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Protective and Therapeutic Role of Ginkgo Biloba Extract Through TRPV1 Channels in an in Vitro Alzheimer's Disease Model

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ABSTRACT

Introduction: The effect of Ginkgo biloba (GB) on mitochondria-dependent TRPV1 ion channels in neuroblastoma cells was investigated by creating an Alzheimer's disease (AD) model.

Methods: Okadaic acid was applied on SH-SY5Y cells to create an AD model. After cellular differentiation, the study was organized with the seven main groups, examining the effect of GB on calcium depended TRPV1 channels in neuroblastoma cells AD, has been established in vitro.

Results: The higher Ca^{2}+ concentration was detected in the GB+AD, AD and AD+GB groups when compared with the control ($p<0.001$). The Ca^{2}+ level was lower in GB+AD and AD+GB groups than in the AD group ($p<0.001$). Also, cytosolic Ca^{2}+ concentration was lower in the GB+AD than in the AD+GB group ($p<0.05$), the apoptosis and intracellular reactive oxygen species (ROS) values were higher in the GB+AD, AD and AD+GB groups than in the control ($p<0.001$). The apoptosis and intracellular ROS values were higher in AD group than in the GB+AD and AD+GB group ($p<0.001$) and the apoptosis level was higher in AD+GB group than GB+AD group ($p<0.001$) and the mitochondrial depolarization, caspase 3 and caspase 9 levels were higher in the GB+AD, AD and AD+GB groups when compared to the control group ($p<0.001$). Also, the values were lower in the GB+AD group, AD group and AD+GB groups when compared with the GB+AD+capsazepine group, AD+capsazepine group and AD+GB+capsazepine respectively ($p<0.001$).

Conclusion: These results show us that GB has a protective effect besides its therapeutic effect in Alzheimer's disease via TRPV1 channel.

Keywords: Alzheimer disease, Ginkgo biloba, neuroblastoma cells, ROS (reactive oxygen species), TRPV1.

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INTRODUCTION

Alzheimer's disease (AD) is a common cause of dementia that classified as neurodegenerative disorder. Based on the number of AD patients and related societal costs, certain clinical studies focused on the potential benefits of natural drugs (1). Researchers employed various synthetic or herbal compounds to determine their potential effects in AD (2).

It is believed that Ginkgo biloba originated on the remote mountainous valleys at Zhejiang province in eastern China (3). Standardized Ginkgo biloba extract (GBE) is derived from the dried Ginkgo leaves and could be therapeutic in the management of dementia and memory disorders, as well as the Alzheimer's disease (AD). The medicinal use of Ginkgo biloba has been documented in China for nearly 5,000 years, namely to treat asthma (4,5).

Crucial effects for AD have been reported primarily on the apoptotic pathway and mitochondrial functions, such as energy metabolism improvements, mitochondrial membrane stabilization, cytochrome C inhibition, anti-apoptotic Bcl-2 protein upregulation, and pro-apoptotic Bax protein downregulation, the reduction of caspase 9 and 3 levels and apoptotic necrosis in oxidative stress. Several basic and preclinical researches demonstrated that standardized Ginkgo biloba extract could be helpful in the treatment and prevention of AD, as well as other neurodegenerative disorders in senior citizens (6).

Highlights

- **Ginkgo biloba has TRPV1 channels-via antiapoptotic effect in vitro model of AD.**
- **Ginkgo biloba reduces intracellular Ca2+ and ROS levels mediated by TRPV1 channels.**
- **Ginkgo biloba has a disease-protective effect when administered before AD is formed.**

The transient receptor potential channel vanilloid type 1 (TRPV1) expressed in trigeminal ganglia sensory neurons and dorsal root which is a kind of is a non-selective cation channel. Apolymodal channel, TRPV1 is activated by capsaicin, protons, and noxious heat. Anoxious stimuli sensor, the TRPV1 channel was described as a key contributor to pain signals. The inhibition of TRPV1 dependent ROS production significantly

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reduces the adverse effects of activated microglia and the inflammatory response induced by astrocytes after stimulation by the Aβ peptide (7). It was demonstrated that excessive ROS production and increased Ca^{2+} concentration can play an important role in the progression of neurodegenerative diseases. Antioxidants and Ca^{2+} channel blockers can regulate the imbalance and aid in normal cellular functions (8).

The current study aimed to examine in vitro the effect of Ginkgo biloba on the TRPV1 channels in neuroblastoma cells that AD was established experimentally.

