Drug Design, Development and Therapy

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ORIGINAL RESEARCH

RETRACTED ARTICLE: Coffee and caffeine potentiate the antiamyloidogenic activity of melatonin via inhibition of Aβ oligomerization and modulation of the Tau-mediated pathway in N2a/APP cells

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Abstract: There is an increasing prevalence of Alzheimer's disease (AD), which has become a public health issue. However, the underlying mechanisms for the pathogenesis of AD are not fully understood, and the current therapeutic drugs cannot produce acceptable efficacy in AD patients. Previous animal studies have shown that coffee (Coff), caffeine (Caff), and melatonin (Mel) have beneficial effects on AD. Disturbed circadian rhythms are observed in AD, and chronotherapy has shown promising effects on AD. In this study, we examined whether a combination of Coff or Caff plus Mel produced a synergistic/additive effect on amyloid-β (Aβ) generation in Neuro-2a (N2a)/amyloid precursor protein (APP) cells and the possible mechanisms involved. Cells were treated with Coff or Caff, with or without combined Mel, with three different chronological regimens. In regimen 1, cells were treated with Coff or Caff for 12 hours in the day, followed by Mel for 12 hours in the night. For regimen 2, cells were treated with Coff or Caff plus Mel for 24 hours, from 7 am to 7 am the next day. In regimen 3, cells were treated with Coff or Caff plus Mel with regimen 1 or 2 for 5 consecutive days. The extracellular Aβ40/42 and Aβ oligomer levels were determined using enzyme-linked immunosorbent assay (ELISA) kits. The expression and/or phosphorylation levels of glycogen synthase kinase 3β (GSK3β), Erk1/2, PI3K, Akt, Tau, Wnt3α, β-catenin, and Nrf2 were detected by Western blot assay. The results showed that regimen 1 produced an additive antiamyloidogenic effect with significantly reduced extracellular levels of Aβ40/42 and Aβ42 oligomers. Regimen 2 did not result in remarkable effects, and regimen 3 showed a less antiamyloidogenic effect compared to regimen 1. Coff or Caff, plus Mel reduced oxidative stress in N2a/APP cells via the Nrf2 pathway. Coff or Caff, plus Mel inhibited GSK3β, Akt, PI3K p55, and Tau phosphorylation but enhanced PI3K p85 and Erk1/2 phosphorylation in N2a/APP cells. Coff or Caff, plus Mel downregulated Wnt3α expression but upregulated β-catenin. However, Coff or Caff plus Mel did not significantly alter the production of T helper cell (Th)1-related interleukin (IL)-12 and interferon (IFN)-γ and Th2-related IL-4 and IL-10 in N2a/APP cells. The autophagy of cells was not affected by the combinations. Taken together, combination of Caff or Coff, before treatment with Mel elicits an additive antiamyloidogenic effects in N2a/APP cells, probably through inhibition of Aβ oligomerization and modulation of the Akt/GSK3β/Tau signaling pathway.

Keywords: Alzheimer's disease, Aβ oligomer, coffee, caffeine, melatonin, Tau, Nrf2, chronotherapy, Akt

Introduction

Alzheimer's disease (AD) is a primary type of dementia (60%–80%) that is characterized by progressive loss of memory and cognition, brain atrophy, and accumulation of

amyloid plaques and neurofibrillary tangles in the cortex, ultimately leading to complete debilitation and death.[1,2] Typical clinical symptoms of AD include progressive short-term memory loss, impaired linguistic function, emotional dysfunction, impaired cognition, and dementia. AD can be further subdivided into early-onset (<65 years old, familial) and late-onset (>65 years old, sporadic) groups. Patients with either sporadic or familial AD share common clinical and neuropathological features.[3,4] The Delphi study estimated that there were 24.3 million people with dementia in the world in 2001 and predicted that this would increase to 42.3 million in 2020 and 81.1 million by 2040.[5] The countries or regions with the largest number of affected individuals are the People's Republic of China and the developing Western Pacific, Western Europe, and the USA. There were about 25 million people with AD in 2010, and now nearly 36 million people have AD or a related dementia, worldwide.[4,6] It is estimated that the number of AD patients will double every 5 years, and the number is expected to be 65.7 million in 2030 and 115.4 million worldwide in 2050 if we cannot find a cure. A recent meta-analysis based on 89 published reports has estimated that the number of people with AD increased from 1.9 million in 1990 to 5.7 million in 2010 in the People's Republic of China.[7] In the USA, an estimated 5.2 million Americans have AD in 2014 (ie, one in nine Americans over 65 has AD) and ~500,000 people die from AD each year, which makes AD the sixth leading cause of death.[6,8,9] By 2050, it is expected that more than 13.8 million Americans will have AD. AD represents a major public health concern, and its increasing prevalence gives rise to a heavy burden to family and society.[1,6,10] For example, the direct costs to American society of caring for those with AD will total an estimated $214 billion, including $150 billion in costs to Medicare ($11 billion) and Medicaid ($37 billion) in 2014.[6] The global cost of AD and dementia is estimated to be $605 billion, which is equivalent to 1.0% of the entire world's gross domestic product.[12]

The etiology of AD is not fully deciphered, and its pathogenesis is complicated. The most important risk factor for AD is age, and other AD risk factors include hypertension, stroke, heart diseases, depression, arthritis, type II diabetes, estrogen supplements, and smoking.[1,2] There is a line of evidence that amyloid-β (Aβ) and the Tau protein play a key role in the development and progression of AD.[1,13–17] Aβ peptides are proteolytic fragments of the transmembrane amyloid precursor protein (APP), whereas Tau is a brain-specific, axon-enriched microtubule-associated protein. APP is primarily processed by the aspartyl proteases β- and γ-secretase, and the β-secretase 1 (known as β-site APP cleaving enzyme 1 [BACE1], also named memapsin and Asp2) that is predominantly expressed in the brain as an integral membrane protein, with its carboxyl terminal end associated with the membrane in endosomes.[18–20] In the nonamyloidogenic pathway, the α-secretase (ie, a disintegrin and metalloproteinase domain protein 10 [ADAM10]) prevents formation of toxic Aβ peptides from APP and alternatively catalyzes the formation of neuroprotective and neurotrophic soluble fragments (APPs-α) and membrane-bound fragment, C83.[18–20] C83 is subsequently cleaved by the γ-secretase complex to yield the 3 kD fragment, P3, and an APP intracellular domain. Accumulation of Aβ leads to plaque development, and hyperphosphorylation and aggregation of Tau protein, resulting in the formation of tangles.[1,11–15] The behavioral symptoms of AD correlate with the aggregation of Aβ and phosphorylation level of Tau.[17] Moreover, Aβ has been considered to be the upstream regulator of Tau in AD pathogenesis that triggers the conversion of Tau from a normal to a toxic state. On the other hand, it was shown that Tau enhanced Aβ toxicity via a feedback loop.[1,11–15] Given the critical role of Aβ and Tau in the pathogenesis of AD, both Aβ- and Tau-associated pathways are the central targets for the development of new therapies aimed to ameliorate the development and progression of AD.[15,21–23] To date, there is no cure for AD. Currently available therapeutic drugs for AD therapy approved by the US Food and Drug Administration include donepezil, galantamine, memantine, rivastigmine, and tacrine. Only memantine is an N-methyl-D-aspartate receptor antagonist, and the others are all cholinesterase inhibitors. These drugs are effective for about 6 to 12 months for about half of the patients who take them. However, these drugs have intrinsic limitations, including poor efficacy for chronic use and side effects.[15,21–23] Therefore, there is an urgent need to develop novel therapeutic agents for the treatment of AD with improved outcomes and reduced side effects.

It has been recognized that coffee (Coff), caffeine (Caff) (1,3,7-trimethylxanthine or 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione) (Figure 1), and melatonin (Mel) (N-acetyl-5-methoxytryptamine) possess a variety of pharmacological activities, including antioxidative, antiapoptotic, antiautophagic, and neuroprotective effects.[24] Caff is the most widely consumed psychoactive substance and acts as an antagonist of adenosine A_1 receptors (A_1Rs) and A_{2A} receptors (A_{2A}Rs) at nontoxic doses.[25] At high concentrations, Caff, like theophylline, acts as a nonselective phosphodiesterase inhibitor, thereby leading to higher levels of the intracellular second messenger cyclic adenosine monophosphate. Mel is synthesized mainly by the pineal gland during the dark

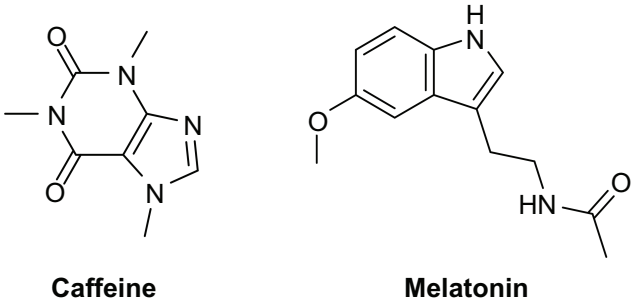


Figure 1 Chemical structures of caffeine and melatonin.

phase of the circadian cycle and exhibits a number of physiological activities, including regulating circadian rhythms, inhibiting the oxidation of biomolecules and clearing free radicals, regulating intestinal motility and metabolism, and enhancing immunity.[26,27] These effects are considered to be mediated by two specific Mel receptor subtypes, namely MT_1 and MT_2, which belong to G-protein-coupled receptors.[28] MT_1 receptors modulate neuronal firing, arterial contraction, and reproductive and metabolic functions, while MT_2 receptors regulate circadian rhythms of neuronal firing in the suprachiasmatic nucleus, induce vasodilation, and enhance immune responses.[27,28] Mel-mediated responses elicited by activation of MT_1 and MT_2 receptors depend on circ... time, duration, and mode of exposure to endogenou... or exogenous Mel, and on functional recept... sensitiv... Studies show that Mel levels are signific...tly lo...er in A... patients compared with those in age-...tched c...trol sub... jects, and therefore, it is thought th... a Me... licit is closely associated with aging and age-...ted disease... such as AD and Parkinson's disease.[29] Anim... studies ha...e shown that Coff, Caff, and Me... can prevent a...-related cognitive impairment, delay t... progre...sion of AD, and ameliorate AD symptoms.[31–3... Our pr...ious studies also demonstrated that Mel im...ved m...chondri... function and directly bound to Aβ to i...bit A... gregation in AD mice,[35] and that th... was a ...elation between Caff/Coff intake and the risk o... ...ementia or delayed onset.[32,36,37] We have found that mild cogn...ive impairment patients with higher plasma Caff levels had delayed onset or lower risk of dementia during a 2–4 year follow-up period.[37] Both Caff and Mel act as blockers of BACE1 and γ-secretase and thus, reduce Aβ production.[37–39]

Circadian rhythms are endogenously generated rhythms, with a periodicity of approximately 24 hours, that are driven by an endogenous circadian timekeeping system located in the suprachiasmatic nucleus of the hypothalamus.[40–42] Circadian rhythms are generated as an output of the clock gene cycle, produced by a series of interlocking transcriptional feedback/feed forward loops of a series of clock genes (eg, *PER1/2*, *CRY1.2*, *CLOCK,* and *BMAL1*). Such cycles drive the rhythmic expression of clock-controlled genes, and ultimately, such molecular cycles are translated into physiological and behavioral circadian rhythms. Circadian rhythms have regulatory effects on cell proliferation, cell metabolism, cell senescence, and cell death, involving a number of functional proteins, such as cyclin D1 and sirtuin 1.[25,26] Circadian dysfunction also impacts negatively on immune, metabolic, and cardiovascular systems.[43,44] Not...bly, increasing evidence indicates that circadian rhythms ...lay an im...rtant role in the pathogenesis of many ailme... including ...urodegenerative diseases.[41,45,46] It ha... been rec...nized th... the circadian rhythms have a stron... association wi... ...h... macotherapeutic effects, so called "...ronot...erapy".[43,44,47] This refers to the administratio... of drug... at a cert... time of day, resulting in the hi... efficacy an... th... lowest side-effects, which profoundly influ...ces the therapeutic outcomes in clinical set...g...[41] With circ...dian rhythms significantly disturbed in ...D, they may have a direct link to the pathogenesis of AD, ...nd accumul...ing evidence shows that chronotherapeutic a...roaches ...ay generate benefits in the treatment of AD.[46] Thus, ...e studies are needed to elucidate the role of circa... rhythms in the pathogenesis and therapy of AD.

To date, the effects of the combination of Coff or Caff and Mel on AD remain unclear, and the underlying mechanism for the beneficial effects of Coff, Caff, and Mel on AD is not fully understood. It has been suggested that reactive oxygen species (ROS), inflammation, and autophagy play a casual role in the development and progression of AD, with the involvement of glycogen synthase kinase 3β (GSK3β), Tau, and Wnt3α/β-catenin, resulting in Aβ accumulation and neuronal death.[1,13,14,48–50] Therefore, we examined whether a combined use of Coff or Caff with Mel could give a synergistic or additive effect on Aβ generation in neurons via the regulation of ROS generation, inflammation, and autophagy.

Materials and methods

Molecular docking

We employed the Discovery Studio program 3.1 designed by Accelrys Inc. (San Diego, CA, USA) to dock Caff and Mel into the active sites of ten human BACE1 structures (protein data bank [PDB] identification codes: 1FKN, 2OHU, 1W51, 2P4J, 2VKM, 4DH6, 3DV5, 2QU3, 3U6A, and 4FRS) as previously described by us.[51–55] The crystal structure of human BACE1 was obtained from the Protein Data Bank

(http://www.rcsb.org/pdb/). Before defining and editing the binding site, BACE1 was cleaned, modified, and prepared. During the preparation for Caff and Mel, the duplicate structures were deleted, and ionization change, Tautomer or isomer generation, Lipinski filter, and three-dimensional (3D) generator were all set true. Following the ligand preparation, Caff and Mel were docked into the binding site of BACE1. Electrostatic energy and van der Waals forces were considered during the docking process. For each defined van der Waals force or electrostatic probe, the interactions with all protein atoms were stored at predetermined grid points. For ligand atoms located between grid points, a trilinear interpolation was used to approximate the energies. A harmonic potential with the force constant of 300 kcal/mol was applied outside the grid boundary.[51–55]

Chemicals and reagents

Caff and Mel were purchased from Sigma-Aldrich Corp (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Corning Cellgro Inc. (Manassas, VA, USA). G418 (geneticin, a selective antibiotic), Dulbecco's phosphate-buffered saline (PBS), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), ethylenediaminetetraacetic acid (EDTA), and fetal bovine serum (FBS) were purchased from Sigma-Aldrich Corp. The autophagy detection kit was bought from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from EMD Millipore (Billerica, MA, USA). Western blot substrate was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Primary antibodies against human Nrf2, LC3-I/II, Beclin 1, Wnt3α, β-catenin, phosphorylated (p)-Tau/Tau, p-GSK3β/GSK3β, p-Akt/Akt, p-PI3K/PI3K (p85/55), and p-Erk1/2/Erk1/2 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The antibody against human β-actin was obtained from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

Preparation of Coff, Caff, and Mel

Maxwell House® regular Coff (Kraft Foods, Northfield, IL, USA) was purchased commercially and dissolved in autoclaved water. Briefly, 40 g of ground Coff was added to 300 mL autoclaved water. Coff solution was then heated to boiling, kept at boiling for 2 minutes, then filtered with a Coff filter into an autoclaved container. The concentration of Coff was determined according to the content of Caff. The concentration of 10 μM Coff is equivalent to 10 μM Caff. For "unconcentrated" Coff solution, 1.5 mL aliquots (containing 1.5 mg/mL Caff) were transferred into 2 mL tubes and stored at −20°C. Caff (7 mg) was dissolved in 1 mL PBS, and the stock solution was stored at −20°C. Coff and Caff were freshly diluted to predetermined concentrations with medium before use. Mel (10 mg) was dissolved in 1 mL ethyl alcohol and diluted to 1, 5, and 10 μM with fresh medium before use (final ethyl alcohol concentration <0.01%, v/v). The chemical structures of Caff and Mel are shown in Figure 1.

Cell culture

Neuro-2a (N2a)/APP cells were derived from stably transfected N2a cells with Swedish mutant APPsw and were maintained in a DMEM supplemented with 10% FBS and 0.2 g/L G418 in 5% CO2 at 37°C.

