Protective effects of ginkgolide on a cellular model of Alzheimer's disease via suppression of the NF-κB signaling pathway

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Abstract

NF-κB signaling has been reported to play a key regulatory role in the pathogenesis of Alzheimer's disease (AD). The purpose of this study is to investigate the effects of ginkgolide on cell viability in an AD cellular model involving an APP/PS1 double gene-transfected HEK293 cell line (APP/PS1-HEK293) and further explored the mechanisms of action related to NF-κB signaling. The optimal time point and concentration of ginkgolide for cell proliferation were screened using a cell counting kit-8 assay. Based on the results, an in vitro study was performed by co-culture of APP/PS1-HEK293 with different dosages of ginkgolide, followed by an enzyme-linked immunosorbent assay to measure the levels of supernatant tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, as well as western blotting and real-time polymerase chain reaction to detect intracellular protein and mRNA expression of NF-κB p65, IκBa, Bcl-2 and Bax. APP/PS1-HEK293 cells exhibited the highest cell viability at a concentration of 100 μg/ml after 48 h of treatment with ginkgolide. The supernatant levels of TNF-α, IL-1β and IL-6 in the high-dosage ginkgolide-treated groups were lower than those in the control group. Compared with the control group, there were decreased intracellular protein and mRNA expression of NF-κB p65 and Bax, but increased protein and mRNA expression of IκBa in both high-dosage and low-dosage group. Ginkgolide may enhance cell viability, indicative of its neuroprotective effects on AD, at least partially via suppression of the NF-κB signaling pathway involving anti-apoptosis and anti-inflammation mechanisms. Therefore, ginkgolide might be a promising therapeutic agent against AD.

Introduction

As one of the most prevalent neurodegenerative disorders, Alzheimer's disease (AD) is characterized by irreversible cognitive impairment and memory loss, with the accumulation of amyloid-beta (Aβ) and neurofibrillary tangles, which represent typical pathological features [1]. Increasing evidence has substantiated that the Aβ-induced inflammatory response plays a crucial role in the neurodegenerative process of AD, and Aβ-mediated neuroinflammation is predominantly regulated via the nuclear factor kappa B (NF-κB) signaling pathway [2–4]. As a ubiquitously expressed transcription factor in eukaryotic cells of the nervous system, NF-κB family transcription factors function as master regulators of immune development, immune response, inflammation, cancer, and apoptosis [5, 6]. Such activities are mediated through homo-or heterodimerization of NF-*κ*B subunits RelA/p65, RelB, c-Rel, p50, and p52, of which RelA/p65 is the most abundant subunit [7]. Proteins of the inhibitory *κ*B (I*κ*B) family function as inhibitors and regulators of NF-*κ*B activity. Members of the I*κ*B family include the classical I*κ*B proteins (I*κ*Bα, I*κ*Bβ, and IκBε), NF-*κ*B precursor proteins (p100 and p105), and nuclear IκBs (I*κ*Bζ, Bcl-3, and I*κ*BNS). Upon stimulation of innate immunity receptors, such as Toll-like receptors and the cytokine receptor tumor necrosis factor receptor superfamily, a series of membrane-proximal events cause the activation of IκB kinase (IKK). Phosphorylation of I*κ*Bs contributes to their proteasomal degradation, the release of NF-*κ*B for nuclear translocation and activation of gene transcription, consequently leading to inflammation and immune response [8, 9]. Recent studies have confirmed that NF-*κ*B signaling has a key

regulatory role in the pathogenesis of AD, and hence, it has been considered a compelling target for therapeutic intervention [10, 11].

Currently, both cholinesterase inhibitors (donepezil, carbazatin, and galanthamine) and N-methyl-D-aspartate receptor antagonists (memantine) are recommended to treat AD. However, these medications are not ideal because their therapeutic effects are accompanied by adverse effects. Although numerous new agents for the treatment of AD have been developed, such as aducanumab and crenezumab [12], they have not been fully implemented in clinical practice because of a lack of definitive therapeutic effects. GV-971, a marine algae-derived oral oligosaccharide, has been approved for clinical use, but its efficacy remains disputable [13]. In the past decade, botanical preparations with multi-target treatment and high-level safety have become a new trend in the research and development of therapeutic drugs for AD. Various *in vivo* and *in vitro* studies have reported the interventional effects of botanicals, such as resveratrol, *Rhodiola sachalinensis*, curcumin, and natural polyphenols on AD, suggesting their therapeutic potential for the prevention and treatment of AD [14–17].