METHOD

Cell Culture and Differentiation

SH-SY5Y (human neuroblastoma cell line) cells were procured from the American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in F12 with HAM and Dulbecco's Modified Eagle Medium at 1:1 ratio that included 10% FBS (fetal bovineserum) (Fisher Scientific), and 1% pen. /strep, Antibiotic combination in 8–10 flasks (5 ml, 25 cm^2 sterile with filter cap). The cells were incubated at 37°C at 5% CO_2 in a humidified incubator. For differentiation, cells were maintained in growth medium with 5 µM retinoic acid (Sigma) for 7 days (9). After the differentiation period, neuroblastoma cells were incubated with GB extract and okadaic acid, as described in the groups section. The previous reports have described the formation of Alzheimer's disease models in SH-SY5Y (Human Neuroblastoma cell line) by incubating with Okadaic acid. The cells were examined daily for contamination evidence. After the incubation, the cells were split into the sterilized falcon tubes for analyses. Our work was carried out in accordance with the Helsinki Declaration.

Reagents/Stains

Fura 2 (AM) florescent calcium stain was procured from Calbiochem (Darmstadt, Germany), Pluronic® F-127 was procured from Biovision (San Francisco, USA). Probenecid, and a mitochondrial stain 5.50, 6.60-tetrachloro-1.10.3.30-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was procured from Santa Cruz (Dallas, Texas, USA). Caspase 3 (AC-DEVD-AMC) and 9 (AC-LEHD-AMC) substrates were procured from Enzo (Lausanne, Switzerland). APO percentage assay with a release buffer was procured from Biocolor (Belfast, Northern Ireland). Dihydrorhodamine-123 (DHR 123) was procured from Sigma Aldrich (St. Louis, MO).

Groups

After cellular differentiation, the study was planned with the following seven principal groups:

Group 1 (Control): No medicine was administered and SH-SY5Y was kept in a flask under the same cell culture.

Group 2 (GB+AD): Cells were incubated with 25 µg/ml Ginkgo biloba extract for 24 hrs and then incubated with 30 nm okadaic acid for 24 hrs (10,11).

Group 3 (GB+AD+Cpz): Cells in this group were incubated with 25 µg/ml Ginkgo biloba extract for 24 hrs, incubated with 30 nm okadaic acid for 24 h, and incubated with the TRPV1 channel antagonist Capsazepine (Cpz, 0.1 mM, 30 min).

Group 4 (AD): Cells in this group were incubated with 30 nm okadaic acid for 24 hrs (10).

Group 5 (AD+Cpz): Cells in this group were incubated with 30 nm okadaic acid for 24 hrs and subsequently incubated with the TRPV1 channel antagonist epin (Cpz, 0.1 mM, 30 min).

Group 6 (AD+GB): Cells in the group were incubated with 30 nm okadaic acid for 24 hrs and subsequently incubated with 25 µg/ml Ginkgo biloba extract for 24 hrs (10,11).

Group 7 (AD+GB+Cpz): Cells in the group were incubated with 30 nm okadaic acid for 24 hrs and subsequently incubated with 25 µg/ml Ginkgo biloba extract for 24 hrs and then incubated with the TRPV1 channel antagonist Capsazepine (Cpz, 0.1 mM; 30 min).

To determine apoptosis, intracellular reactive oxygen species, mitochondrial depolarization, and caspase 3 and caspase 9 levels, the cells were further with the TRPV1 channel agonist Capsaicin (Cap, 0.1 mM; 10 min) to activate the TRPV1 channel before the analysis. In calcium signalling analysis (Fura-2/AM), the cells were stimulated in the 20th cycle with 0.1 mM Cap in the presence of 1.2 mM calcium and calcium free buffer in the extracellular medium.

Intracellular Calcium Measurements and Fura-2 Load

Intracellular calcium levels of SH-SY5Y cells were measured by using Fura 2 AM (acetoxymethyl ester) dye. The cells were incubated with 1.2 mM $CaCl_2$ and calcium free HEPES-buffered saline [HBS; 5 mM KCl, 10 mM D-glucose, 145 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES and 0.1% (w/v) bovine serum albumin (BSA; pH 7.4) that included 5 µM Fura-2 AM and 0.05% (w/v) Pluronic F-127 for 60 min at 37°C and in the dark. These cells were laved twice with HBS and the surface was covered with 1 ml HBS and 2.5 mM probenecid for at least 20 min in the dark at 37°C to allow de-esterification of the Fura-2 AM. Fluorescence intensity (emission) was detected in individual wells at 510 nm with a plate reader that included an automated injection system (SynergyTM H1; Biotek, USA) at alternate excitation wavelengths (340 and 380 nm) in 50 acquisition cycles every 3 seconds. During the intracellular calcium signalling measurement, TRPA1 channels were stimulated with the automatic injector with CPx (0.1 mM) on 20th cycle. Ca^{2+} measurement with staining modification was conducted based on the method proposed by Martinez et al. (12,13).