Drug treatment

We adopted three different chronological administration regimens in this study. N2a/APP cells were treated with Caff at 10 μM, 10 μM Coff (equivalent to 10 μM Caff), or Mel at 1, 5, or 10 μM alone or in combination. As shown in Figure 2, cells were treated with Coff or Caff at 10 μM for 12 hours in the day, followed by Mel at 1, 5, or 10 μM, for 12 hours in the night in regimen 1 (Figure 2). The medium was washed out after every 12-hour treatment. For regimen 2, cells were treated with Coff or Caff at 10 μM or Mel at 1, 5, and 10 μM, alone or in combination, for 24 hours, from 7 am to 7 am the next day. In regimen 3 (Figure 2), cells were treated with Coff or Caff or Mel for 5 consecutive days with regimen 1 or 2. N2a/APP cells were treated with Coff or Caff at 10 μM for 12 hours in the day, followed by Mel at 10 μM for 12 hours in the night and continued for 5 consecutive days. When regimen 2 was incorporated in regimen 3, the cells were treated with the drugs for 24 hours. The medium was changed at every 12- or 24-hour drug treatment.

Cell viability assay

The viability of N2a/APP cells was examined using the MTT assay. Briefly, the N2a/APP cells (8,000/well) were seeded into 96-well plates and treated with 10 μM Coff or Caff alone for 12 hours in the day, or with 1, 5, or 10 μM Mel for 12 hours in the night, or in combination. Following the treatment, 10 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 hours at 37°C. The medium was replaced with 100 μL dimethyl sulfoxide (DMSO), and the absorbance was measured using a Synergy™ H4 Hybrid Microplate Reader (BioTek Inc., Winooski, VT, USA) at a wavelength of 570 nm.

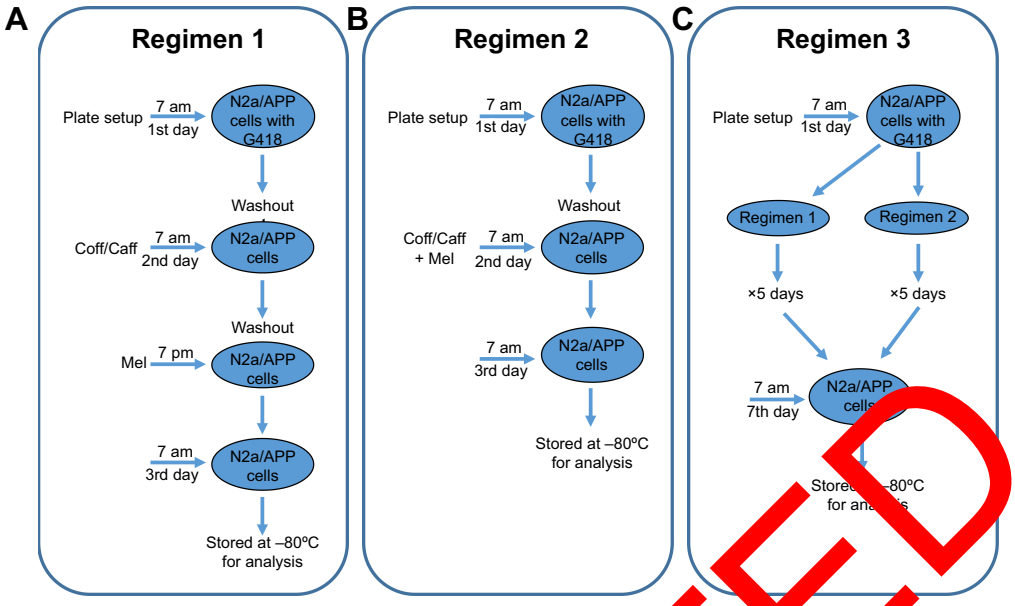


Figure 2 Flowcharts for the three chronological therapeutic regimens in N2a/APP cells.
Notes: (**A**) Regimen 1, including groups of cells treated with Coff/Caff at 10 μM for 12 hours in the d[illegible]owed by Mel at [illegible] 10 μM for 12 hours in the night. The medium was washed out after every 12-hour treatment; (**B**) regimen 2, including groups of cells tre[illegible] with C[illegible]/Caff at 10 μM a[illegible] Mel at 1, 5, or 10 μM for 24 hours, from 7 am to 7 am the next day; and (**C**) regimen 3, including groups of cells treated with Coff/Caff at 10 μM and Mel[illegible]0 μM with regimen 1 or 2 for 5 consecutive days.
Abbreviations: APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; G418, geneticin; [illegible]elatonin; N2a, [illegible]uro-2a.

Enzyme-linked immunosorbent assay (ELISA)

The concentration of Aβ40/42 was measured using the Aβ40/42 Human ELISA kit (catalog numb[illegible] KHB34[illegible] KHB3442; Life Technologies Corp, Ca[illegible]bad, [illegible]A, USA) according to the manufacturer's ins[illegible]ctions. The assay can recognize both natural and s[illegible]thetic forms of human Aβ40/42, and the antibodies [illegible] not cross-r[illegible]ct. According to the manufacturer's i[illegible]structi[illegible] we used the kits to measure the extracellul[illegible] levels of A[illegible] in N2a/APP cells. The concentration o[illegible] Aβ oligomer was measured using an Aggregated β-Amy[illegible]id H[illegible]an ELISA kit (catalog number KHB3491; Li[illegible]fe Techn[illegible]gies Co[illegible]) according to the manufacturer's instructions. The culture medium was collected after N[illegible]/APP [illegible] were treated with indicated regimens. The samp[illegible]were centrifuged at 100,000× *g* for 1 hour at 4°C prior to [illegible]alysis. This oligomeric form of Aβ (also known as amyloid β–derived diffusible ligand [ADDL]) can be separated from fibrillar and protofibrillar forms of aggregated Aβ by high speed centrifugation (ie, 100,000× *g* for 1 hour) or by size exclusion methods, as previously described.[56] Sample preparation should therefore be carefully considered when using this assay. Centrifugation at 14,000× *g* for 10 minutes has been shown to minimize fibrils in aggregated Aβ-containing samples, while centrifugation at 100,000× *g* for 1 hour at 4°C has been shown to minimize [illegible]rils and pr[illegible]ofibrils.[56,57] Size exclusion methods, such as gel p[illegible]on chromatography or ultrafiltration, may also [illegible]prove assay performance. The concentrations of interleukin (IL)-4, IL-12, and IL-10 were measured using ELISA kits (catalog number KHC0041/KHC0121/KAC1321; Life Technologies Corp). The concentration of interferon (IFN)-γ was determined using a Human IFN-γ ELISA Kit (catalog number EHIFNG; Thermo Fisher Scientific). The absorbance was detected at wavelength of 450 nm, using the Synergy™ H4 Hybrid Microplate Reader.

Western blot analysis

N2a/APP cells were washed with precooled PBS after treatment with indicated regimens and lysed with a lysis buffer consisting of 50 mmol HEPES at pH 7.5, 150 mmol NaCl, 10% glycerol, 1.5 mmol $MgCl_2$, 1% Triton™ X-100, 1 mmol EDTA at pH 8.0, 10 mmol sodium pyrophosphate, 10 mmol sodium fluoride, and the protease inhibitor cocktail. The supernatant was collected after the cell lysate was centrifuged at 14,000× *g* for 15 minutes at 4°C. Protein concentrations were measured using the BCA Protein Assay Kit. Equal amount of protein sample (30 μg) was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer and denatured for 10 minutes at 95°C. Subsequently, the samples were electrophoresed on 7%–12% SDS-PAGE minigel and transferred onto

Immobilon® PVDF membrane at 200 mA for 3 hours, at 4°C. Membranes were probed with indicated primary antibodies overnight at 4°C and then blotted with respective horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody. Visualization was performed using a Bio-Rad ChemiDocTM XRS system (Bio-Rad Inc., Hercules, CA, USA) with enhanced-chemiluminescence substrate. Protein level was normalized to the matching densitometric value of the internal control, β-actin.

ROS measurement

Intracellular level of ROS was measured using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Invitrogen™; Life Technologies Corp, Carlsbad, CA, USA). Briefly, N2a/APP cells were seeded into black 96-well plates and treated with indicated regimens at different concentrations and different time points. The mean intensity of green fluorescence of 2′,7′-dichlorofluorescein (DCF) was determined by the Synergy H4 Hybrid Microplate Reader, at 485 nm for excitation and 525 nm for detection. Fluorescence was measured every 3 minutes for 45 minutes using the Synergy H4 Hybrid Multi-Mode Microplate Reader. The protein concentration was measured to normalize the intracellular level of ROS.

Determination of autophagy using flow cytometry

To further explore the regulatory effect of Coff, Caff and Mel on N2a/APP cell death, we determined the autophagy using flow cytometry. N2a/APP cells were plated in 6-well plates (Corning Inc, Corning, NY, USA) at an intensity of 4×10^5 cells/well. The cells were treated with fresh medium, or Coff/Caff at 10 μM, for 12 hours in the day, followed by Mel at 1, 5, and 10 μM for 12 hours in the night. At the end of the treatment, the cells were trypsinized and centrifuged at 3,000× *g* for 5 minutes, to pellet the cells. The cells were suspended in 1× assay buffer containing 5% FBS (Enzo Life Sciences Inc.) and collected by centrifugation. Following that, cells were resuspended in 250 μL of phenol red–free culture medium (Invitrogen; Life Technologies Corp) containing 5% FBS. The diluted Cyto-ID® Green stain solution (250 μL) (Enzo Life Sciences Inc.) was added to each sample and mixed well. Cells were incubated for 30 minutes at 37°C, in darkness. After the incubation, cells were washed with 1× assay buffer and resuspended in 500 μL fresh 1× assay buffer. Thereafter, cells were analyzed using the green (FL1) channel of a flow cytometer (BD LSR II Analyzer; BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

Data were presented as mean ± standard deviation. Comparisons of multiple groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedure. $P<0.05$ was considered statistically different.

Results

Molecular interactions between Caff/Mel and BACE1

Both Caff and Mel are known blockers of BACE1 and reduce Aβ production.[37–39] Cleavage of APP at the N-terminus of the Aβ region by BACE1 and at the C-terminus by γ-secretase is the first and key amyloidogenic pathway from the substrate APP to generate Aβ peptides.[13,15] To explore how Caff and Mel inhibit human BACE1, we first carried out docking experiments using the Discovery Studio program 3.1. After docking Caff or Mel into the active sites of ten BACE1 structures, ten positions were generated for each compound–enzyme interaction. CDOCKER interaction energy ranges from around 17.6–34.6 kcal/mol (Figures 3 and 4, Table 1). Each compound–enzyme complex with the highest CDOCKER interaction energy was selected, and the two-dimensional (2D) and 3D pictures of them were collected. When Caff and Mel were docked into the active sites of the ten selected BACD1 structures, we observed different intermolecular interactions (Figures 3A–D and 4A–F, Table 1). The data showed that Caff bound to 1FKN and 3DV5 via the formation of a hydrogen bond at the site of Thr72 and Thr232, respectively. Caff formed three hydrogen bonds with 2OHU via Lys238, Lys239, and Lys246, and generated two hydrogen bonds via Gln73 and Thr232. No electrostatic and π-π stacking interactions were observed with Caff and the BACE1 structures.

Mel could be readily docked into the active sites of the ten BACE1 structures (Figure 4A–F). Mel formed two hydrogen bonds with 2OHU via Phe108 and Thr232. It also formed multiple hydrogen bonds with 1W51 (via Gly13), 2P4J (via Gly230 and Thr232), 2VKM (via Asp32, Thr72, and Gly230), 3DV5 (via Asp32 and Gly230), 3U6A (via Gly11, Ser229, and Arg307), and 4FRS (via Asp93, Gly95, and Tyr259). Notably, Mel formed π-π stacking with Tyr71 of 2VKM.

These data demonstrate that both Caff and Mel could readily bind to the active sites of BACE1, mainly via hydrogen bond formation and less commonly, via π-π stacking with the key residue Tyr71. Many of the residues involved in hydrogen bond formation with Caff and Mel are located in the active

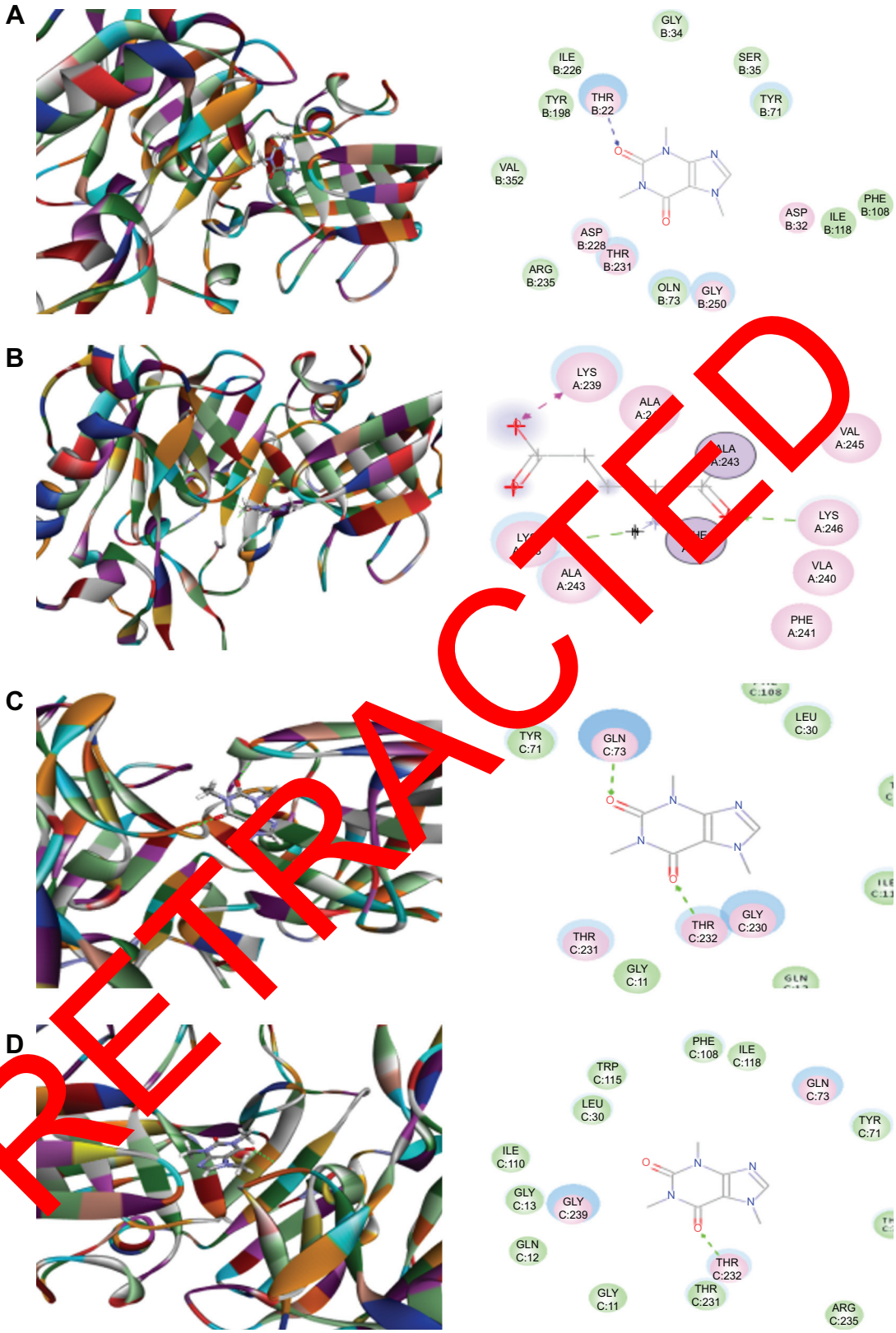


Figure 3 Molecular interactions between caffeine and the active site of BACE1 structures.
Notes: (**A**) Caffeine binds to the active site of 1FKN via hydrogen bond formation with Thr72; (**B**) Caffeine binds to the active site of 2OHU via hydrogen bond formation with Lys238, Lys239 and Lys246; (**C**) Caffeine binds to the active site of 2VKM via hydrogen bond formation with Gln73 and Thr232; and (**D**) Caffeine binds to the active site of 3DV5 via hydrogen bond formation with Thr232. The Discovery Studio 3.1 program (Accelrys Inc., San Diego, CA, USA) was used to dock caffeine into the active site of human BACE1 structures.
Abbreviation: BACE, β-site amyloid precursor protein cleaving enzyme.