As a botanical agent, Ginkgo biloba (GB) extract has been widely used to treat cerebrovascular diseases because of its multiple biological and pharmacological activities, such as antioxidative, anti-inflammatory, and anti-allergic effects as well as free radical scavenging and platelet aggregation suppression [18, 19]. Interestingly, several recent studies have revealed that GB extracts also exhibited certain therapeutic effects on dementia. Given these findings, GB extract has been recommended for the treatment of AD patients, especially for those who have failed to benefit from other treatments [20]. Currently, the international standard extract of GB is EGb 761, produced according to the German Schwabe patent process. EGb 761 is a well-defined plant extract product of Ginkgo biloba leaves. The extract contain two main active substances: flavonoid glycosides (24–26%) and terpene lactones (6–8%) consisting of ginkgolides A, B, C, and bilobalide [21]. Furthermore, based on technological advances, a new product of ginkgolide (Baiyu®), which is composed of ginkgolide ABCJ and bilobalide, has been recently developed and approved for the treatment of ischemic cerebrovascular disease; however, its therapeutic efficacy in the context of AD remains unclear. Hence, we performed a preliminary study to investigate the effects of ginkgolide (Baiyu®) and its components (ginkgolide B and bilobalide) on cell viability in an AD cellular model involving an APP/PS1 double gene-transfected HEK293 cell line (APP/PS1-HEK293) and further explored the related mechanisms of action.

Materials And Methods

Reagents

Ginkgolide (Baiyu®) (Catalog Number: Z20110035), bilobalide (Catalog Number: 20190730), and ginkgolide B (Catalog Number: 20170324) were kindly provided by Baiyu Pharmaceutical Co. Ltd (Chengdu, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). The cell counting kit-8 (CCK-8) was purchased from Tongren Company (Japan). Human tumor necrosis factor (TNF)-α enzyme-linked immunosorbent assay (ELISA)

kit, human interleukin (IL)-6 ELISA kit, and human IL-1β ELISA kit were purchased from eBioscience (San Diego, CA, USA). RIPA total protein extraction kit, phenylmethylsulfonyl fluoride, and bicinchoninic acid (BCA) protein assay kits were purchased from Sigma (St. Louis, MO, USA). Goat anti-rabbit IgG (H + L) HRP and goat anti-rabbit IgG (H + L) HRP were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was purchased from Ruierkang Biotech Co. Ltd. (Tianjin, China). Rabbit anti-I*κ*Ba antibody, anti-NF-κB p65 antibody, anti-Bcl-2 antibody, and anti-Bax antibody were purchased from Abcam (Cambridge, UK). TRIzol reagent was obtained from Tiangen Biotech Co.,Ltd. (Beijing, China). PrimeScript™ room temperature (RT) reagent kit with gDNA Eraser, SYBR® Premix Ex Taq™ II, ROX plus, and DL2000 DNA marker were purchased from TaKaRa Baoshengwu (China). Electrochemiluminescence (ECL) was obtained from Pierce (Wisconsin, USA).

Cell culture

APP/PS1-HEK293 cells were purchased from Hanbio (Shanghai, China). Cells were stimulated with ginkgolide (Baiyu®) in the absence or presence of different concentrations (100, 200, 300 and 400 μg/ml), and detection was carried out at 0, 24, 48, and 72 h posttreatment. Briefly, screening via CCK-8 assay was performed to ascertain the optimal time point and concentration for cell proliferation as follows: (1) After removing the DMEM, 100 μL CCK-8 solution per well was added into 96-well cell culture plates at a concentration of 10%, and the plates were incubated for 2 h in a 37°C incubator; (2) The optical density of each well was then determined using a fully automatic multifunctional microplate reader (wavelength: 450 nm). The drug concentration range was further reduced to 25, 50, 75, 100, 125 and 150 μg/ml for observation to obtain additional confirmation of the optimal concentration. Based on these experiments, an *in vitro* study was carried out using untreated APP/PS1-HEK293 as the control group, while APP/PS1-HEK293 cells were treated with high dosage (100 μg/ml) and low dosage (50 μg/ml) ginkgolide, as well as its components ginkgolide B (100 μg/ml) and bilobalide (100 μg/ml) formed the interventional groups. After 48 h of co-culture, cells were harvested on the third day for further analysis.