Apoptosis Assay and Intracellular ROS Production Measurement

APOPercentageTM (cell apoptosis assay) was employed to determine and quantify apoptosis. The APOPercentage stain actively binds to phosphatidyl-serine lipids and transferred to the cells, while the apoptotic cells are stained in red color. The analysis of apoptosis was established according to the described instructions by the producer (Öz et al.; 13,14). SH-SY5Y cells were analyzed to determine the apoptotic cells with spectrophotometry (multiplate reader) at 550 nm (SynergyTM H1, Biotek, USA), and the results are presented as fold change over the baseline before treatment (experiment/control).

The cell membrane penetration was provided easily by using non-charged and non-fluorescent dye [Dihydrorhodamine-123 (DHR-123)], inside the SH-SY5Y cells, DHR-123 is oxidized into cationic rhodamine-123 (Rh-123), localized in the mitochondria and observed as green fluorescence dye. The cells (106 cells/ml in each group) were incubated with 20 µm DHR-123 florescent oxidant stain at 37°C for 25 min (14). SynergyTM H1 automatic microplate reader was employed to determine Rh-123 fluorescent intensity. Analyzes were conducted at 488 nm excitation and 543 nm emission wavelengths. The data are presented as fold change over the baseline before treatment.

Caspase 3 and 9 Activity Assays and Mitochondrial Membrane Potential (JC-1) Analyses

Caspase 3 (AC-DEVD-AMC) and 9 (AC-LEHD-AMC) substrate were determined with the SynergyTM H1 microplate reader (Biotek, USA) at wavelengths (excitation/emission) of 360 nm and 460 nm. Caspase

3–9 activities were analyzed based on previously reported methods (14). The findings are presented as fluorescent units/mg protein and as change in multiples of the baseline level (experimental/control).

Mitochondrial membrane potential fluorescence dye [JC1 (1 μM)] intensity was checked under 485 nm (green) excitation wavelength and 535 nm emission wavelength, the red signal at 540 nm (excitation) and 590 nm (emission) wavelengths (SynergyTM H1, Biotek, USA) (13,14). The study data are presented as emission ratio (590/535). While, potential mitochondrial membrane changes were specified quantity as the integral of the decrement in JC1 fluorescence ratio in the experimental/control groups.

Statistical Analyses

All findings are presented as mean ± standard deviation (SD). The statistical comparison of the groups was made with one-way ANOVA. Statistical analyses were conducted with GraphPad and Prism version 7.04 for Windows (GraphPad Software, San Diego California, USA). P<0.05 was considered as significant.

RESULTS

The Effects of GB on Cytosolic Calcium Levels in Okadaic Ocid Induced in Vitro AD Model

The effect of GB administration on neuroblastoma cell cytosolic calcium levels are demonstrated in Figure 1. The TRP Vanilloid 1 channel stimulator capsaicin and blocker capsazepine were used to evaluate intracellular Ca^{2+} increase through the TRPV1 channels in in vitro AD model. As seen in Figure 1b, increased Ca^{2+} concentration in SH-SY5Y was detected in the GB+AD, AD and AD+GB groups according to the basic levels in control (p<0.001). The Ca^{2+} level was lower in GB+AD and AD+GB groups when compared to the AD group (p<0.001). Also, lower cytosolic Ca^{2+} concentration was detected in the GB+AD than in the AD+GB group (p<0.05).

Effects of GB on Apoptotic Activity and Intracellular ROS Values in Okadaic Acid Induced in vitro AD Model

The effect of GB administrations on apoptosis and intracellular ROS levels in neuroblastoma cells are demonstrated in Figures 2 and 3. Apoptosis and intracellular ROS were higher in the GB+AD, AD and AD+GB groups than the basal levels in the control (p<0.001). The intracellular ROS values and apoptotic activity were higher in AD group than in the GB+AD and AD+GB group (p<0.001) and the apoptosis level was higher in AD+GB group than GB+AD group (p<0.001). Also, the findings were lower in the GB+AD group, AD group and AD+GB groups when compared with the GB+AD+capsazepine group, AD+capsazepine group and AD+GB+capsazepine respectively (p<0.001).