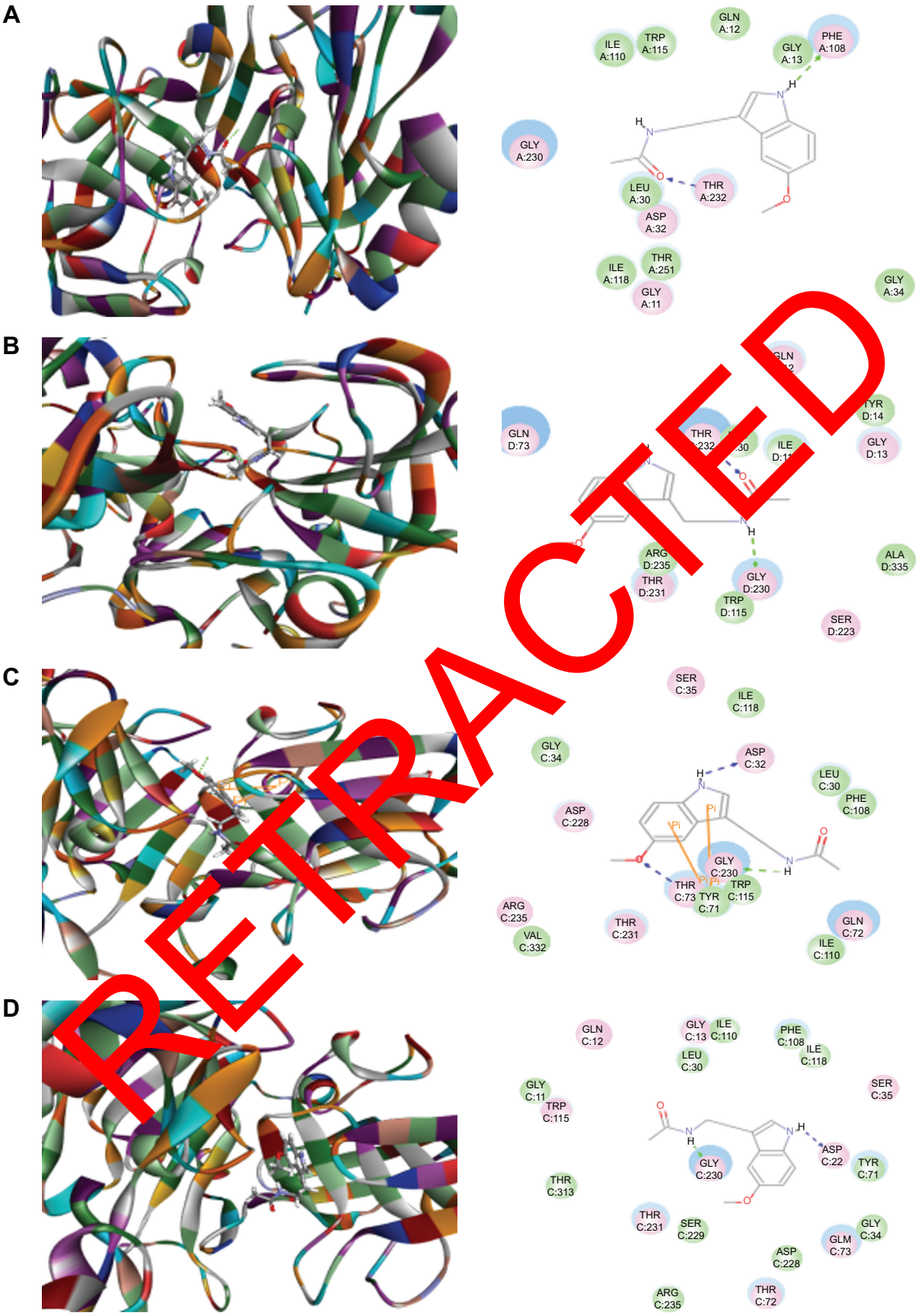


Figure 4 (*Continued*)

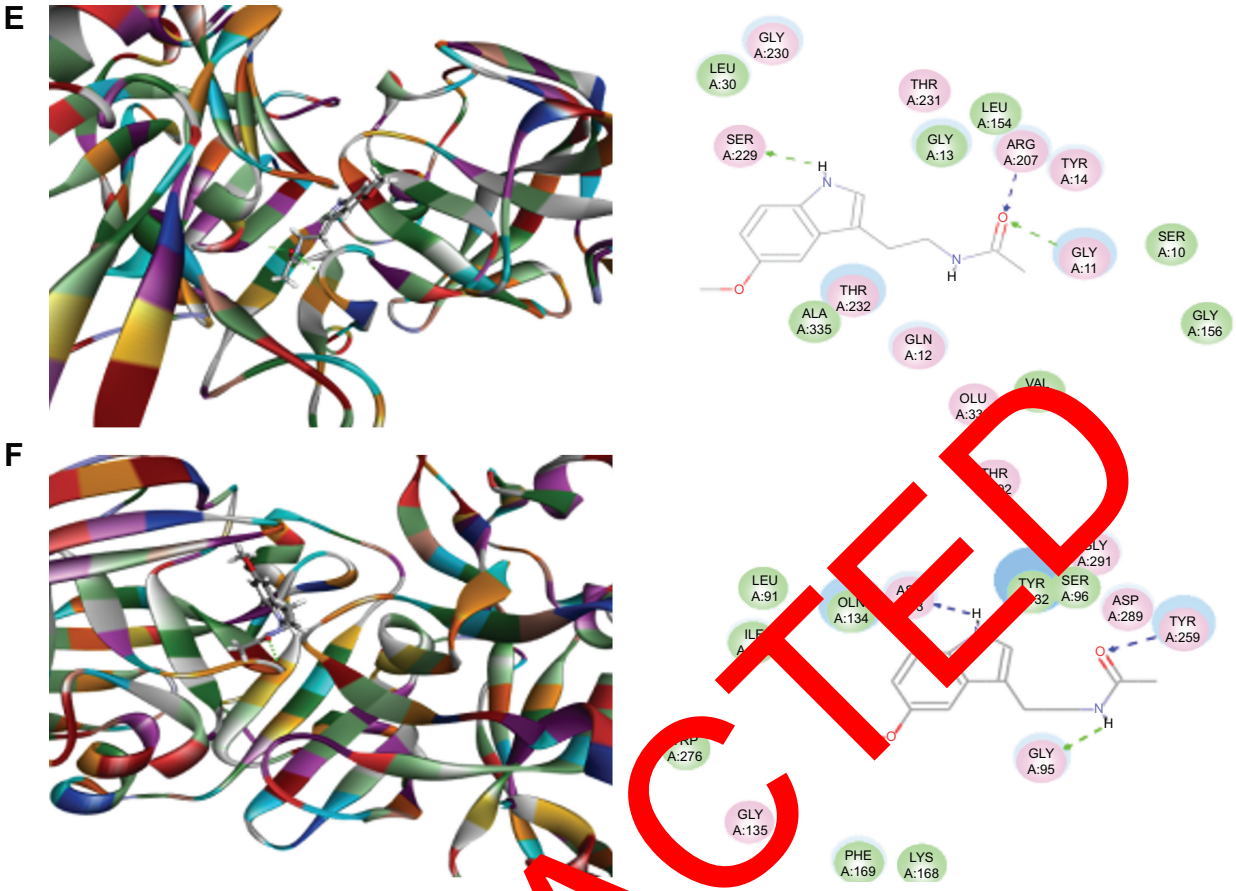


Figure 4 Molecular interactions between melatonin and the active site of human BACE1 structures.
Notes: (**A**) Melatonin binds to the active site of 2OHU via hydrogen bond formation with Phe108 and Thr232; (**B**) melatonin binds to the active site of 2P4J via hydrogen bond formation with Gly230 and Thr232; (**C**) melatonin binds to the active site of 2VKM via hydrogen bond formation with Asp32, Thr72, and Gly230, and π-π stacking with Tyr71; (**D**) melatonin binds to the active site of 3DV5 via hydrogen bond formation with Asp32 and Gly230; (**E**) melatonin binds to the active site of 3U6A via hydrogen bond formation with Gly11, Ser229, and Arg307; and (**F**) melatonin binds to the active site of 4FRS via hydrogen bond formation with Asp93, Gly95, and Tyr259. The Discovery Studio 3.1 program (Accelrys Inc., San Diego, CA, USA) was used to dock melatonin into the active site of human BACE1 structures.
Abbreviation: BACE, β-site amyloid precursor protein cleaving enzyme.

site of BACE1, and thus, it is not a surprise that both Caff and Mel can act as competitive inhibitors of BACE1.

Effect of Coff[illegible] Caff, plus Mel on N2a/APP cell viability

After treatment of N2a/APP cells with Coff or Caff at 10 μM for 12 hours, combination with Mel at 10 μM for another 12 hours, we measured the cell viability, using the MTT assay. Compared with the vehicle control (medium), Mel at 1 μM alone increased, by 18.4%, cell viability, and Coff (10 μM) plus Mel at 1 or 5 μM increased cell viability, by 25.0% and 35.7%, respectively ($P<0.001$, by one-way ANOVA) (Figure 5A). Incubation of cells with the combination of Caff with Mel at 1, 5, or 10 μM resulted in a 34.3%, 42.0%, and 21.7% increase in cell viability, respectively ($P<0.001$). In addition, treatment of cells with 10 μM Caff increased 34.4% in cell viability ($P<0.001$), but Coff at 10 μM did not affect cell viability (Figure 5B). These results demonstrated that Caff alone or in combination with Mel, and Mel at a low concentration, promoted the proliferation of N2a/APP cells.

Dynamic change of basal extracellular level of Aβ40/42 in N2a/APP cells

N2a/APP cells were treated with G418 to induce Aβ40/42 production prior to the treatment with different chorological regimens. We observed varying extracellular levels of Aβ40/42 over 5 days and a peak at 24 hours (Figure 6). The extracellular level of Aβ40 was 234.3, 1,801.7, and 56.6 pg/μL when cells were treated with G418 for 12 hours, 24 hours, and 5 days, respectively, with a maximum fold change of 31.8. For Aβ42, the maximum fold change was 10.0. These findings demonstrate the dynamic and chronological changes of Aβ40/42 in N2a/APP cells.

Table 1 Molecular interactions between Caff and Mel with human BACE1

Compound	CDOCKER interaction energy (kcal/mol)	H-bond number	Residues involved in H-bond formation	Charge interactions	Residues involved in charge interactions	π-π stacking	Residues involved in π-π stacking
Caff							
1FKN	26.7893	1	O-Thr72	0	–	0	–
2OHU	20.8538	3	H-Lys238 O-Lys239 O-Lys246	0	–	0	–
1W51	19.9758	0	–	0	–	0	–
2P4J	24.65	0	–	0	–	0	–
2VKM	27.7626	2	O-Gln73 O-Thr232	0	–	0	–
4DH6	22.18	0	–	0	–	0	–
3DV5	23.2694	1	O-Thr232	0	–	0	–
2QU3	17.6425	0	–	0	–	0	–
3U6A	19.6014	0	–	0	–	0	–
4FRS	22.5468	0	–	0	–	0	–
Mel							
1FKN	34.4816	0	–	0	–	0	–
2OHU	29.1	2	O-Phe108 O-Thr232	0	–	0	–
1W51	31.3981	1	O-Gly13	0	–	0	–
2P4J	33.3171	2	H-Gly230 O-Thr232	0	–	0	–
2VKM	36.5899	3	H-Asp32 O-Thr72 H-Gly230	0	–	2	Tyr71
4DH6	32.1519	0	–	0	–	0	–
3DV5	34.1243	2	H-Asp32 H-Gly230	0	–	0	–
2QU3	26.2322	0	–	0	–	0	–
3U6A	31.441	3	-Gly11 H-Ser22 O-Ar 07	0	–	0	–
4FRS	34.5751	3	H-A 93 H-Gly O-Tyr259	0	–	0	–

Note: –, means none.
Abbreviations: BACE, β-site amyl precursor protein cleavi enzyme; Caff, caffeine, Mel, melatonin.

Effect of Coff or C , plus el on the extracellul l of β40/42 in N2a/AP cells

The extracel level of Aβ40/42 in N2a/APP cells treated wi regimen 1

First, we checked the ffect of regimen 1 on the extracellular levels of Aβ40/42 in N2a/APP cells. When the cells were incubated with Mel alone at concentrations of 1, 5, or 10 μM for 12 hours, there was a concentration-dependent increase in the extracellular level of Aβ40 in N2a/APP cells. Incubation of cells with 5 or 10 μM Mel elevated the extracellular level of Aβ40 by 1.6- and 2.3-fold, respectively ($P<0.001$, by one-way ANOVA) (Figure 7A and C). On the other hand, treatment of cells with Coff or Caff alone showed distinct effects on the extracellular level of Aβ40. When N2a/APP cells were treated with Coff at 10 μM alone for 12 hours, the extracellular level of Aβ40 was increased in comparison with the control cells (312.2 vs 234.3 pg/mL) (Figure 7A). When the cells were treated with Caff at 10 μM alone for 12 hours, the extracellular level of Aβ40 was only slightly decreased compared with the control cells (160.1 vs 234.3 pg/mL) ($P>0.05$) (Figure 7C).

We treated N2a/APP cells with the combination of 10 μM each of Coff/Caff for 12 hours in the day, followed by another 12-hour treatment with Mel at 1, 5, or 10 μM during the night. The extracellular level of Aβ40 was

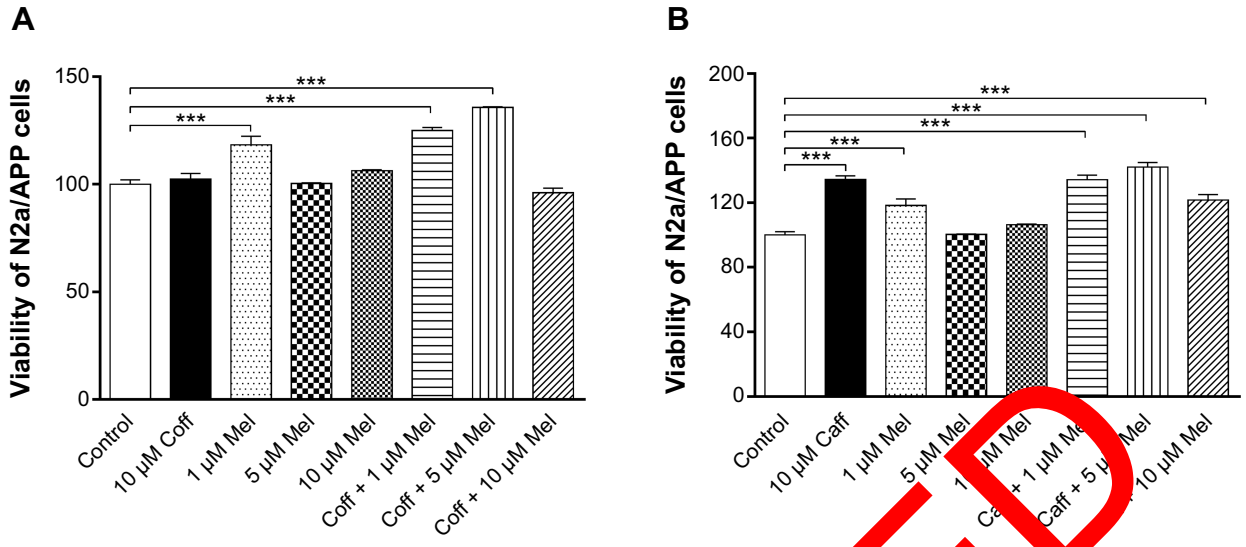


Figure 5 Effects of Coff/Caff plus Mel on N2a/APP cell viability.
Notes: The cells were treated with Coff (**A**) or Caff (**B**) at 10 μM for 12 hours in the day, followed by Mel at 1, 5, and 10 μM for a further 12 hours in the night. The cell survival was determined by the MTT assay. ***$P<0.001$, by one-way ANOVA.
Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; Mel, melatonin; MTT, 3-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; N2a, Neuro-2a.

concentration-dependently increased. Compared with the control cells, incubating cells with Coff plus 5 or 10 μM Mel increased the extracellular level of Aβ40 by 1.3- and 2.0-fold, respectively ($P<0.001$, by one-way ANOVA) (Figure 7A). Treatment of cells with Caff plus 5 or 10 μM Mel resulted in a 2.0- and 4.0-fold increase in the extracellular level of Aβ40, respectively ($P<0.001$, by one-way ANOVA) (Figure 7C). Incubation of the cells with Caff for 12 hours in the day followed by Mel for 12 hours in the night more significantly increased Aβ40 levels than with Mel alone. In addition, exposure of N2a/APP cells to 5 or 10 μM Mel with Caff increased the extracellular level of Aβ42 1.2- and 1.7-fold, respectively, compared with exposure to Mel alone ($P<0.001$, by one-way ANOVA) (Figure 7C).