ELISA

Supernatant levels of TNF-α, IL-1β and IL-6 were measured using an ELISA kit according to the manufacturer's instructions. All assays were performed in a blinded manner.

Western blotting

The cells from the control and interventional groups were disposed in the culture dish. Total protein was extracted by cell lysis and measured using a BCA assay kit (Sigma, St. Louis, USA) and stored at -80°C until use. Briefly, 13 μg of total protein/well was separated on a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane (Amersham, UK). The membrane was blocked for 1 h using 3% BSA, TBS-Tween-20 (TTBS), 0.2% azide at pH 7.4, washed three times by TTBS containing 50 mM Tris, 0.5 M NaCl, 0.05% Tween 20 at pH 7.4. It was then hybridized with rabbit anti-I*k*Ba antibody, anti-NF-*κ*B p65 antibody,

anti-Bcl-2 antibody, or anti-Bax antibody, as well as anti-GAPDH antibody (1:10000 dilution) as an internal control overnight at 4℃, followed by incubation with HRP-conjugated goat anti-rabbit IgG (1:20000 dilution) for 30 min at 25℃. After washing six times with TTBS, 3 ml ECL was added to the membrane for 3–5 min at 25℃. The membrane was exposed for 10 s to 5 min, and the integrated density value was calculated as above. The gray value of each band was analyzed using image software G, and a semi-quantitative analysis of protein expression in each group was carried out using an independent normalization method.

Quantitative reverse transcription-polymerase chain reaction (PCR)

Total ribonucleic acid (RNA) was extracted from APP/PS1-HEK293 cells in each group using TRIzol reagent. Following the manufacturer's instructions, 2 μl of RNA was reverse transcribed into cDNA using a PrimeScript™ RT reagent kit with a gDNA Eraser. Primers were synthesized by Invitrogen, USA (Table 1), and real-time PCR was performed in an ABI 7500Fast Sequence Detector. All amplifications were carried out in triplicate for each sample. Amplifications were performed at 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 40 s. The 2−ΔΔCT (comparative threshold cycle or CT) method was then applied to calculate the mRNA expression levels of each gene, as described by the manufacturer (Technical Bulletin 2; Applied Biosystems).

Table 1
Primers used in this study.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
NF-κB p65	CTGCAGTTTGATGATGAAGA	TAGGCGAGTTATAGCCTCAG
IκBa	TGGTGTCCTTGGGTGCTG	GCTGTATCCGGGTGCTTG
bcl-2	GCCTTCTTTGAGTTCGGTGGG	GCCGGTTCAGGTACTCAGTCATC
Bax	GACGAACTGGACAGTAACATGGAGCT	CGGCCCCAGTTGAAGTTGC
β-actin	ACTTAGTTGCGTTACACCCTT	GTCACCTTCACCGTTCCA

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). All data are presented as the mean ± standard deviation (proliferative activities by CCK-8 assay; supernatant TNF-α, IL-1β and IL-6; NF-κB p65, IκBa, Bcl-2 and Bax mRNA). Data were analyzed by one-way analysis of variance (ANOVA) with the Student-Newman-Keuls post hoc test. Statistical significance was set at $P < 0.05$.

Results

Effects of ginkgolide on cell proliferation

The proliferative activities associated with each dosage group (100 μg/ml, 200 μg/ml, 300 μg/ml and 400 μg/ml) demonstrated a downward-upward-downward trend after 24, 48, and 72 h of treatment with ginkgolide and cell viability at 48 h posttreatment was significantly higher than that at 0 h and 24 h posttreatment ($P < 0.01$). Furthermore, at 48 h posttreatment, the cell viability at different dosages was significantly increased compared to the control group, particularly at a concentration of 100 μg/ml (Table 2 and Fig. 1). Based on the above findings, 48 h was selected as the best time point for promoting cell proliferation, and the drug dose range (25, 50, 75, 100, 125 and 150 μg/ml) was further narrowed down to determine the optimal dosage. As a result, the different dosage groups exhibited a marked increase in cell viability compared to the control group ($P < 0.01$, $P < 0.05$), especially at the concentration of 100 μg/ml (Table 3 and Fig. 2). Finally, 100 μg/ml and 48 h posttreatment with ginkgolide were chosen as the optimal concentration and time point for cell proliferation.

Table 2
Effects of different doses of ginkgolide on proliferative activities at different time points.