Effects of GB on Mitochondrial Depolarization, Caspase 3 and Caspase 9 in Okadaic Acid Induced in Citro AD Model

The effect of GB administrations on mitochondrial depolarization, caspase 3 and 9 levels in neuroblastoma cells are shown in Figure 4, 5 and 6. The mitochondrial depolarization, caspase 3 and caspase 9 levels were higher in the GB+AD, AD, and AD+GB groups when compared tothe control group (p<0.001). The mitochondrial depolarization, caspase 3 and caspase 9 values were higher in AD group than in the GB+AD and AD+GB groups (p<0.001). Caspase 3 level was lower in GB+AD group when compared to the AD+GB group (p<0.001) but in the same groups, there were no statistically difference in mitochondrial

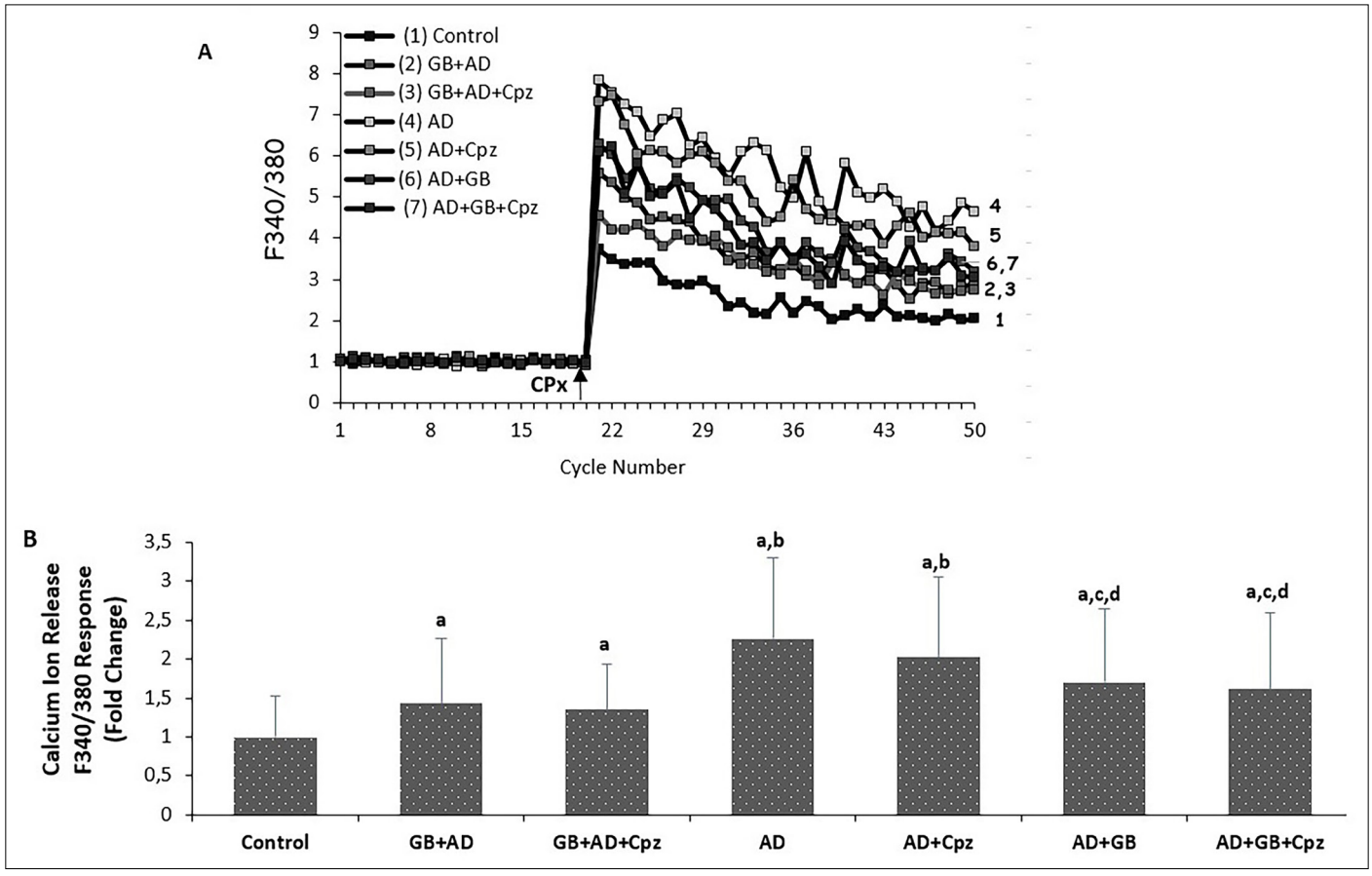

Figure 1. a-b. The effect of okadaic acid (AD, 30 nM, 24 hrs) and Ginkgo biloba extract (25 μg/ml, 24 hrs) on intracellular free calcium increase through the TRPV1 channels in vitro AD model. (n=3 and mean ± SD). Capsaicin (Cap and 0.1 mM on the 20th cycle) was used for the stimulation of cells and the inhibition was provided with using capsazepine (Cpz and 0.1 mM for 30 min). (mean ± SD and n=3). GB:Gingko Biloba, AD:Alzheimer Disease, Cpz: capsazepine