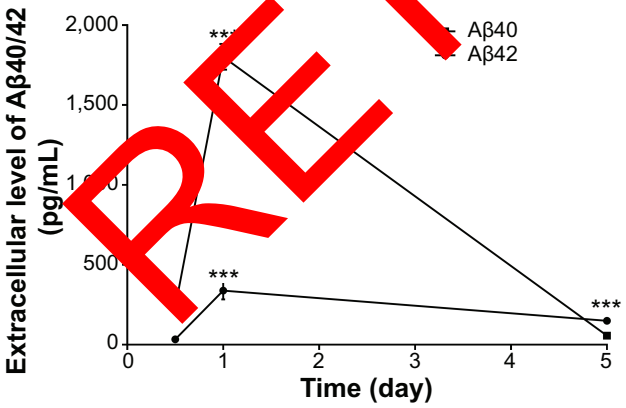


Figure 6 Changes in extracellular basal levels of Aβ40/42 levels in N2a/APP cells over 5 days.
Notes: The cells were treated with the selective antibiotic G418 and the supernatants collected for Aβ determination using ELISA kits. ***$P<0.001$, by one-way ANOVA.
Abbreviations: Aβ, amyloid-β; ANOVA, analysis of variance; APP, amyloid precursor protein; ELISA, enzyme-linked immunosorbent assay; G418, geneticin; N2a, Neuro-2a.

Mel alone significantly increased the extracellular level of Aβ42 in a concentration-dependent manner in N2a/APP cells (Figure 7B and D). Compared with the control cells, incubation of cells with 5 or 10 μM Mel resulted in a 5.8- and 8.0-fold increase in the extracellular Aβ42 level, respectively (195.5 vs 33.9 pg/mL, and 270.4 vs 33.9 pg/mL) ($P<0.001$, by one-way ANOVA) (Figure 7B). However, treating cells with Coff/Caff alone for 12 hours led to a decrease in the extracellular level of Aβ42 compared with the control cells (26.2/17.0 vs 33.9 pg/mL) (Figure 7B and D). Furthermore, we treated N2a/APP cells with Coff/Caff for 12 hours in the day, followed by Mel for another 12 hours in the night. There was a 3.9- and 4.0-fold increase in the extracellular level of Aβ42 after cells were treated with 10 μM Coff followed by 5 or 10 μM Mel, respectively ($P<0.001$, by one-way ANOVA) (Figure 7B). Moreover, incubation of cells with 10 μM Caff followed by 5 or 10 μM Mel resulted in a 3.8- and 6.0-fold increase in the extracellular level of Aβ42, respectively ($P<0.001$, by one-way ANOVA) (Figure 7D). These results demonstrated that Mel alone considerably increased the extracellular levels of Aβ40/42 in N2a/APP cells and

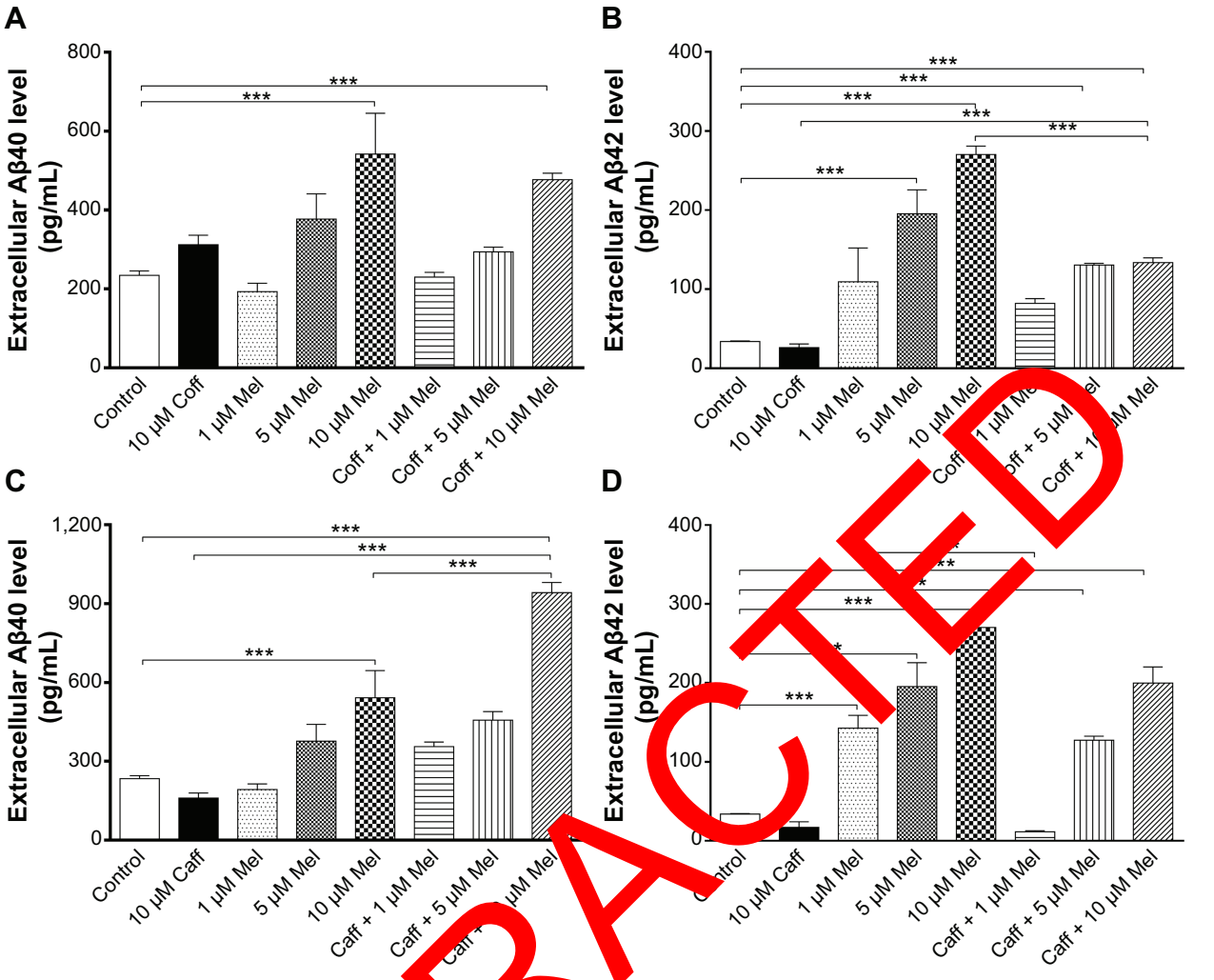


Figure 7 Effects of Coff/Caff plus Mel on the extracellu[lar] Aβ4[0]/42 l[evels] in N2a/AP[P] cells treated with regimen 1.
Notes: N2a/APP cells were treated with Coff (**A** an[d] **B**) or Caff ([**C**] and **D**) at 10 μM for 12 hours in the day, followed by Mel at 1, 5, or 10 μM for another 12 hours in the night. The extracellular level of Aβ40/42 was det[ected] by ELISA. ***[P]<0.001, by one-way ANOVA.
Abbreviations: Aβ, amyloid-β; ANOVA, an[alysis] of [variance]; APP, amyl[oid] precursor protein; Caff, caffeine; Coff, coffee; ELISA, enzyme-linked immunosorbent assay; Mel, melatonin; N2a, Neuro-2a.

that Coff or Caff at rela[tively] high concentration (10 μM) decreased extracellular [levels] of Aβ40/42 to a greater extent, than did Mel at 1 [or] 5 μM.

The extra[cellular] l[evel] of Aβ40/42 in N2a/APP cells treated [with] regimen 2

Next, in order to e[xamine] the effect of different chorological regimens of Coff/Caff plus Mel, N2a/APP cells were treated with Coff/Caff plus Mel for 24 hours, from 7 am to 7 am the next day (regimen 2). In comparison with the control cells, there was no significant difference in the extracellular level of Aβ40 and Aβ42 when cells were exposed to Coff, Caff, or Mel alone for 24 hours ($P>0.05$) (Figure 8A–D). However, incubation of cells with 10 μM Coff plus 5 or 10 μM Mel increased by 1.3- and 1.2-fold the extracellular level of Aβ40, respectively (2,359 and 2,173 vs 1,802 pg/mL) ($P<0.001$, by one-way ANOVA), and by 1.5-fold that of Aβ42 (491.5 vs 314.6 pg/mL) ($P<0.001$, by one-way ANOVA) (Figure 8A and B). Treating cells with 10 μM Caff plus 5 or 10 μM Mel resulted in a 1.2-fold increase in the extracellular level of Aβ40 (2,224 and 2,117 vs 1,802 pg/mL) ($P<0.001$, by one-way ANOVA), respectively, and 1.33-fold that of Aβ42 (451.7 vs 314.6 pg/mL) ($P<0.001$, by one-way ANOVA) (Figure 8C and D).

The extracellular level of Aβ40/42 in N2a/APP cells treated with regimen 3

Furthermore, we treated cells with Coff/Caff with or without Mel combination, for 5 consecutive days with regimen 1 or 2. The data for the changes of the extracellular levels of Aβ40/42 are shown in Figure 9A–H. The extracellular level of Aβ40/42 was lower than that in regimens 1 and 2.

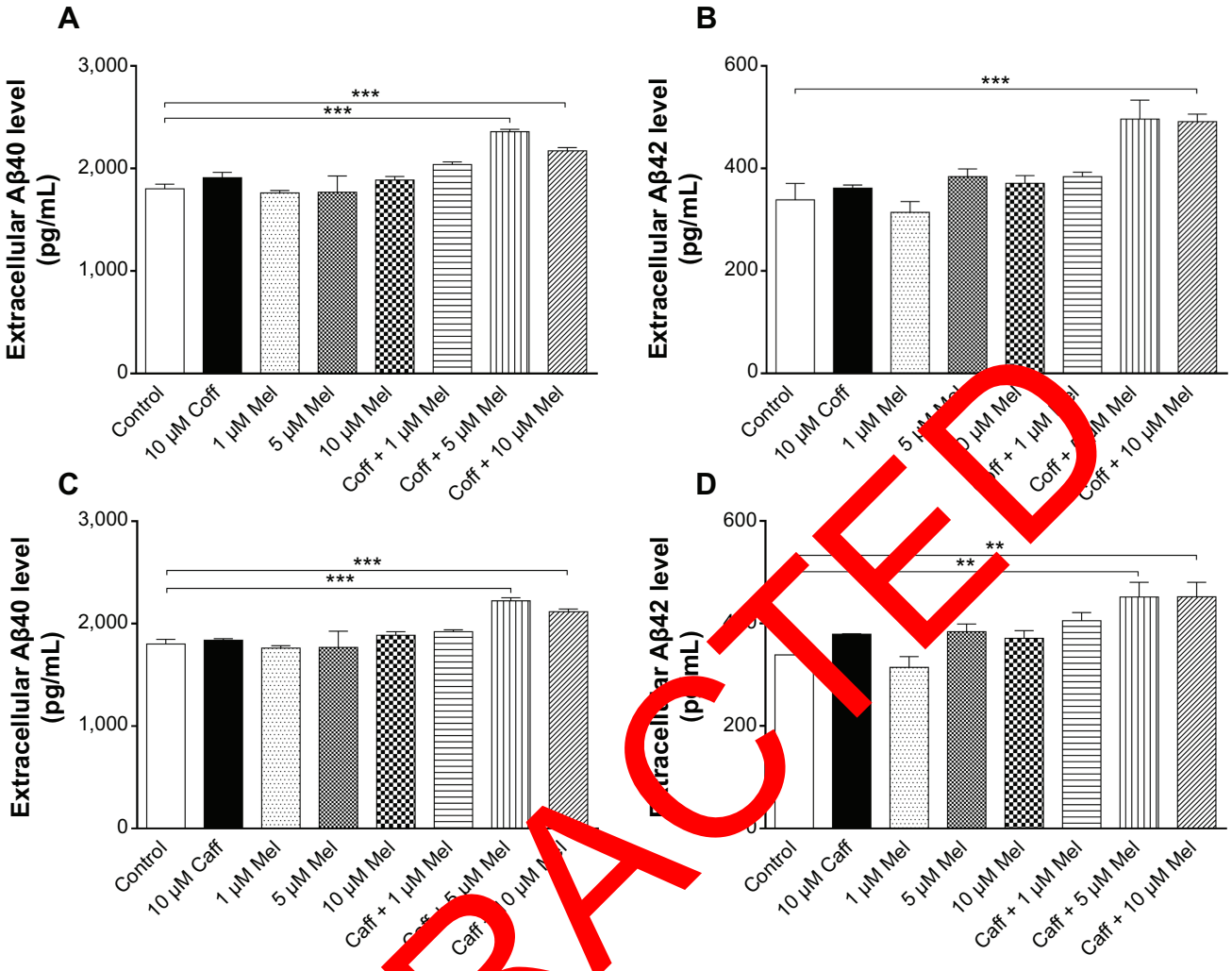


Figure 8 Effects of Coff/Caff plus Mel on the extracellular Aβ40/42 levels in N2a/APP cells treated with regimen 2.
Notes: N2a/APP cells were treated with Coff (**A** and **B**) or Caff (**C** and **D**) at 10 μM, plus Mel at 1, 5, or 10 μM for 24 hours, from 7 am to 7 am the next day. The extracellular level of Aβ40/42 was determined by ELISA. $^{**}P<0.01$ and $^{***}P<0.001$, by one-way ANOVA.
Abbreviations: Aβ, amyloid-β; ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; ELISA, enzyme-linked immunosorbent assay; Mel, melatonin; N2a, Neuro-2a.

Incubation of cells with 10 μM Mel for 12 hours in the night following 10 μM Coff for 12 hours in the day for 5 days significantly reduced, by 9%, the extracellular level of Aβ42 (126.9 vs 149.2 pg/mL) ($P<0.001$, by one-way ANOVA) (Figure 9B). Treatment of cells with 10 μM Mel for 12 hours in the night following Caff 10 μM for 12 hours in the day for 5 days also significantly decreased, by 8% and 13%, the extracellular level of Aβ40 (52.7 vs 56.9 pg/mL) ($P<0.001$, by one-way ANOVA) (Figure 9E) and Aβ42, respectively (130.5 vs 149.2 pg/mL) ($P<0.05$, by one-way ANOVA) (Figure 9F). There were only slight changes in the extracellular Aβ40 level when N2a/APP cells were treated with Caff, Coff, and Mel alone (Figure 9A–H). No significant difference was observed in the extracellular level of Aβ40/42 in N2a/APP cells treated with Coff/Caff plus Mel together for 24 hours over 5 days (Figure 9C, D, G, and H).