	0 h (%)	24 h (%)	48 h (%)	72 h (%)
Control group (n = 3)	100 ± 2.74	91.55 ± 3.84	114.88 ± 1.44**	109.55 ± 3.70
100 μg/ml (n = 3)	100 ± 2.86	94.09 ± 2.35	131.06 ± 1.97**##	123.14 ± 4.27
200 μg/ml (n = 3)	100 ± 1.13	89.70 ± 0.81	120.65 ± 2.07**	115.42 ± 1.62
300 μg/ml (n = 3)	100 ± 0.83	95.41 ± 2.64	124.92 ± 4.03**	116.43 ± 1.74
400 μg/ml (n = 3)	100 ± 0.92	93.56 ± 0.96	120.60 ± 3.00**	115.59 ± 1.47
**$P < 0.01$, compared with cell viability at 0 h and 24 h post-treatment indicated by post hoc test.				
##$P < 0.01$, compared with control group at 48 h post-treatment indicated by post hoc test.				

Table 3
Effects of different doses of ginkgolide on cell proliferative activities at 48 h post-treatment.

	Cell viability (%)	*P value*
Control group (n = 3)	107.41 ± 3.79**	< 0.0001
25 μg/ml (n = 3)	125.10 ± 0.60*	0.0114
50 μg/ml (n = 3)	125.85 ± 3.99*	0.0194
75 μg/ml (n = 3)	119.55 ± 2.52**	0.0003
100 μg/ml (n = 3)	135.06 ± 2.45	-
125 μg/ml (n = 3)	124.37 ± 2.76**	0.0068
150 μg/ml (n = 3)	122.21 ± 5.08**	0.0015
***P*< 0.01 or **P*< 0.05, compared with ginkgolide (100 μg/ml) indicated by post hoc test.		

Supernatant TNF-α, IL-1β and IL-6 levels

The supernatant levels of TNF-α, IL-1β and IL-6 in the high-dosage (100 μg/ml) ginkgolide-, ginkgolide B- and bilobalide-treated groups were lower than those in the control group (P < 0.01), but higher in the low dosage (50 μg/ml) ginkgolide-treated group (P < 0.01) (Table 4).

Table 4
Effects of different doses of ginkgolide and its components (ginkgolide B and bilobalide) on supernatant levels of TNF-α, IL-1β and IL-6.

	TNF-α (pg/ml)	IL-1β (pg/ml)	IL-6 (pg/ml)
Control group (n = 3)	67.78 ± 2.05**	18.31 ± 0.24**	172.75 ± 4.42**
Low-dosage ginkgolide (n = 3)	100.54 ± 3.82**	20.89 ± 0.06**	195.77 ± 2.71**
High-dosage ginkgolide (n = 3)	57.81 ± 3.74**	12.45 ± 0.19**	118.43 ± 2.08**
Ginkgolide B (n = 3)	41.37 ± 2.93**	8.29 ± 0.16**	105.92 ± 0.32**
Bilobalide (n = 3)	48.42 ± 1.68**	10.48 ± 0.16**	111.96 ± 0.67**
***P*< 0.01, compared with control group indicated by post hoc test.			

Intracellular protein expression levels of NF-κB p65, IκBa, Bcl-2 and Bax

Compared to the control group (Fig. 2), there was a decrease in the intracellular protein expression levels of NF-*κ*B p65 and Bax (P < 0.01 and P < 0.05), but an increase in the protein expression levels of I*κ*Ba and

Bcl-2 (P < 0.01) in both high-and low-dosage ginkgolide-, ginkgolide B- and bilobalide-treated groups (Fig. 3).

Intracellular mRNA expression levels of NF-κB p65, IκBa, Bcl-2 and Bax

Compared with the control group, there was a decrease in the intracellular mRNA expression levels of NF-*κ*B p65 and Bax (P < 0.01, P < 0.05), but an increase in the mRNA expression levels of I*κ*Ba (P < 0.01 and P < 0.05) in both high-and low-dosage ginkgolide-, ginkgolide B- and bilobalide-treated groups, as well as an increase in the mRNA expression levels of Bcl-2 in the high- and low-dosage ginkgolide-treated groups (P < 0.05) (Table 5).

Table 5
Effects of different doses of ginkgolide and its components (ginkgolide B and bilobalide) on the intracellular expression of NF-*κ*B p65, I*κ*Ba, bcl-2 and Bax mRNA.