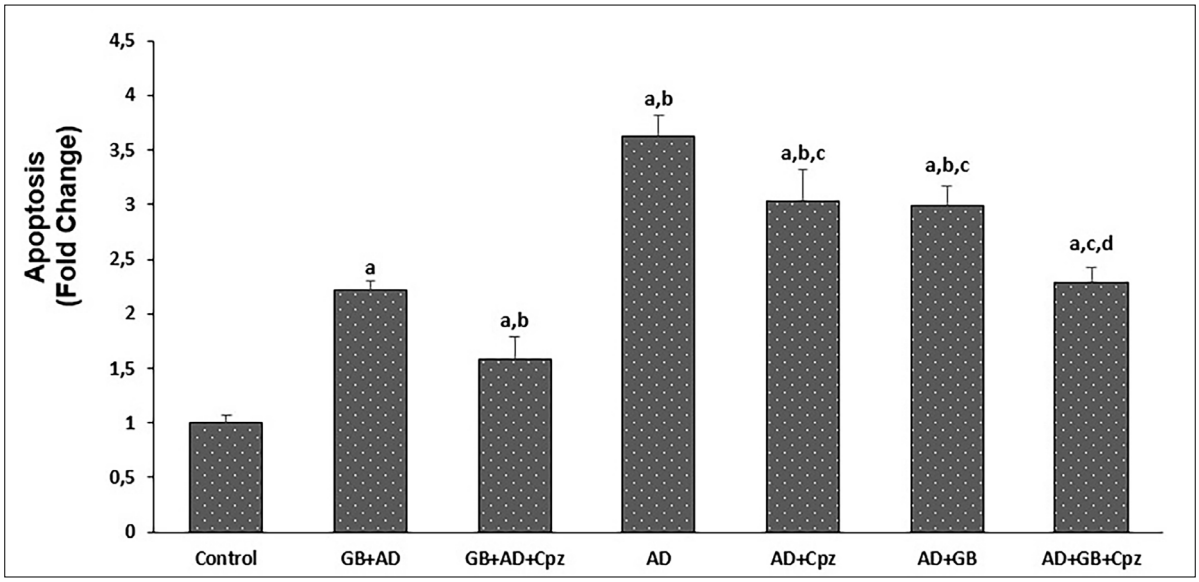

Figure 2. The effect of okadaic acid (AD, 30 nM, 24 hrs) and Ginkgo biloba extract (25 µg/ml, 24 hrs) on programmed cell death levels in vitro AD model. The cells were inhibited by using capsazepine (Cpz and 0.1 mM for 30 min) and stimulation was provided by using capsaicin (Cap and 0.1 mM for 10 minute) (mean ± SD and n=10). GB:Gingko Biloba, AD:Alzheimer Disease, Cpz: capsazepine

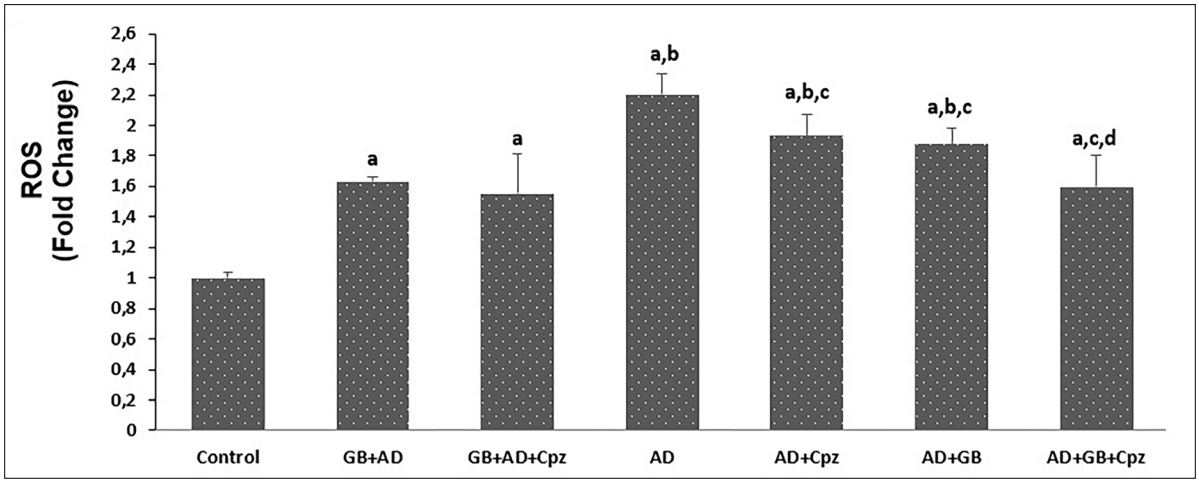

Figure 3. The effect of okadaic acid (AD, 30 nM, 24 hrs) and Ginkgo biloba extract (25 µg/ml, 24 hrs) on reactive oxygen species levels in vitro AD model. The cells were inhibited by using capsazepine (Cpz and 0.1 mM for 30 min) and stimulation was provided by using capsaicin (Cap and 0.1 mM for 10 minute) (mean ± SD and n=10). GB:Gingko Biloba, AD:Alzheimer Disease, Cpz: capsazepine