Effect of Coff or Caff, plus Mel on the extracellular level of Aβ oligomers in N2a/APP cells

Age-dependent accumulation of Aβ in the brain from "normal brain aging" to AD is a complicated process. It has been proposed that the intracellular level of Aβ oligomers may indicate the progression of AD.[58] Therefore, we examined the extracellular level of Aβ oligomers in N2a/APP cells. Our data showed that there was only a slight reduction in the level of Aβ oligomers in N2a/APP cells treated with Coff for 12 hours, followed by Mel for 12 hours (689.8 vs 759.3 pg/mL) (Figure 10A). Incubation of cells with 10 μM Mel for 12 hours in the night following Caff 10 μM for 12 hours in the day reduced, by 22%, the extracellular level of Aβ oligomers in N2a/APP cells (589.5 vs 759.3 pg/mL)

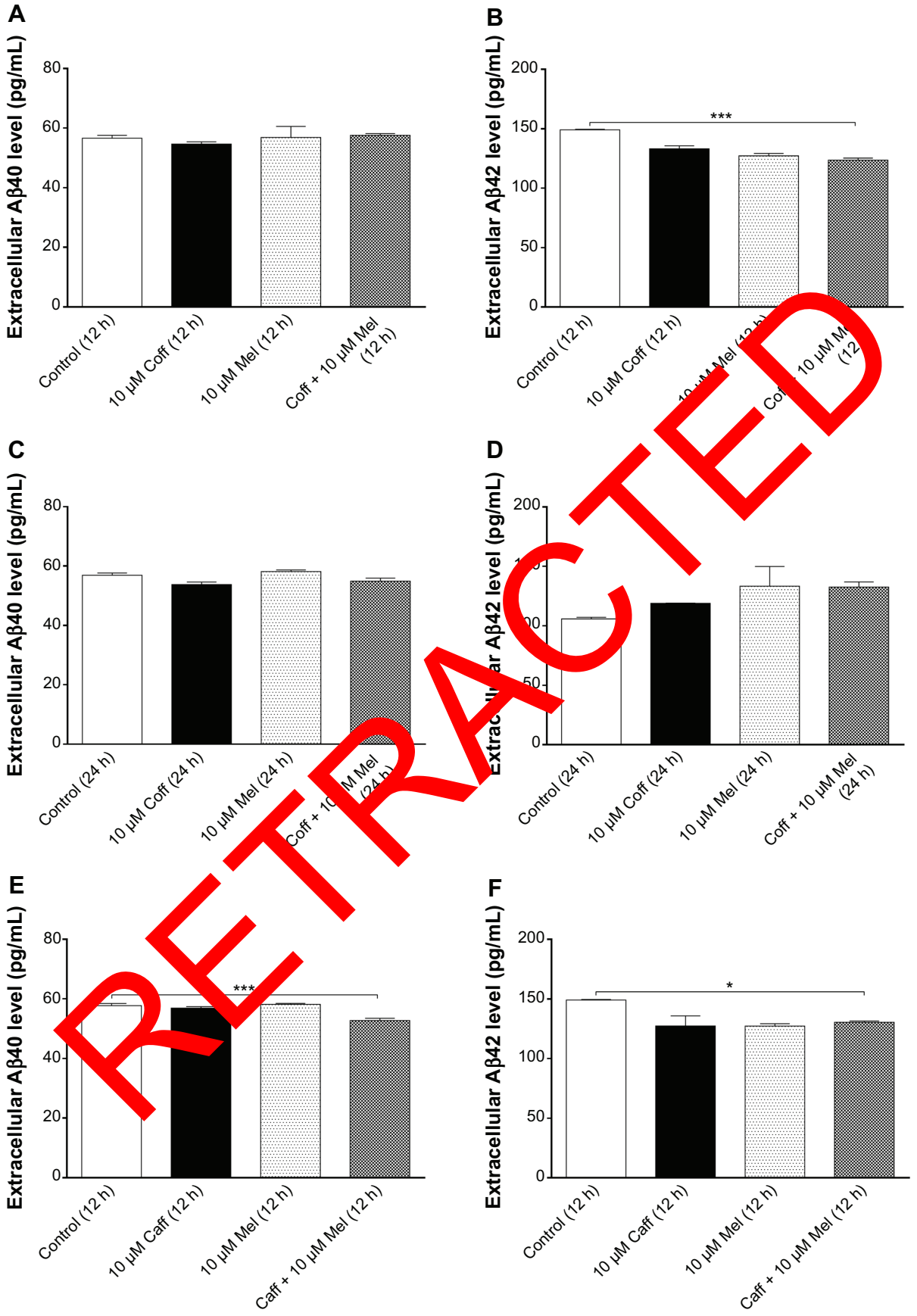


Figure 9 (*Continued*)

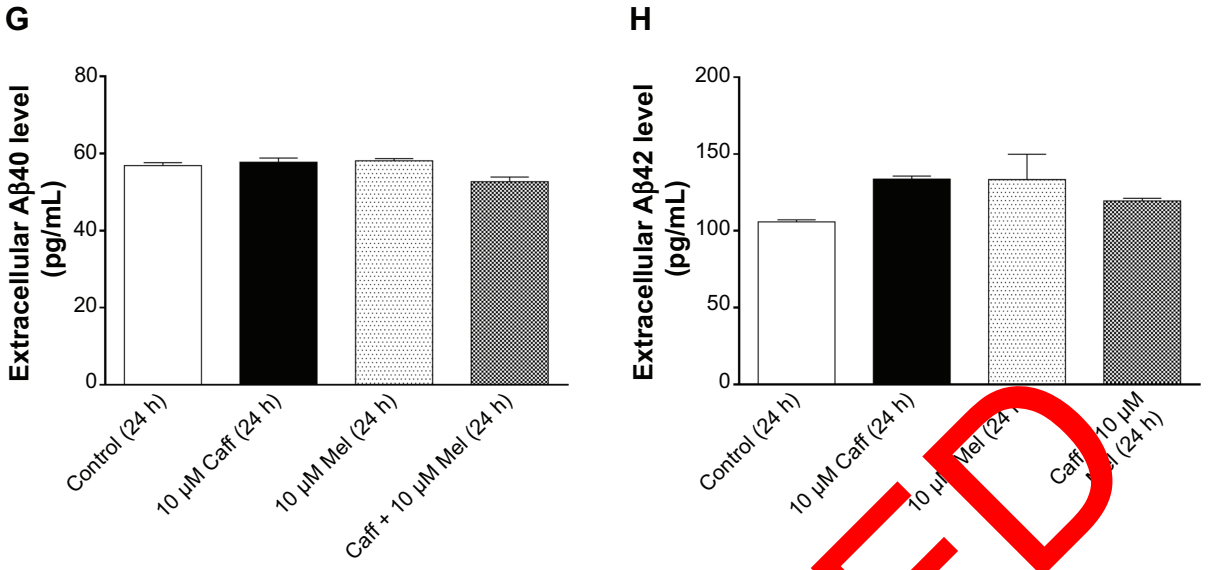


Figure 9 Effects of Coff/Caff plus Mel on the extracellular Aβ40/42 levels in N2a/APP cells treated with Regimen 3.
Notes: N2a/APP cells with 10 μM Coff (**A**–**D**) or Caff (**E**–**H**) for 12 hours or 24 hours, followed by 10 μM Mel for 12 or 24 hours for 3 consecutive days. The extracellular level of Aβ40/42 was determined by ELISA. *$P<0.05$ and ***$P<0.001$, by one-way ANOVA.
Abbreviations: Aβ, amyloid-β; ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; ELISA, enzyme-linked immunosorbent assay; Mel, melatonin; N2a, Neuro-2a.

($P<0.001$, by one-way ANOVA) (Figure 10B). However, there was only a slight reduction of extracellular Aβ oligomer levels in N2a/APP cells treated with Caff alone (658.3 vs 759.3 pg/mL) ($P>0.05$), whereas Coff elicited an opposite effect on the level of Aβ oligomers (Figure 10A and B).

Coff or Caff, plus Mel did not change the expression levels of cytokines in N2a/APP cells

The buildup of Aβ aggregates in AD is followed by the formation of intracellular neurofibrillary tangles and activation of neuroinflammatory responses.[2] Mel and Coff/Caff may prevent the cellular damage induced by the exposure of neurons to Aβ, which is associated with neuroinflammation.[37] Therefore, we next determined the production of T helper cell (Th)1-related IL-12 and IFN-γ and Th2-related IL-4 and IL-10 in N2a/APP cells. Treatment of N2a/APP cells with Coff or Caff at 10 μM, plus Mel at 1, 5, or 10 μM did not have a significant effect on the levels of these cytokines in N2a/APP cells ($P>0.05$, by one-way ANOVA) (Figure 11A–H).

Coff or Caff, plus Mel had no effect on autophagy of N2a/APP cells

Both autophagy and apoptosis are basic physiologic processes contributing to the maintenance of cellular homeostasis, involving a sequential set of events, including double membrane formation, elongation, vesicle maturation, and finally, delivery of the targeted materials to the lysosomes.[60–62] Autophagy can remove and eliminate damaged organelles to protect cells against cell death. Dysregulation of autophagy plays a pivotal role in the etiology and progress of neurodegenerative disorders.[49,50] It has been reported that Mel-induced autophagy protected against neuronal apoptosis in rats.[63] In order to further unveil the underlying mechanisms for the beneficial effects of Caff, Mel, and Coff on AD, the autophagic effect was examined using flow cytometry. In N2a/APP cells treated with regimen 1, there was only a slight increase in cellular autophagy (Figure 12A–C), and there was no significant alteration in the expression of the autophagy-associated markers Beclin 1 and LC3-I/II (Figure 12D–H). These results suggest that autophagy may play a negligible role in the beneficial effects of Caff, Mel, and Coff on AD.

Coff or Caff, plus Mel reduced oxidative stress in N2a/APP cells via Nrf2 pathway

Oxidative stress has a notorious role in the development and progression of AD;[1,21,64,65] oxidative stress interacts with macromolecules, such as DNA and proteins, resulting in cellular dysfunction and eventually leads to cell death. Antioxidation is proposed to be capable of ameliorating the symptoms of AD.[66] As such, the antioxidative effects of Coff, Caff, and Mel were examined in N2a/APP cells. Treatment of cells with 10 μM Coff or Caff for 12 hours in the day, combined with 10 μM Mel for 12 hours in

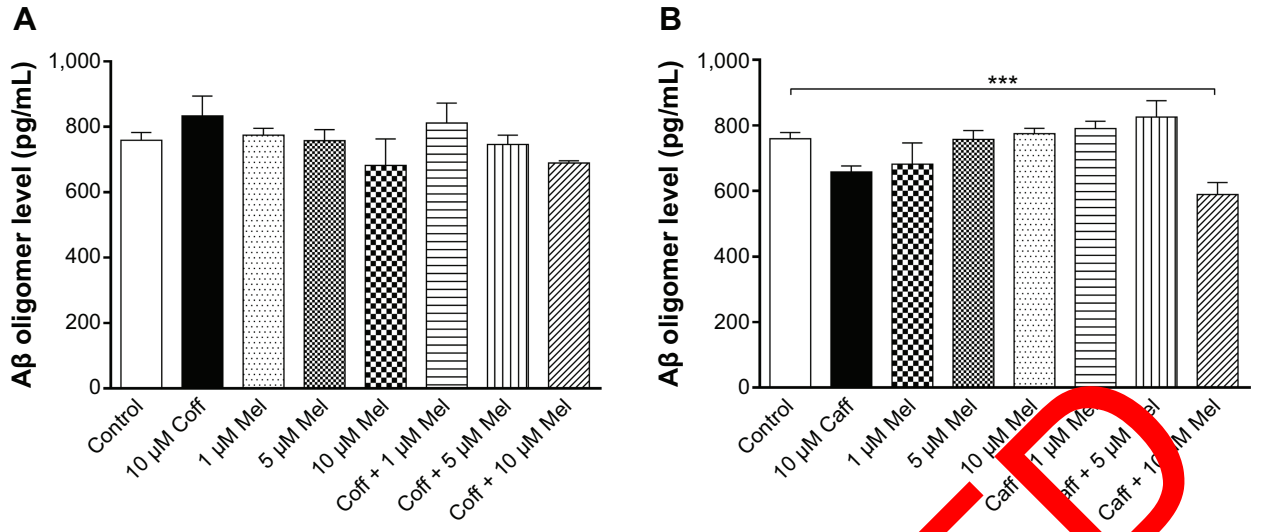


Figure 10 Effects of Coff/Caff plus Mel on extracellular Aβ oligomer levels in N2a/APP cells treated with regimen 1.
Notes: N2a/APP cells were treated with Coff (**A**) or Caff (**B**) at 10 μM for 12 hours in the day, followed by Mel at 1, 5, or 10 μM for another 12 hours in the night. The extracellular level of Aβ oligomer was determined by ELISA. ***$P<0.001$, by one-way ANOVA.
Abbreviations: Aβ, amyloid-β; ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; ELISA, enzyme-linked immunosorbent assay; Mel, melatonin; N2a, Neuro-2a.

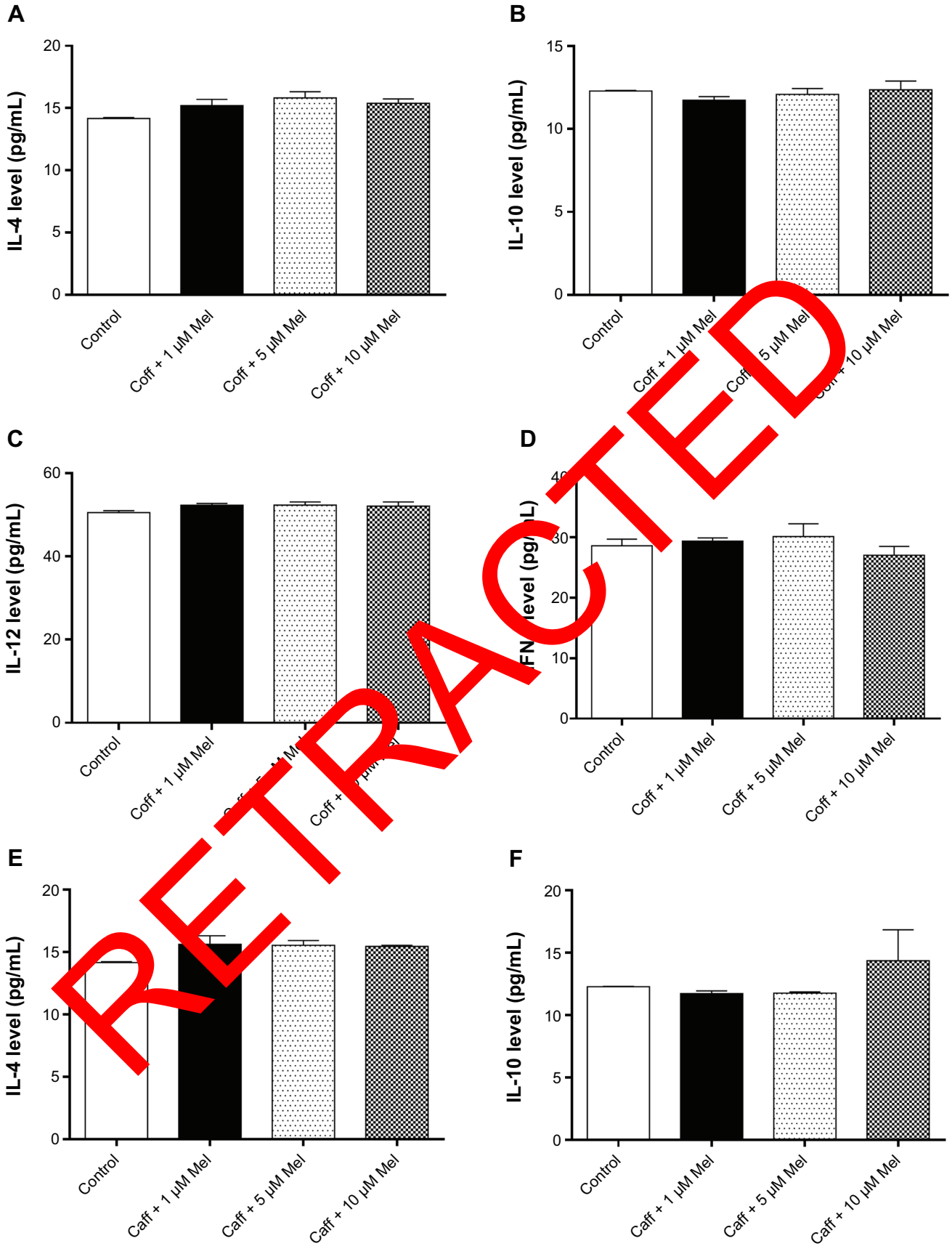
the night significantly reduced, by 20.2% and 17.5%, the intracellular levels of ROS, respectively ($P<0.05$ and $P=0.001$, respectively, by one-way ANOVA) (Figure 13A and B). Since Nrf2 controls the basal and induced expression of an array of antioxidant response element-dependent genes to regulate the physiological and pathophysiological outcomes of oxidant exposure,[67,68] we further determined the effects of Coff/Caff and Mel on its expression in N2a/APP cells. We found that incubation of cells with 10 μM Coff/Caff in the day and 10 μM Mel in the night significantly decreased, by 72.7% and 4[illegible]9%, the expression level of Nrf2, respectively ($P<0.0001$, by one-way ANOVA) (Figure 14A–C). These results suggest that the beneficial effects of Coff/Caff and Mel on AD may be ascribed, at least in part, to antioxidant activity and the involvement of Nrf2-mediated signaling pathways.