	NF-*κ*B p65	**I*κ*Ba**	**bcl-2**	**Bax**
Control group (n = 3)	1.008 ± 0.153	1.064 ± 0.053	1.002 ± 0.074	1.002 ± 0.756
Low-dosage ginkgolide (n = 3)	0.328 ± 0.373*	1.558 ± 0.005*	11.264 ± 7.626*	0.339 ± 0.288**
High-dosage Ginkgolide (n = 3)	0.302 ± 0.252*	1.721 ± 0.060**	16.110 ± 10.870*	0.280 ± 0.254**
Ginkgolide B (n = 3)	0.207 ± 0.195**	1.603 ± 0.306*	1.664 ± 0.442	0.260 ± 0.086**
Bilobalide (n = 3)	0.092 ± 0.061**	1.701 ± 0.306**	2.168 ± 1.290	0.351 ± 0.189**
***P*< 0.01 or **P*< 0.05, compared with control group indicated by post hoc test.				

Discussion

In our study, APP/PS1-HEK293 cells showed markedly increased proliferative activity at 48 h post-treatment with ginkgolide, as well as lower and higher expression of NF-*κ*B p65 and IκBa, respectively, indicating that ginkgolide could improve cell viability by suppressing the activation of intracellular NF-*κ*B signaling. Furthermore, component analysis of ginkgolide revealed that ginkgolide B downregulated the production of NF-*κ*B p65, while bilobalide upregulated the expression of IκBα, suggesting that these two components might exert distinctive regulatory effects on the NF-*κ*B signaling pathway. Nevertheless, future research is required to determine their exact roles.

Increasing evidence has substantiated the crucial role of the Aβ-induced inflammatory response in the neurodegenerative process of AD. Once microglia are activated by Aβ, there is a resultant secretion of proinflammatory cytokines, such as IL-6, IL-1β, and TNF-α [22], and IL-1β reduces the number of synaptic

connections, resulting in synaptic degeneration and neuronal loss [23]. Aβ also induces neurotoxicity and the sustained release of neurotoxic factors leading to neurodegeneration, many of which are deemed to be microglia-derived, including TNF-α, nitrogen oxide (NO), IL-1β, and reactive oxygen species, which consequently accelerate the development of AD [22]. Several studies have confirmed that ginkgolide reduces the production of TNF-α, IL-1β and IL-6 by suppressing oxidative stress, mitochondrial dysfunction, and endothelial dysfunction [24–26]. In keeping with these findings, our study demonstrated that high-dose ginkgolide significantly downregulated the production of TNF-α, IL-1β and IL-6, in contrast to low-dose ginkgolide, suggesting that a dose-dependent pattern might exist in terms of the regulatory effects of ginkgolide on inflammation. Furthermore, the component analysis showed that both ginkgolide B and bilobalide lowered the production of the aforementioned inflammatory cytokines to the same extent, suggesting the existence of their synergistic anti-inflammatory effects of these cytokines.

Aβ accumulation can accelerate neuronal apoptosis, although the exact molecular mechanism remains uncertain. It is well known that Aβ-induced neuronal apoptosis causing neuronal loss plays a critical role in the development of AD [27, 28]. Intriguingly, a recent study showed that Aβ1–42 could lead to cerebral vascular damage by prompting oxidative stress, inducing mitochondrial dysfunction and apoptosis of cerebral endothelial cells in a mouse model of AD [29], suggesting that the inhibition of apoptosis is beneficial for halting the progression of AD. Notably, Xiao et al. recently reported that Aβ25-35-induced apoptosis was attenuated by ginkgolide B via the upregulation of brain-derived neurotrophic factors when the cells were subjected to Aβ25–35 insult [30]. In line with this finding, our study showed that ginkgolides downregulated the expression of the apoptosis protein Bax and upregulated the expression of the anti-apoptotic protein Bcl-2, which once again validated the anti-apoptotic effects of ginkgolide in an AD cell model. In parallel with ginkgolide, ginkgolide B and bilobalide decreased the expression of Bax and increased the expression of Bcl-2 to some extent, suggesting that they may exert anti-apoptotic effects through synergistic action against apoptosis.

In conclusion, we found that ginkgolide significantly enhanced the viability of APP/PS1-HEK293 cells, strongly indicative of its neuroprotective effects on AD, at least partially via suppression of the NF-κB signaling pathway involving anti-apoptosis and anti-inflammation mechanisms. However, this issue needs to be further addressed by *in vivo* studies on animal models and, more importantly, human clinical trials. Nevertheless, our findings shed light on novel treatment options for AD and ginkgolide might be a promising therapeutic agent against this disease.