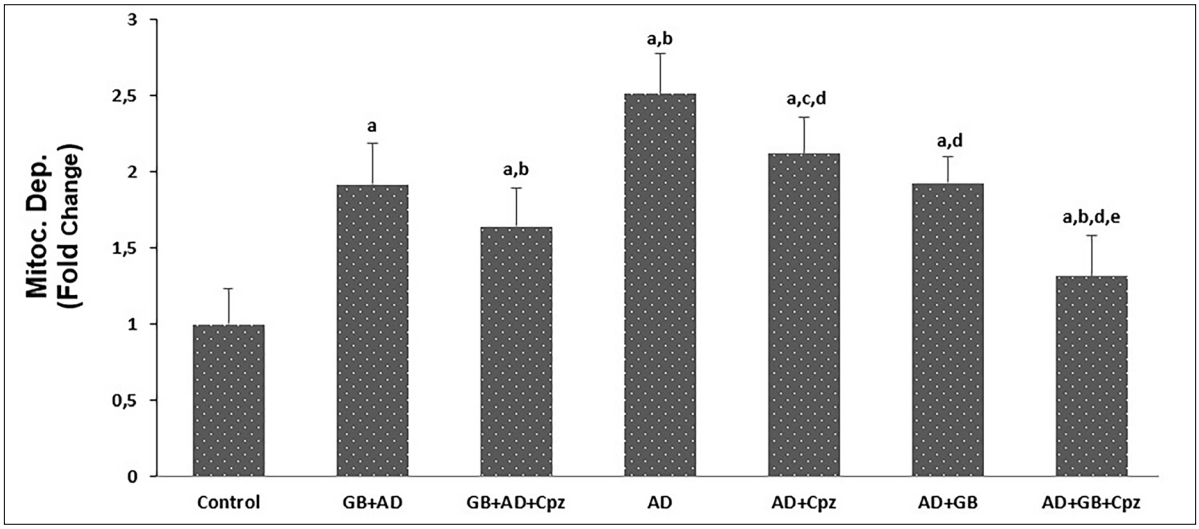

Figure 4. The effect of okadaic acid (AD, 30 nM, 24 hrs) and Ginkgo biloba extract (25 µg/ml, 24 hrs) on mitochondrial depolarization levels in vitro AD model. The cells were inhibited by capsazepine (Cpz and 0.1 mM for 30 min) and stimulated by capsaicin (Cap and 0.1 mM for 10 minute) (mean ± SD and n=10). GB:Gingko Biloba, AD:Alzheimer Disease, Cpz: capsazepine