Coff or Caff plus Mel downregulates Wnt3α expression but upregulated β-catenin expression in N2a/APP cells

Dysfunctional Wnt/β-catenin signaling plays an important role in the pathogenesis of AD.[69] In the present study, we examined the effect of the combinations of Coff/Caff with Mel on the expression of Wnt3α and β-catenin in N2a/APP cells. The data showed that treatment of cells with Coff/Caff plus 1, 5, or 10 μM Mel decreased, by 20.3%, 29.3%, and 28.0%, and 23.8%, 67.0%, and 77.0%, the expression of Wnt3α, respectively, compared with the control cells ($P<0.001$ or 0.001, by one-way ANOVA) (Figure 15A–E). There was an increase in the expression of β-catenin when cells were treated with the two combinations. In comparison with the control cells, Coff plus 1, 5, or 10 μM Mel increased, by 2.7%, 53.0%, and 33.3%, respectively, the expression of β-catenin; and Caff plus 5 or 10 μM Mel increased, by 27.9% and 41.3%, respectively, the expression of β-catenin ($P<0.001$, by one-way ANOVA) (Figure 15A–E). These data clearly demonstrate that Coff/Caff plus Mel or Mel alone concentration-dependently downregulated the expression of Wnt3α/β-catenin in N2a/APP cells, probably contributing to the protective effects of Coff/Caff and Mel on AD.

Coff or Caff, plus Mel inhibited GSK3β, Akt, PI3K p55, and Tau phosphorylation but enhanced PI3K p85 and Erk1/2 phosphorylation in N2a/APP cells

GSK3 is a ubiquitously expressed serine/threonine protein kinase that plays a key role in the pathogenesis of AD.[70] GSK3 phosphorylates Tau at serine and threonine, contributing to Aβ production and Aβ-mediated neuronal death.[70] Our data showed that the phosphorylation level of GSK3β was significantly reduced, by 17.2% with Coff, and by 33.5% in Caff at 5 μM; and by 61.5% with Coff, and by 52.7% with Caff at 10 μM for 12 hours in the day plus Mel at 10 μM for 12 hours in the night compared with the control cells, respectively ($P<0.001$, by one-way ANOVA) (Figure 16A–C).



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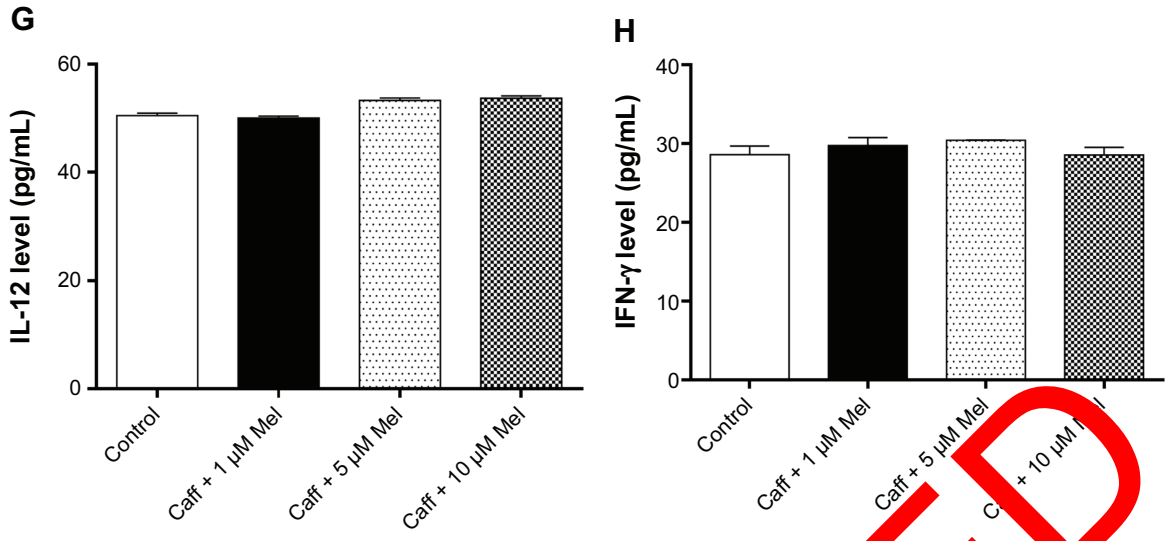


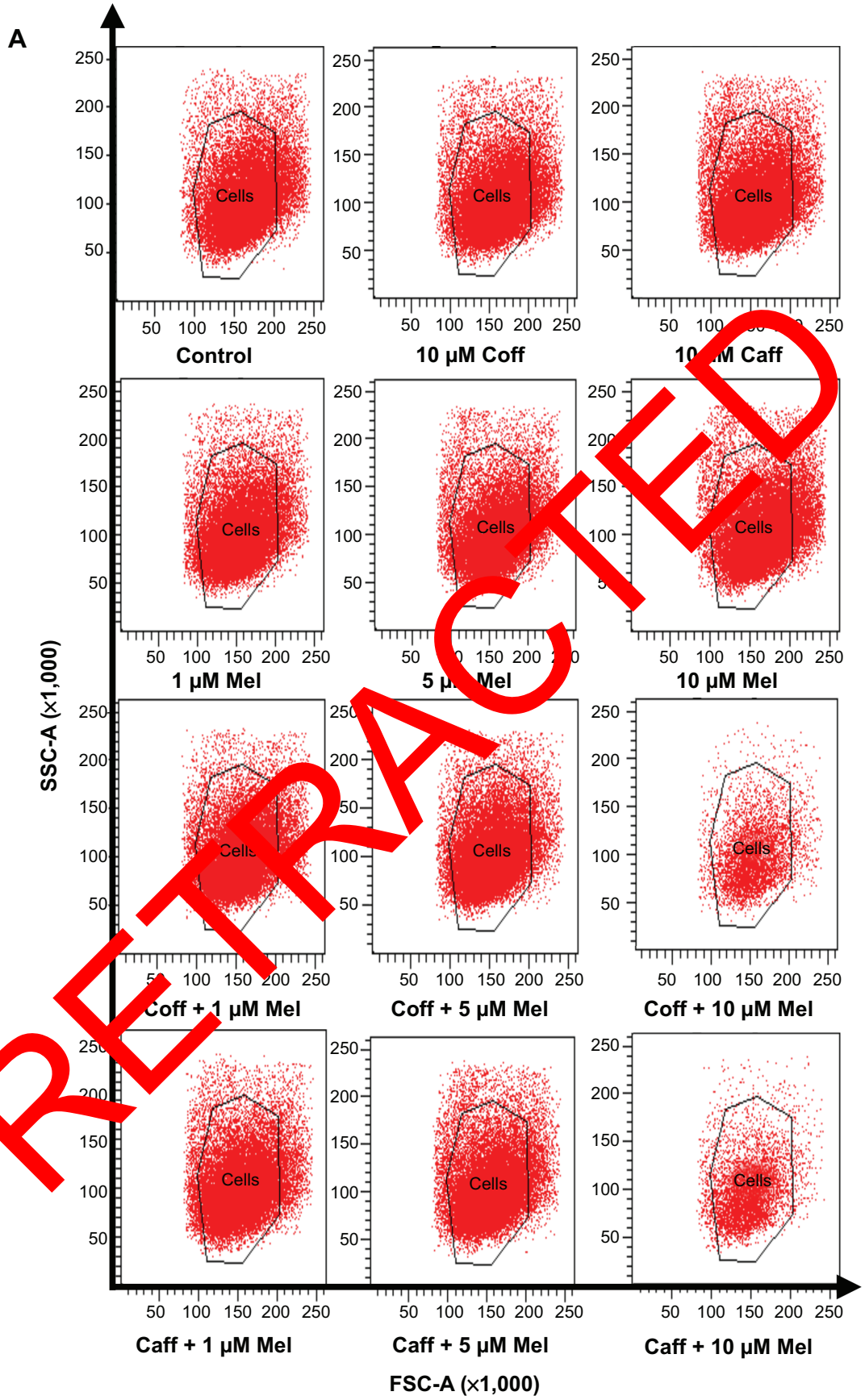
Figure 11 Effects of Coff/Caff plus Mel on cytokine levels in N2a/APP cells treated with regimen 1.
Notes: N2a/APP cells were treated with Coff (**A**–**D**) or Caff (**E**–**H**) at 10 μM for 12 hours in the day, followed by Mel at [illegible] μM for another 12 hours in the night. The cytokine levels were determined by ELISA.
Abbreviations: Aβ, amyloid-β; ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; [illegible] coffee; ELISA, [illegible]yme-linked immunosorbent assay; IFN, interferon; IL, interleukin; Mel, melatonin; N2a, Neuro-2a.

Coff (10 μM) plus 5 or 10 μM Mel elevated 19.6% and 7.0% of the ratio of p-PI3K over PI3K p85, respectively; and Caff (10 μM) plus 1, 5, or 10 μM Mel increased 51.0%, 80.3%, and 80.4% of the ratio of p-PI3K over PI3K p85, respectively ($P<0.001$, by one-way ANOVA) (Figure 16A–C). Coff at 10 μM plus 1 μM Mel elevated 51.7% of the ratio of p-PI3K over PI3K p55, but this ratio was decreased by 31.4% and 73.1% when the concentration of Mel was increased to 5 or 10 μM, respectively ($P<0.001$, by one-way ANOVA) (Figure 16A–C). In addition, Caff at 10 μM plus 1 μM Mel increased 47.5% of the ratio of p-PI3K over PI3K p55, but Caff at 10 μM plus 10 μM Mel decreased 23.5% of the ratio ($P<0.001$, by one-way ANOVA) (Figure 16A–C). However, Caff at 10 μM plus 5 μM Mel only slightly increased the ratio of p-PI3K over PI3K p55 ($P>0.05$).

Exposure of cells to the combination of Coff/Caff with Mel led to an increase in the phosphorylation of Erk1/2 ($P<0.001$, by one-way ANOVA) (Figure 16A–C). On the other hand, the phosphorylation level of Akt was significantly reduced, 34.8%/30.1% for Coff/Caff plus Mel at 1 μM; 27.5%/43.6% for Coff/Caff plus Mel at 5 μM; and 42.9%/52.0% for Coff/Caff plus Mel at 10 μM, in N2a/APP cells treated with Coff/Caff at 10 μM for 12 hours in the day plus Mel at 1, 5, or 10 μM for 12 hours in the night compared with the control ($P<0.001$, by one-way ANOVA) (Figure 16A–C). In addition, incubation with 5 or 10 μM Mel in the night, followed by Coff/Caff at 10 μM in the day significantly reduced 51.5%/53.3% for Coff/Caff plus Mel at 5 μM; and 61.5%/89.2% for Coff/Caff plus Mel at 10 μM [illegible] phosphorylation levels of Tau ($P<0.001$, by one-way ANOVA) (Figure 16A–C). The same combination significantly inhibited 42.94% (Coff)/52.2% (Caff) of the phosphorylation level of Akt ($P<0.01$ or 0.001, by one-way ANOVA) (Figure 16A–C). Taken together, these results demonstrated that Coff or Caff plus Mel inhibited GSK3β, Akt, PI3K p55, and Tau phosphorylation but enhanced PI3K p85 and Erk1/2 phosphorylation in N2a/APP cells, probably contributing to the neuroprotective and antiamyloidogenic effect. The reason for the opposing effect of Coff or Caff, plus Mel on PI3K p55 and p85 in N2a/APP cells is unknown, depending on the concentrations of Mel.

Comparison of the neuroprotective and antiamyloidogenicity-enhancing effects between Coff and Caff, plus Mel in N2a/APP cells

Based on above findings, both Coff and Caff chronologically enhanced the antiamyloidogenic activity of Mel in N2a/APP cells. Finally, we compared the beneficial effects of these two combinations (Coff plus Mel vs Caff plus Mel). As shown in Table 2, two combinations showed comparable neuroprotective and antiamyloidogenic effects in N2a/APP cells, but there were some differences in the magnitude of the effects on cell viability, Aβ42 oligomerization, and the phosphorylation of several key regulators. Caff plus Mel significantly increased cell viability but decreased the extracellular level of Aβ oligomers; whereas Coff plus Mel did not exhibit significant effects (Table 2). Caff plus



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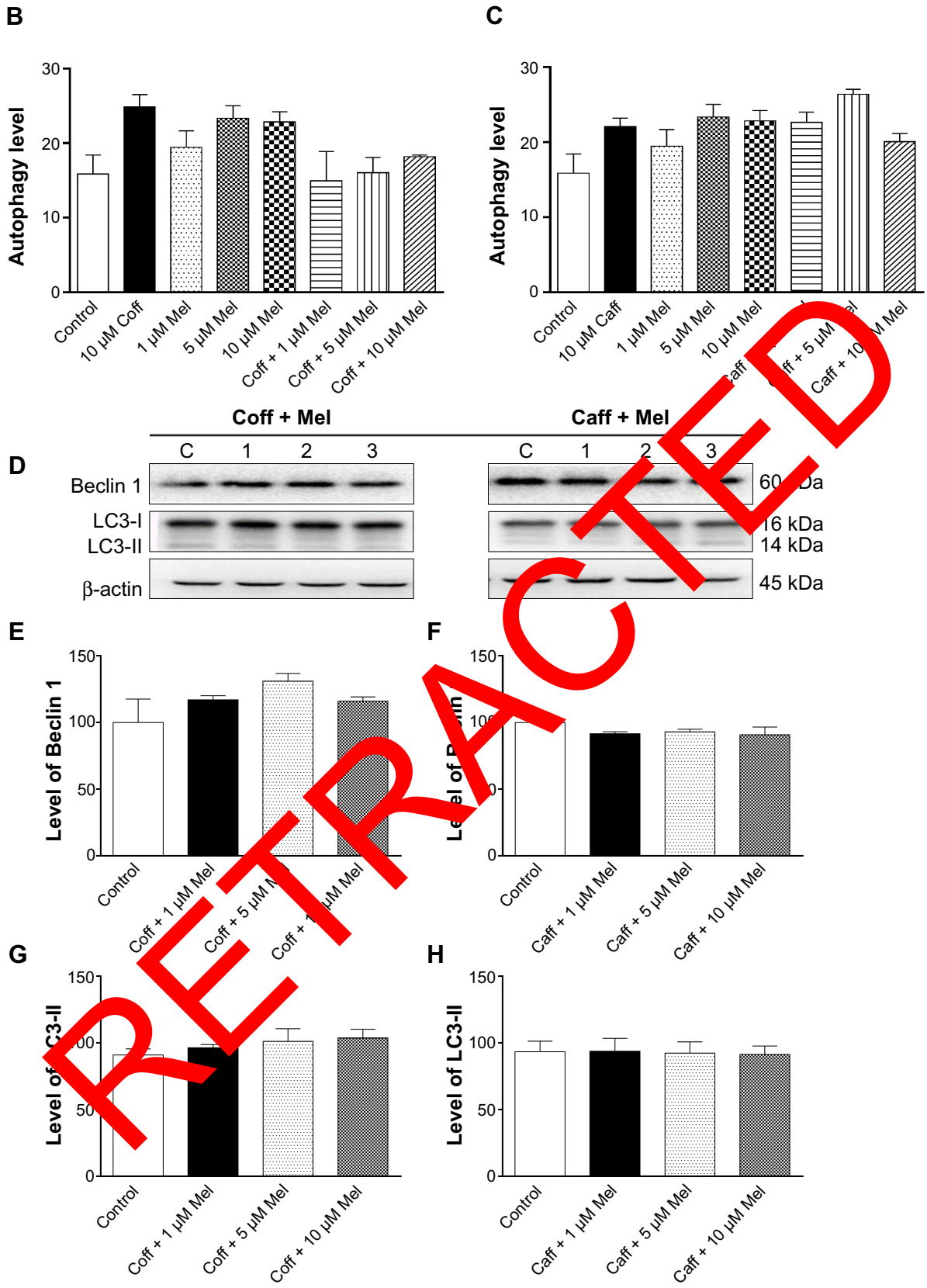

Figure 12 Effects of Coff/Caff plus Mel on autophagy in N2a/APP cells treated with regimen 1.