Declarations

Ethics approval and consent to participate

Yes

Consent for publication

Yes

Authors' contributions

In this study, T.T. N. and G.Z. L. wrote this manuscript; Y. H. and G.Z. L. designed the research; H. Y. performed the research; H.Y. analyzed the data; H.Q. L, Y.S. and T.T. Y. contributed new reagents and /analytical tools.

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Competing interests

The authors report no competing interest.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

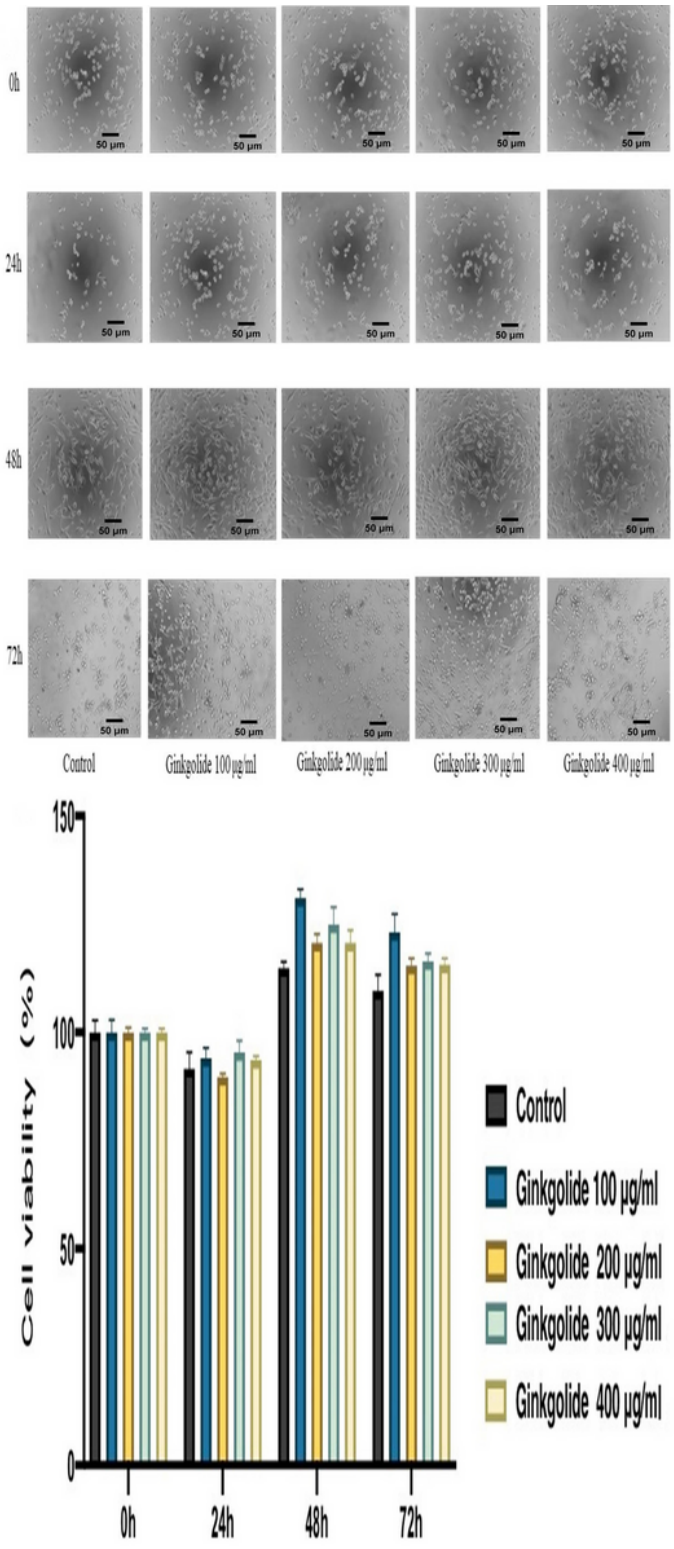

Figure 1

Effects of different dosages of ginkgolide on cell proliferative activity at different time points. Cell viability was measured by a cell counting kit-8 (CCK-8) assay. APP/PS1-HEK-293 cells were treated with different dosages of ginkgolide (0, 100, 200, 300 and 400 μg/ml) for 0, 24, 48 and 72 h, respectively, and then the cells were observed with an inverted microscope (100×).