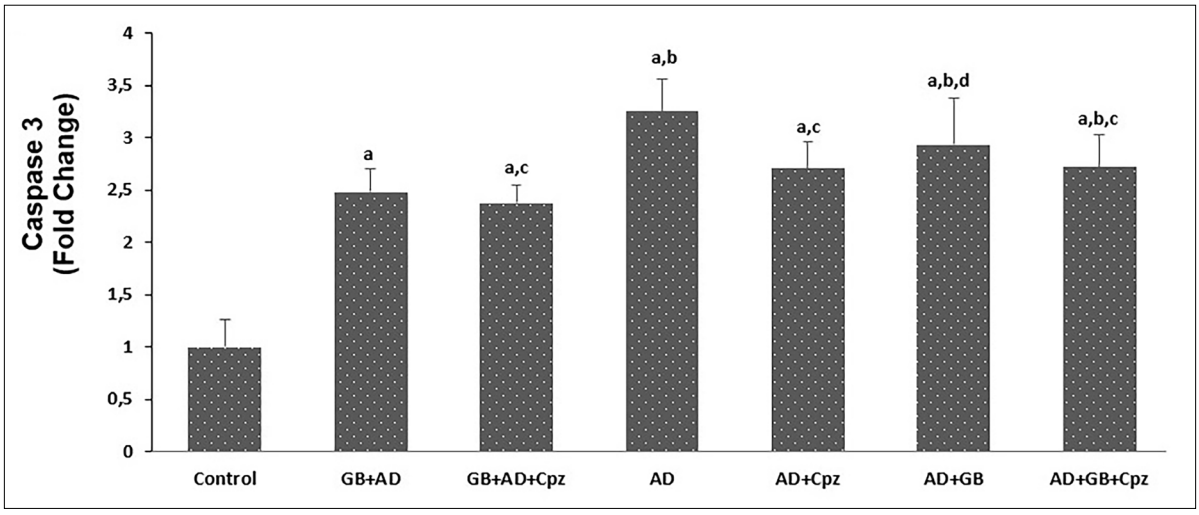

Figure 5. The effect of okadaic acid (AD, 30 nM, 24 hrs) and Ginkgo biloba extract (25 µg/ml, 24 hrs) on caspase 3 levels in vitro AD model. The cells were inhibited by capsazepine (Cpz and 0.1 mM for 30 min) and stimulated by capsaicin (Cap and 0.1 mM for 10 minute) (mean ± SD and n=10). GB:Gingko Biloba, AD:Alzheimer Disease, Cpz: capsazepine

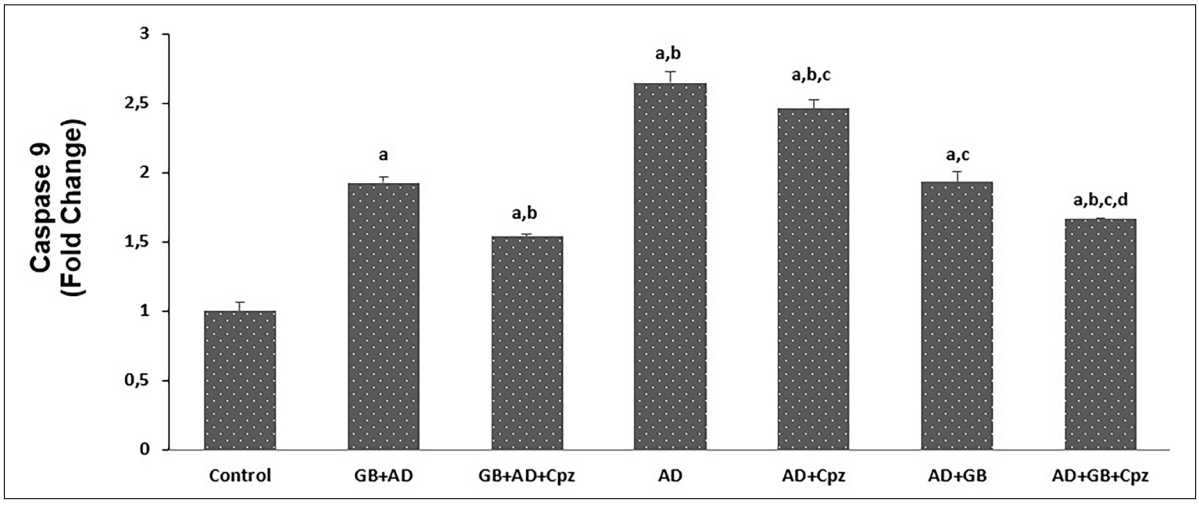

Figure 6. The effect of okadaic acid (AD, 30 nM, 24 hrs) and Ginkgo biloba extract (25 µg/ml, 24 hrs) on caspase 9 levels in vitro AD model. The cells were inhibited by capsazepine (Cpz and 0.1 mM for 30 min) and stimulated by capsaicin (Cap and 0.1 mM for 10 minute) (mean ± SD and n=10). GB:Gingko Biloba, AD:Alzheimer Disease, Cpz: capsazepine

depolarization and caspase 9 results. Also, the values were lower in the GB+AD group, AD group and AD+GB groups when compared with the GB+AD+capsazepine, AD+capsazepine and AD+GB+capsazepine groups, respectively ($p<0.001$; $p<0.05$).

DISCUSSION

Alzheimer's disease is a type of dementia where patients exhibit neurodegeneration and complete loss of cognitive skills. Neurodegenerative process mainly entails the failure of neurons to adequately regulate Ca^{2+} concentrations. In AD, the correlations between the pathological properties of AD (amyloid plaques and neurofibrillary tangles) and perturbed cellular Ca^{2+} homeostasis were determined in human, animal, and cell culture studies. Particularly, high amyloid b-peptide (Ab) levels induce neurotoxic agents such as ROS and cytokines, which inhibit cellular Ca^{2+} homeostasis and increate the vulnerability of the neurons to apoptosis and excitotoxicity (15).

The TRPV1 is the most popular mammalian TRP channel investigated in many research disciplines. In addition to its role as a nociceptive ion channel expressed in afferent sensory neurons (16), several studies have revealed that the TRPV1 is also expressed in the brain (17,18) involved in the modulation of synaptic plasticity and cognitive functions (19). Neurodegenerative diseases are characterized by increased oxidative stress with an accumulation of Ca^{2+} entry through the instability of mitochondrial membrane mediated by the TRPM2 and the TRPV1 channel activity (20). Our results confirm that GB prevents oxidative injury-related excitotoxicity via the TRPV1 in Alzheimer's modelled neuroblastoma cells.