Notes: N2a/APP cells were treated with Coff or Caff at 10 μM for 12 hours in the day, followed by Mel at 1, 5, or 10 μM for another 12 hours in the night. (**A**) Flow cytometric analysis of autophagy; bar graphs showing the autophagy level with Coff and Mel (**B**), or Caff and Mel (**C**); (**D**) representative blots of LC3-I/II and Beclin 1; and bar graphs showing the relative expression level of Beclin 1 (**E, F**) and LC3-II (**G, H**) after treatment with Coff/Mel (**E, G**) or Caff/Mel (**F, H**).

Abbreviations: APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; FSC-A, flow cytometric channel detecting by a photodiode detector; Mel, melatonin; N2a, Neuro-2a; SSC-A flow cytometric channel detecting by a photomultiplier detector.

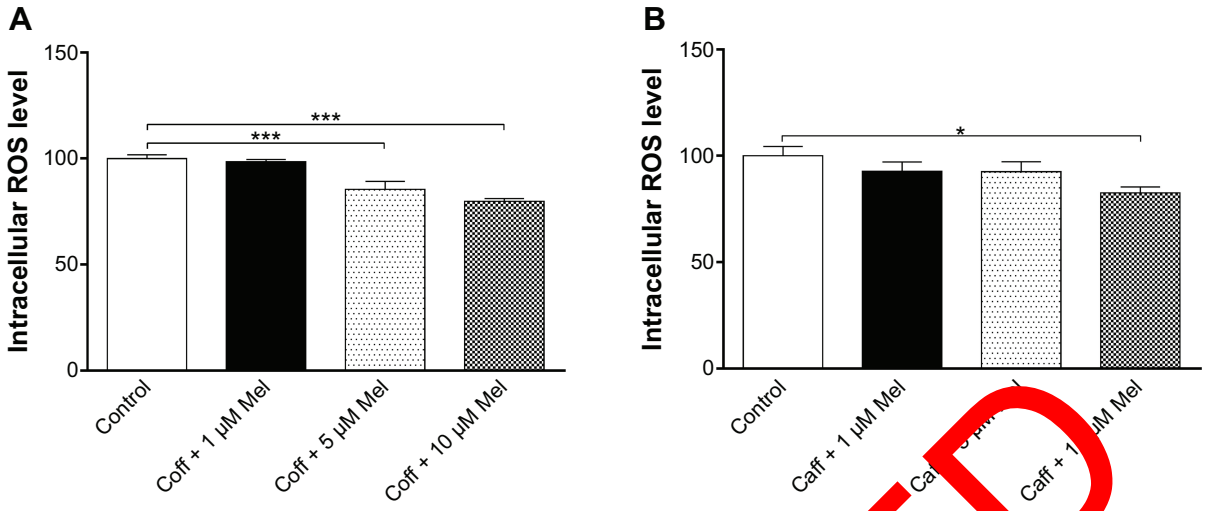

Figure 13 Effects of Coff/Caff plus Mel on intracellular ROS levels in N2a/APP cells treated with regimen 1.
Notes: N2a/APP cells were treated with Coff (**A**) or Caff (**B**) at 10 μM for 12 hours in the day, followed by M at 1, 5, o 0 μM for a er 12 hours in the night. Intracellular ROS level was determined using CM-H_2DCFDA as the probe. *P<0.05 and ***P<0.001, by one-w A /A.
Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; CM-H_2DCF , 6-carb ,7′-dichlor ydrofluorescein diacetate; Coff, coffee; Mel, melatonin; N2a, Neuro-2a; ROS, reactive oxygen species.

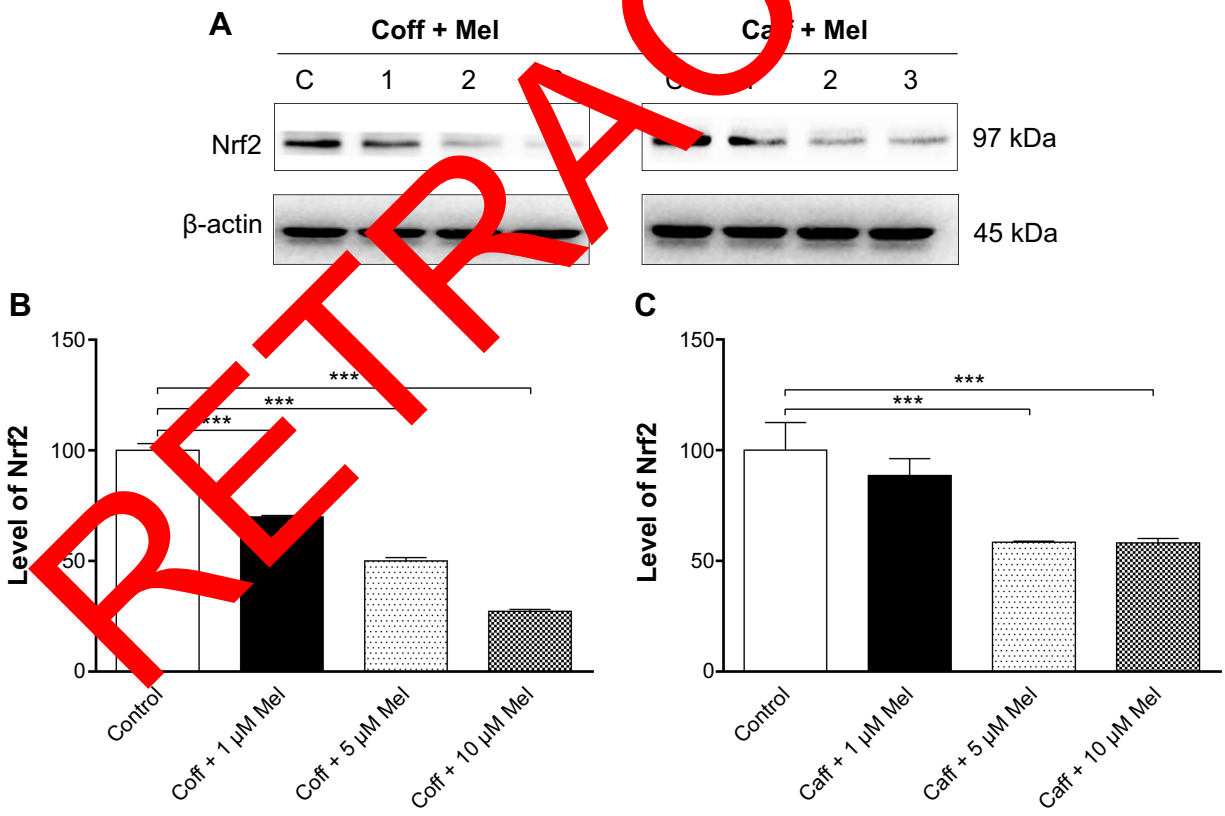

Figure 14 Effects of Coff/Caff plus Mel on the expression level of Nrf2 in N2a/APP cells treated with regimen 1.
Notes: N2a/APP cells were treated with Coff or Caff at 10 μM for 12 hours in the day, followed by Mel at 1, 5, or 10 μM for another 12 hours in the night. Cells were harvested, lysed, and subjected to SDS-PAGE. The target protein was probed using corresponding primary antibody. β-actin was used as the internal control for value normalization. (**A**) Representative blots of Nrf2; and bar graphs showing the relative expression level of Nrf2 in response to Coff and Mel (**B**), and Caff and Mel (**C**). ***P<0.001, by one-way ANOVA.
Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; Mel, melatonin; N2a, Neuro-2a; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

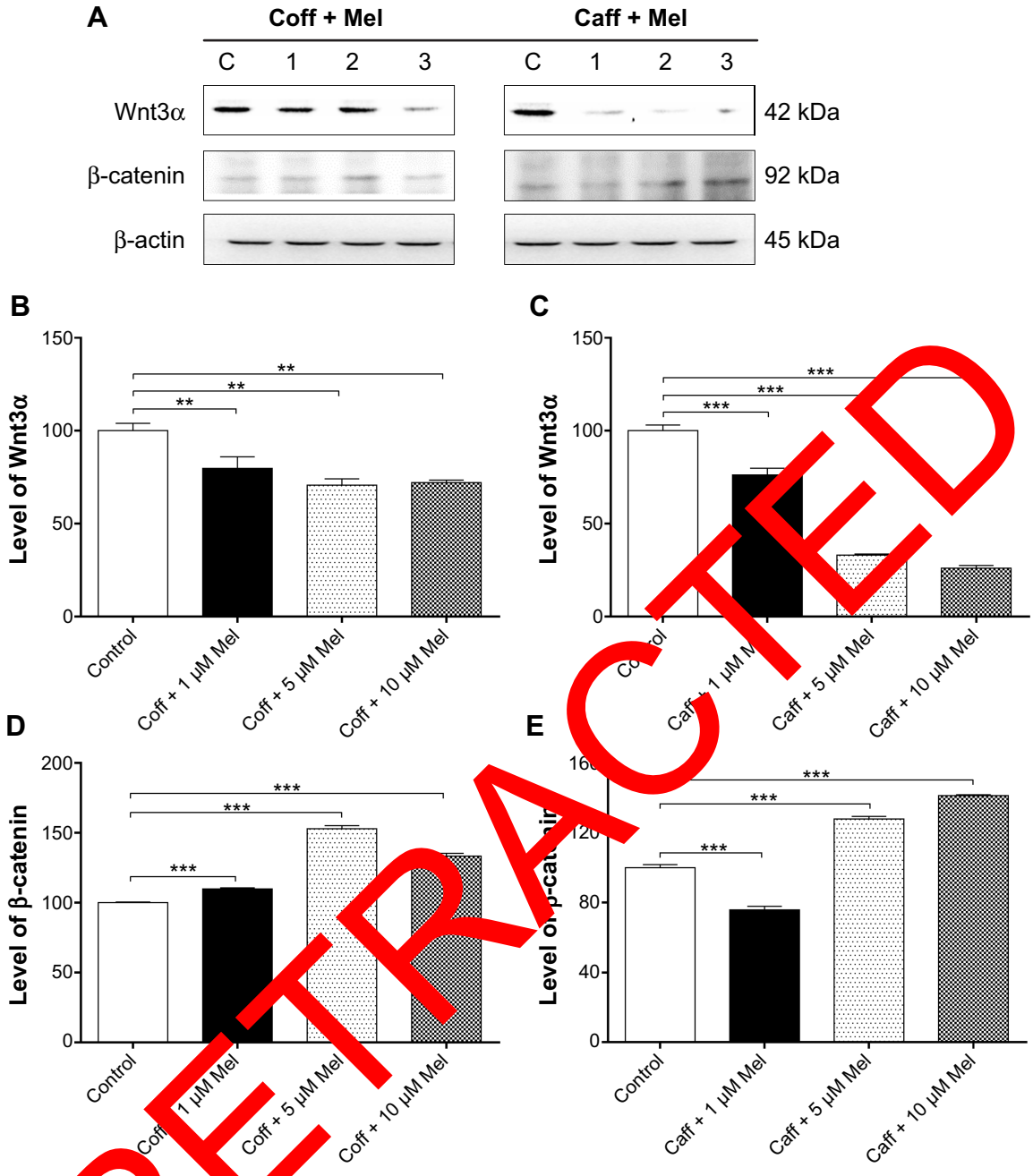

Figure 15 Effects of Coff/Caff plus Mel on the expression of Wnt3α and β-catenin in N2a/APP cells treated with regimen 1.
Notes: N2a/APP cells were treated with Coff or Caff at 10 μM for 12 hours in the day, followed by Mel at 1, 5, or 10 μM for another 12 hours in the night. Cells were harvested, lysed, and subjected to SDS-PAGE. The target proteins were probed using corresponding primary antibodies. β-actin was used as the internal control for value normalization. (**A**) Representative blots of Wnt3α and β-catenin; and bar graphs showing the relative expression level of Wnt3α and β-catenin in response to Coff and Mel (**B**, **D**), and Caff and Mel (**C**, **E**). $^{**}P<0.01$ and $^{***}P<0.001$, by one-way ANOVA.
Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; Mel, melatonin; N2a, Neuro-2a; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Mel more significantly increased the extracellular level of monomeric Aβ42 than did Coff plus Mel. Compared with the combination of Coff plus Mel, Caff plus Mel showed more potent enhancing effects on the phosphorylation of PI3K and Erk1/2. In addition, Caff plus Mel showed a stronger regulatory effect on Wnt3α than did its counterpart. Overall, Caff had a stronger enhancing effect on the antiamyloidogenic activity of Mel than Coff in N2a/APP cells, suggesting that the Caff in Coff was the main active component that enhanced the antiamyloidogenic activity of Mel and that other components in Coff might compromise the antiamyloidogenic effects of Caff.

Discussion

AD is characterized by the presence of numerous extracellular plaques and intracellular neurofibrillary tangles, and

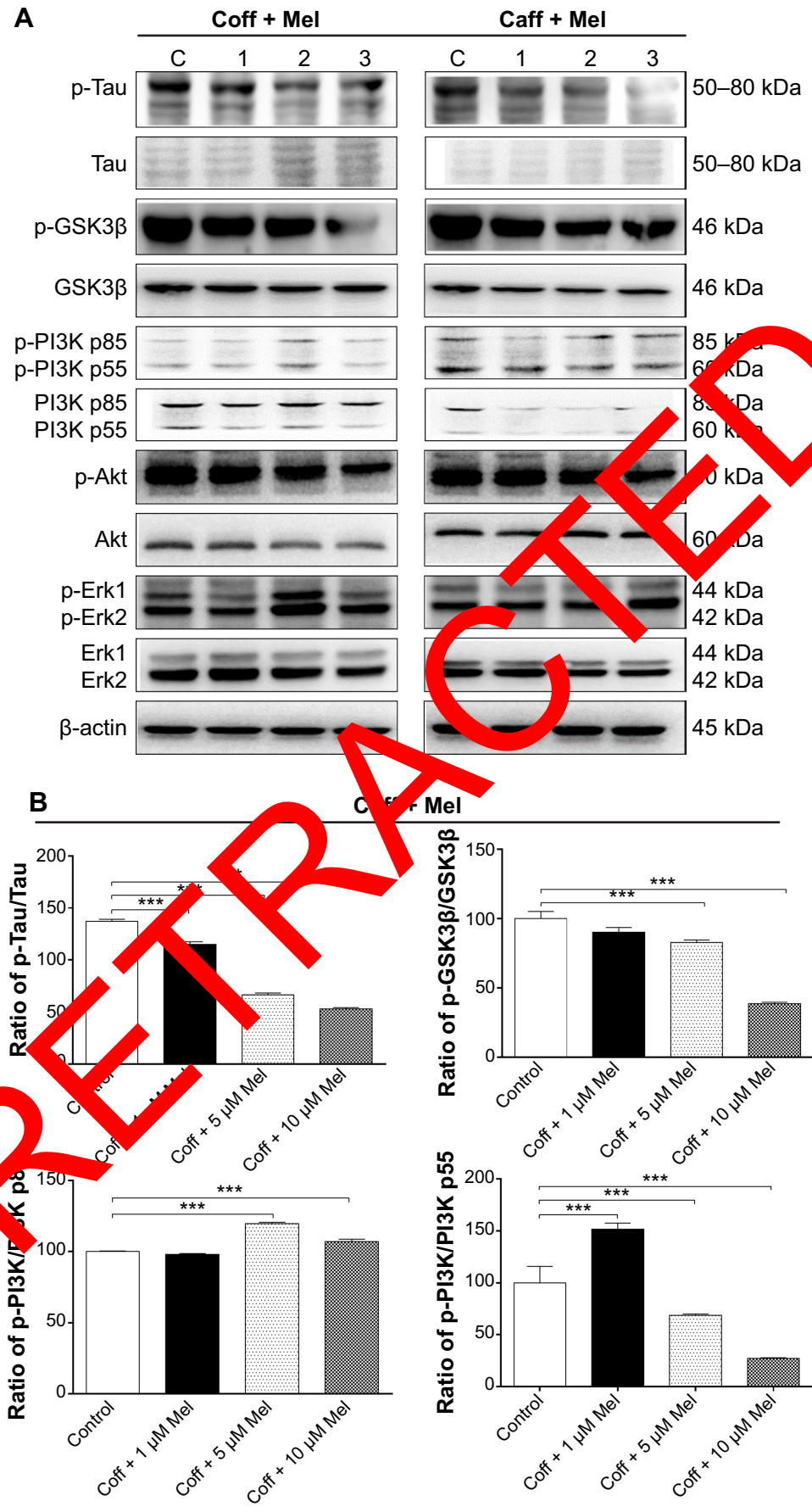

Figure 16 (*Continued*)