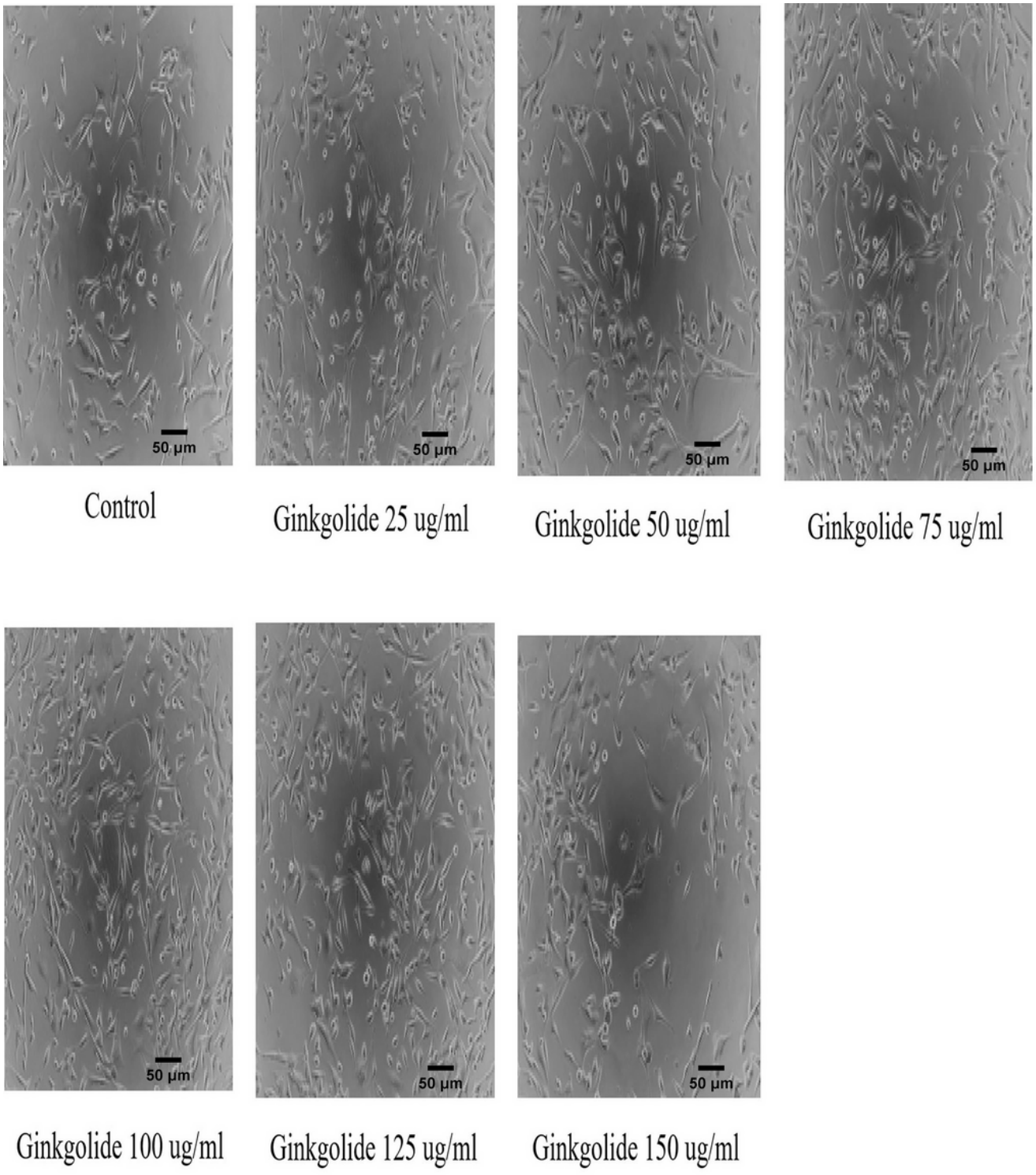

Figure 2

Effects of different doseges of ginkgolide on cell proliferative activity at 48 h posttreatment. APP/PS1-HEK-293 cells were treated with different dosages of ginkgolide (25, 50, 75, 100, 125 and 150 μg/ml) for 48 h, and then the cells were observed with an inverted microscope (100×).

