of EGb761 against Aß (25-35) oligomer-induced cellular apoptosis differ between neuronal cells and glial cells. This result agrees with one result from a previous study on the hippocampal neurons (Bastianetto et al, 2000). In the study of hippocampal, the protective effects on EGb761 were measured in separate cultures of neuronal cells and glial cells and the results showed that the protective effects of Egb761 tend to be more pronounced on glial cells than on neuronal cells. The present study, on the other hand, showed a similar observation in a mixed culture of neuronal cells and glial cells from peripheral nerve system of 10-days chick embryo. Based on the present data, following possible reasons may explain the observed difference. First, the comparison between the boxplots of Aß treatment for neuronal cells and glial cells implied that the toxicity of Aß 25-35 may bring a stronger apoptotic influence on glial cell than on neuronal cells. A diminished apoptotic influence may partially explain why a reduced protective effect of EGb761 was detected in neuronal cells. On the other hand, since neurons from peripheral nerve system were used, the glial cell culture in the present study are Schwann cells. Schwann cells have an important role as the regulator of nerve development (Mirsky et al., 2002). One major function of Schwann cells is to provide the insulating material that allows rapid conduction of electrical signals along the axons as well as a protection through a process called myelination (Kandel et al., 2013). Previous evidence showed that in a primary cell culture of isolated neuronal cells and glial cells, myelination would occur over a course of several weeks (Johnson, Bunge and Wood, 2001). The cell cultures that the present study used were cells of two-day-old when myelination had not occurred yet. This may explain the reason for which there was a higher percentage of the apoptotic neuronal cell compared to glial cells in response to the same treatment. At the same time, the relatively higher sensitivity of glial cells to the protective effects of EGb761 implied the importance of glial cells and neuronal cells together as a system in response to injury or drugs.

Antiapoptotic effects of EGb761 have long been recognized and been under intense research for EGb761's potential as a pharmaceutical drug. Though the investigation of the underlying mechanism for ginkgo extract's protective effects was beyond the scale of present research, a brief discussion of the previously discovered pathways would help to understand the results of present study. Because cellular apoptosis was induced by neurotoxic effects from Aß oligomer, the discussion of the mechanism of EGb761's neuroprotective effects would be against the mechanism of the toxicity of Aß. First of all, Aß induces cellular apoptosis by initiating the generation of reactive oxygen-free species (ROS) in contact with the neuronal membrane (Markesbery, 1996). EGb761, in this sense, has been shown to have an anti-oxidation property by acting directly to inhibit the free radicals. The study done by Jiang et al. has shown an inhibition of H_2O_2 level in spinal neurons upon exposure to EGb761 (2009). H_2O_2 is one major type of the free radicals that is able to enhance the oxidative stress within the nerve system. Besides this free radical pathway rescuer effects, EGb761 also interferes the disturbing mitochondrial pathway. Upon exposure to Aß, the outer membrane integrity of the neuronal cell mitochondrial is disturbed because Aß is able to interact with ions. As a result, mitochondrial experiences swelling and releases cytochrome C (Clementi, et al., 2005). The resale of cytochrome C is one signal that triggers the apoptotic pathway (Goldstein, et al., 2005). In this sense, EGb761 has shown its regulation of cytochrome C and may be able to trigger another intrinsic pathway that counteracts the release of cytochrome C (Shi, et al., 2009). All the above

underlines how EGb761 may be able to protect nerve system against the neurotoxic effects from beta-amyloid.

The present results also implied the potential of EGb761 as a pharmaceutical drug for AD treatment. One feature of the EGb76 constituted drug may distinguish itself from the rest of the western medicine. Most western medicines are manufactured on the basis of isolation of single, purified active ingredients. Not being the case for EGb761, it is a leaf extract with a mixture of active constituents. It is reasonable to believe that a mixture of active constituents may bring EGb761 a so-called "synergistic effects" (Nash and Shah, 2015). Because it is a mixture, each active ingredient within EGb761 may be able to function at different pathways at the same time to create an amplified influence. For example. the active ingredient that functions as an antioxidant may be Ginkgolide A, while the active ingredient that functions as a cytochrome C inhibitor may be Bilobalide B (Nash and Shah, 2015). Both ingredients function at the initiation of apoptotic pathways, but through different pathways. This may contribute to the creation of the synergistic effects. Also, it is not for sure that individual active ingredient could achieve the same antiapoptotic influence.

In summary, the results of the current study support the possibility that there may be difference existing in the potency of neuroprotective effects of EGb761 to glial cells and to neuronal cells. The present study has its pitfall as that the cell viability assay of chosen may not be accurate enough. Trypan blue exclusive method could not distinguish between the apoptotic cell and necrotic cells (Cummings et al., 2013). Though this pitfall would not have changed the experiment results, it may influence the understanding of underlying mechanism because apoptotic cell and necrotic cell undergo different pathways. Also, more data could be gathered to make the statistical test results more powerful even if the data collected in the present study were sufficient to perform meaningful statistical tests. Further research shall be done to pin down the mechanism of EGb761's action by monitoring the change in certain molecular expression level.

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