Ca^{2+} accumulation plays a crucial role in cell survival. However, several studies suggested that this ion can also induce apoptosis in its supraphysiological levels through the mitochondria. Herein, increased Ca^{2+} concentration of mitochondria can activate the apoptotic cascade by inducing the release of several apoptotic proteins and oxidative radicals, such as cytochrome C, caspase 3 and caspase 9, and the ROS (21–23). Our study revealed that GB application significantly ameliorated the mitochondrial depolarization, caspase 3 and caspase 9 activation in vitro AD model (Figure 1–6).

In the seven groups we studied, the effects of GB on cytosolic calcium levels, apoptosis and intracellular reactive oxygen species levels, were described in an in vitro model of okadaic acid-induced AD. The Ca^{2+} concentration was higher in in Groups 1, 2, 4, and 6 when compared to the control ($p<0.001$). The Ca^{2+} level was lower in Group 2 and Group 6 than in Group 4 ($p<0.001$). Also, cytosolic Ca^{2+} concentration was lower

in Group 2 than in the Group 1 and Group 6 group ($p<0.05$) (Figure 1). Apoptosis and intracellular ROS values were higher in Groups 2, 4, and 6 when compared to the control group ($p<0.001$). Apoptosis and intracellular ROS values were higher in Group 4 when compared to Groups 2 and 6 ($p<0.001$), and apoptosis level was higher in Group 6, Group 2 ($p<0.001$) (Figure 2 and 3), and mitochondrial depolarization, caspase 3 and 9 levels were higher in Group 2, Group 4 and Group 6 compared to the control group ($p<0.001$). Also, these values were lower in the GB+AD group, AD group and AD+GB groups when compared with the GB+AD+capsazepine, AD+capsazepine and AD+GB+capsazepine groups, respectively ($p<0.001$; $p<0.05$) (Figure 4–6). This shows us that GB acts through the TRPV1 channels.

Accumulation of Ca^{2+} accumulation causes mitochondrial membrane depolarization through the swelling and rupture of mitochondrial membranes (8). In our study, we found that effects of GB on mitochondrial depolarization, caspase 3 and caspase 9 decreased after GB application (Figure 4–6).

According to Ovey et al., they showed that Hcy causes oxidative stress and apoptosis in the hippocampus of mice, and that MEM treatment indirectly activates the TRPA1, TRPM2 and TRPV1 channels in hippocampal neurons, and Hcy and Memantine play physiologically relevant roles in the regulation of the TRPA1, TRPM2 and TRPV1 channels. They found that Memantine, which is routinely used in the treatment of Alzheimer's disease, indirectly causes the TRPA1, TRPM2 and TRPV1 channel inhibition, as it reduces the amount of cytosolic calcium and reactive oxygen species and decreases NMDA channel inhibition (8). Similar findings were obtained in our study and positive effects on cellular functions, such as alleviation of oxidative stress and cell viability, were found in the in vitro AD model of GB. In addition, the cytosolic Ca^{2+} concentration in GB+AD was lower than in the AD+GB group (Figure 1 A-B), which showed us that the protective effect of GB was more than the therapeutic effect.

In experimental model of memory impairment, Balaban et al. showed that TRPM2 and TRPV1 channels played a key role in neuronal death due to Ca^{2+} entry in hippocampal and DRG (dorsal root ganglion) neurons; meanwhile, reduced channel activity associated with a significant neuroprotective effect against apoptosis and Ca^{2+} entry was described with selenium treatment.

Selenium treatment also increased memory impairment indicators (working memory error and reference memory error), reduced glutathione level and glutathione peroxidase activity in neurons. They reported that scopolamine-induced neuron death and selenium reduced memory impairment via endogenous oxidative stress pathway pathways (20,21,24). These reports were found to be compatible with the fact that GB treatment reduced apoptosis via TRPV1 in our study.

CONCLUSION

In conclusion, our study results show that GB decreases the amount of intracellular cytosolic calcium and reactive oxygen species through TRPV1 channel inhibition and reduces apoptosis in an in vitro AD model. We determined that administration of GB to cells with AH reduced cytosolic Ca^{2+} concentration, apoptosis and intracellular ROS, and that cytosolic Ca^{2+} concentration, apoptosis and intracellular ROS values were more reduced in cells with AH after GB administration. These results show us that GB has a protective effect as well as a therapeutic effect in Alzheimer's disease through the TRPV1 channel.

To the best of our knowledge, there exists no study on the role of Ginkgo biloba in vitro AD model. Hence, the role of the TRPV1 channels should be investigated in an in vitro AD model, in regard to potential modulation by Ginkgo biloba.

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