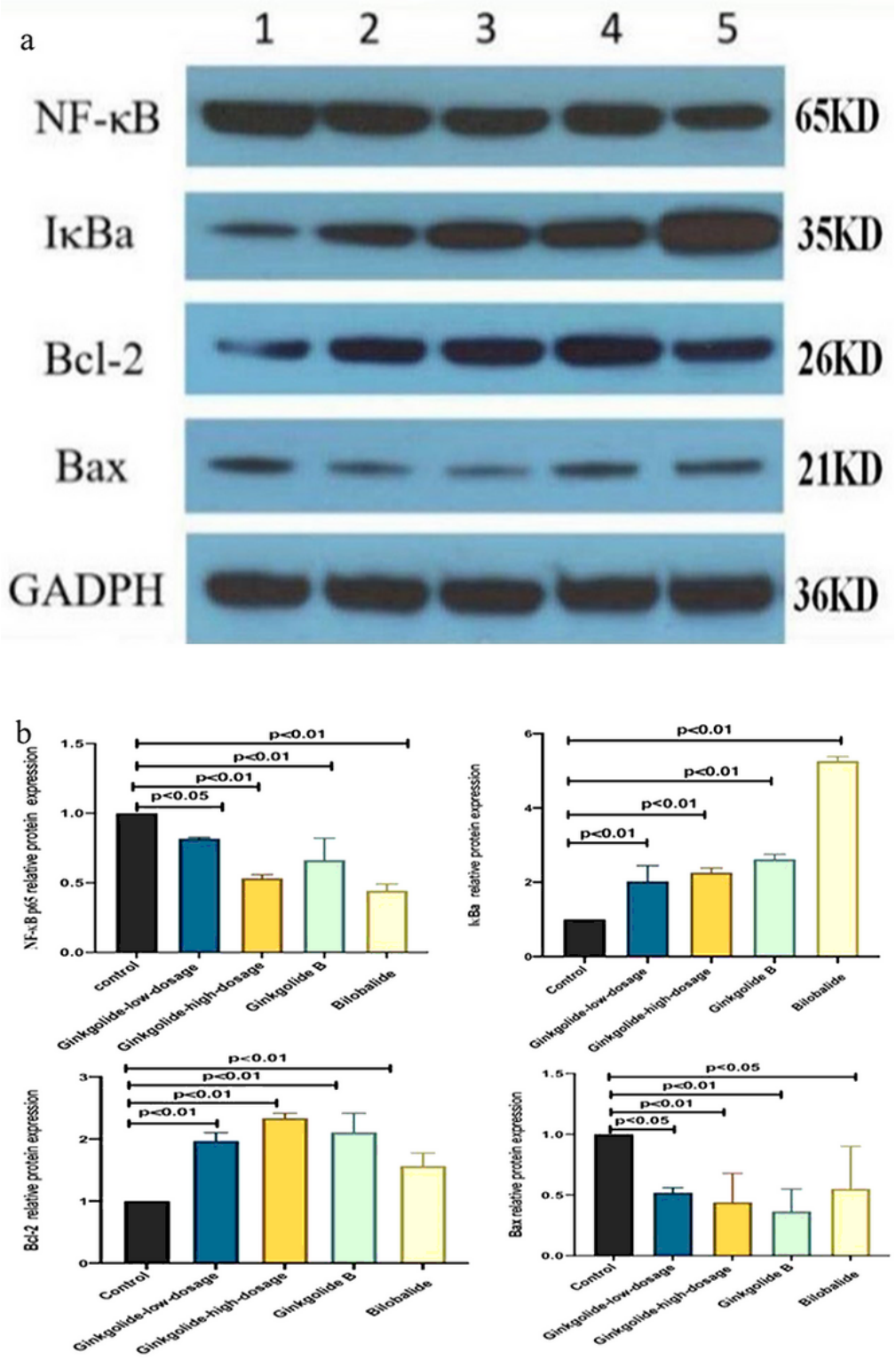

Figure 3

Detection of intracellular protein expression of NF-κB p65, IκBa, Bcl-2 and Bax by Western blotting. (a) Western blotting. Lane 1, control group; lane 2, low-dosage ginkgolide; lane 3, high dosage ginkgolide; lane 4, ginkgolide B; lane 5, bilobalide; (b) Effects of different doses of ginkgolide and its components (ginkgolide B and bilobalide) on intracellular protein expression of NF-κB p65, IκBa, Bcl-2 and Bax.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1NFkBPVDF.tif
- Supplementaryfile2IkBaPVDF.tif
- Supplementaryfile3BaxPVDF.tif
- Supplementaryfile4Bcl2PVDF.tif
- Graphicalabstract.png