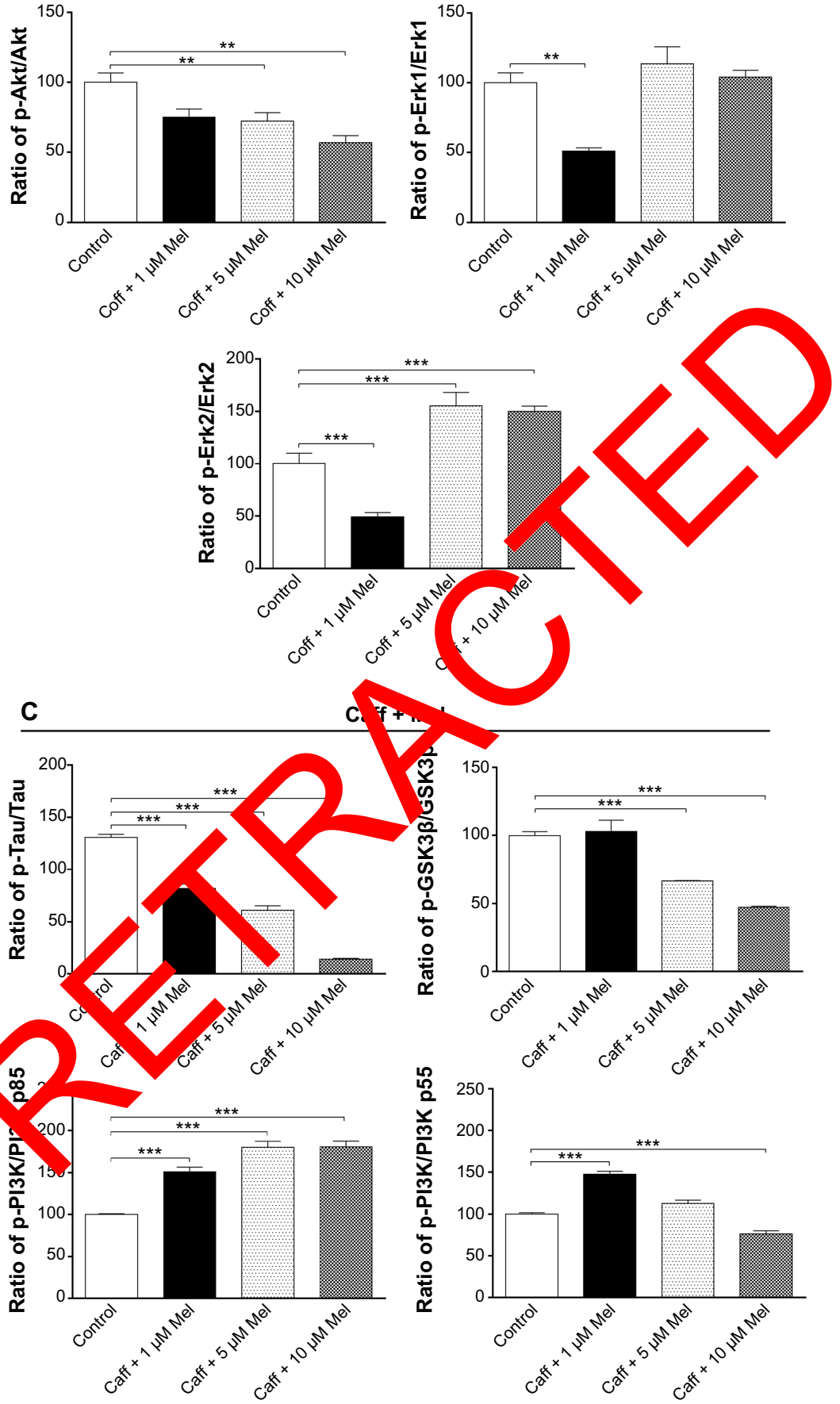

Figure 16 (*Continued*)

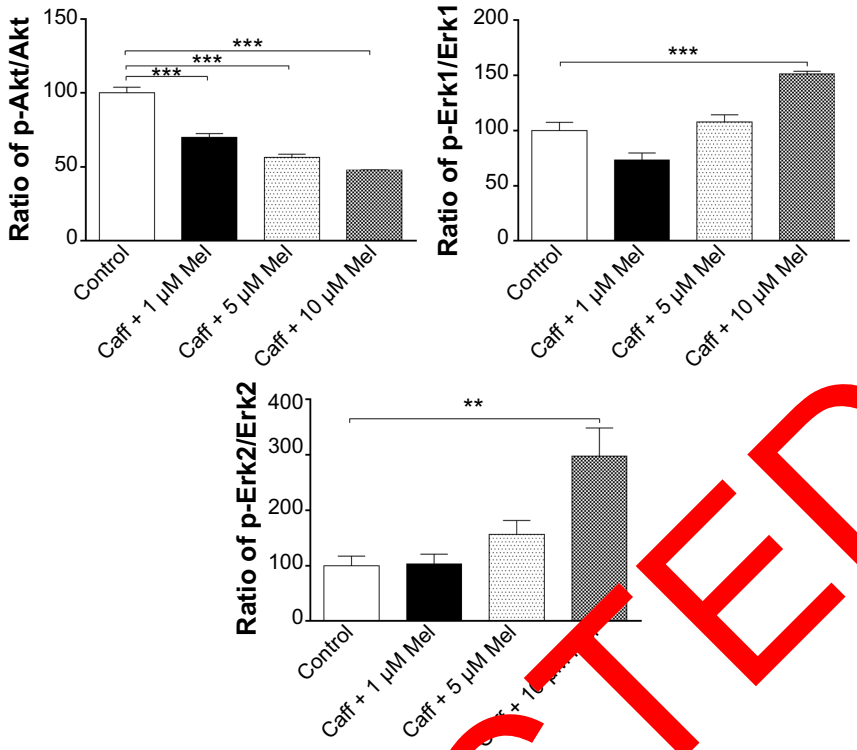

Figure 16 Effects of Coff/Caff plus Mel on the levels of p-Tau/Tau, p-GSK3β/GSK3β, p-PI3K/PI3K p85, p-PI3K/PI3K p55, p-Akt/Akt, p-Erk1/Erk1 and p-Erk2/Erk2 in N2a/APP cells treated with regimen 1.

Notes: N2a/APP cells were treated with Coff or Caff at 10 μM for 12 hours, followed by Mel at 1, 5, and 10 μM for another 12 hours. Cells were harvested, lysed, and subjected to SDS-PAGE. The target proteins were probed using corresponding primary antibodies. β-actin was used as the internal control for value normalization. (**A**) Representative blots of p-Tau/Tau, p-GSK3β/GSK3β, p-PI3K/PI3K p85, p-PI3K/PI3K p55, p-Akt/Akt and p-Erk1/2/Erk1/2, and bar graphs showing the relative expression level of p-Tau/Tau, p-GSK3β/GSK3β, p-PI3K/PI3K, p-Akt/Akt, p-Erk1/Erk1 and p-Erk2/Erk2 after treatment with Coff + Mel (**B**) or Caff and Mel (**C**). **$P<0.01$ and ***$P<0.001$, by one-way ANOVA.

Abbreviations: ANOVA, analysis of variance; APP, amyloid precursor protein; Caff, caffeine; Coff, coffee; Mel, melatonin; N2a, Neuro-2a; p, phosphorylated; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Table 2 A comparison of the antiamyloidogenic effects of Coff or Caff, plus Mel in N2a/APP cells

Regimen	Parameter	Effect	Change (Coff + Mel 10 μM)	Change (Caff + Mel 10 μM)
1	MTT	↓↑	3.95% decrease without statistical significance	21.7% increase with statistical significance***
	Aβ40	↑	2.04-fold***	4.02-fold***
	Aβ42	↑	3.95-fold***	5.91-fold***
	Aβ oligomer	↓	0.09%	22%***
	ROS	↓	20.15%***	17.48%***
	Nrf2	↓	72.65%***	41.87%***
	p-Akt/Akt	↓	42.94%**	52.02%***
	p-GSK3β/GSK3β	↓	61.43%***	52.67%***
	p-Tau/Tau	↓	61.42%***	89.12%***
	p-PI3K/PI3K p85	↑	6.96%***	80.43%***
	p-Erk1/Erk1	↑	4.2%	51.28%***
	p-Erk2/Erk2	↑	50.20%***	197.50***
	Wnt3α	↓	27.9%**	73.9%***
	β-catenin	↑	33.27%***	41.27***
2	Extracellular Aβ40 level	↑	1.21-fold***	1.18-fold***
	Extracellular Aβ42 level	↑	1.45-fold***	1.33-fold**
3	Extracellular Aβ40 level (12 h)	↑	1.02-fold	0.93-fold***
	Extracellular Aβ42 level (12 h)	↑	0.83-fold***	0.87-fold*
	Extracellular Aβ40 level (24 h)	↑	0.96-fold	0.93-fold
	Extracellular Aβ42 level (24 h)	↑	1.25-fold	1.13-fold

Notes: The results are compared with the control cells. *$P<0.05$; **$P<0.01$; and ***$P<0.001$, by one-way ANOVA. Down and up arrows mean decrease and increase, respectively.

Abbreviations: Aβ, amyloid-β; ANOVA, analysis of variance; Caff, caffeine; Coff, coffee; GSK3β, glycogen synthase kinase-3β; Mel, melatonin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p, phosphorylated; ROS, reactive oxygen species.

extracellular Aβ accumulation and Tau hyperphosphorylation and aggregation are the hallmarks of AD.[1,15,21] Excessive extracellular Aβ consequently triggers the hyperphosphorylation and aggregation of Tau protein. Inhibition of the production and increase in the clearance of Aβ are the promising strategies to treat AD.[1,15,21] In the present study, various chronological regimens of the combination of Caff or Coff plus Mel showed inhibitory effects on the formation of Aβ oligomers, ROS generation, and Tau signaling pathway in N2a/APP cells, which may partially explain their neuroprotective effect in AD mice.

Our data clearly show that the combination of Coff or Caff, with Mel had more potent antiamyloidogenic effects than did a single agent, and Caff showed a larger antiamyloidogenic activity than did Coff in N2a/APP cells. There is epidemiological evidence that supports therapeutic benefits of Coff consumption against development of AD. In a population-based study with follow-up of 21 years, people who drank three to five cups of coffee per day during midlife were observed to have a 65% lower risk of developing AD later in life as compared with those who drank little or no Coff.[71] A meta-analysis of pooled epidemiological studies also reported protective effects of Coff consumption against AD.[72] On the other hand, decaffeinated coffee may have limited protective effects against AD, implicating the important role of Caff as the major active component against AD. We have previously observed potentiating effect of other unknown Coff components on Caff's benefits against AD.[37] Further studies are required to investigate and compare the effects of caffeinated and decaffeinated Coff on AD, and the dose-response and dose-toxicity relationships of caffeinated Coff and Caff in the treatment of AD should be established.

BACE1 catalyzes the proteolytic cleavage of APP between Met and Asp residues in the sequence stretch of Lys670-Met671-Asp672.[18] BACE1 is reinternalized from the cell surface to early endosomes and can be recycled back to the cell surface. Considering the long half-life of BACE1 (~9 hours) and the recycling rate, BACE1 moves between the cell surface and the endosomal system many times through the course of its life span.[18–20] A double mutant APP with Asn670-Leu671-Asp672 sequence at the cleavage site, found in Swedish patients with early onset AD, is a more efficient substrate in vitro than the wild-type protein and is the cause for the disease in this family. Transgenic mice carrying these mutations showed memory deficit, with a fivefold increase in Aβ40 and a 14-fold increase in Aβ42/43.[73] Our molecular docking results showed that Caff could bind to BACE1 via the formation of hydrogen bonds at Thr72, Gln73, Thr232, Lys238, Lys239, and Lys246. Mel could also bind to BACE1 via hydrogen bond formation at Gly11, Gly13, Asp32, Thr72, Asp93, Gly95, Phe108, Ser229, Gly230, Thr232, Tyr259, and Arg307. Furthermore, Mel formed π-π stacking with Tyr71 of BACE1. BACE1 is a monomeric protein, with the catalytic site containing the two aspartate residues, Asp32 and Asp228, located between the N- and C-terminal domains. BACE1 has two aspartic protease active site motifs, DTGS (residues 93–96) and DSGT (residues 289–292), and mutation of either aspartic acid renders the enzyme inactive. The active site is covered by a flexible antiparallel β hairpin between Val67 and Glu77 (commonly known as the "flap") that forms a large portion of the binding pocket and offers shielding from the solvent to facilitate efficient enzymatic catalysis.[74] BACE1 becomes activated within the late Golgi compartments and endosomes/lysosomes. The acidic conditions optimal for the enzyme (pH of 4.5–6) indicate that an inhibitor containing a basic amine with a pKa of ≥6.5 would show a high affinity. Tyr71 is a key residue in the flap, which adopts a conformation complementary to the shape and nature of the substrates/inhibitors bound in the active site.[74] The changing position of the flap relative to the catalytic dyad provides a means for the substrates/inhibitors to get access into the active site.[74] The conformation of BACE1 is defined as "closed" in the inhibitor-bound form when the Tyr71 side chain hydroxyl on the flap is within hydrogen bonding distance to the nitrogen of the Trp76 indole side chain (with a distance of 3.3 Å), but the hydrogen bond in the β-strands between Tyr71 and Gly74 is not formed, which physically separates the S1 and S2' subsites. Also, the side chain of Arg128 occupies a space between Arg128 and Tyr198 in the inactive form. In contrast, the conformation of BACE1 is defined as "open" when the flap moves away from the catalytic Asp, the hydrogen bond between the flap residues Tyr71 and Trp76 is not formed, and Tyr71 does not occupy the position between the S1 and S2' subsites.[74] In the open form of BACE1, Tyr71 may adopt a unique orientation to form a hydrogen bond between the backbone carbonyl of Lys107, and the hydrogen bond in the β-strands between Tyr71 and Gly74 is formed.[75] As such, conformational change in the flap must take place upon binding of the substrate/inhibitor to the active site and may participate kinetically in substrate binding in the closed conformation and product release in the open conformation. Both Caff and Mel are known BACE1 inhibitors.[37–39] BACE1 belongs to the pepsin family of the aspartyl protease superfamily and has the most sequence identity with BACE2 (52%), cathepsin D (29%), pepsin (27%), cathepsin E (27%), and renin (24%).[18–20] To date, a number of BACE1 inhibitors

have been developed and tested at the preclinical stage; several of them, including LY2886721, E-2609, MK-8931, RG-7129, HPP-854, and AZD-3839, have been evaluated in Phase I studies. The open form of the BACE1 enzyme appears to offer a more drug-like binding cavity than does the closed form of the enzyme. The inhibitors that target the open-form BACE1 are of low molecular weight; high binding efficiency; low polar surface area with improved metabolic stability, P-glycoprotein efflux, and oral bioavailability, and Aβ-lowering activity, in animal models and in human clinical trials.[20,76]

The monomeric Aβ (molecular weight ~4 kDa) is generated mainly in neurons from APP (molecular weight ~120 kDa) via sequential scission by β- and γ-secretase.[13,17,77,78] Aβ is a heterogeneous mixture of peptides with distinct solubility, stability, and biological and toxic properties. γ-Secretase cleaves APP at different positions, giving rise to a variety of peptides, of which Aβ43, Aβ42, Aβ40, Aβ38, and Aβ37 can be detected in cell culture and body fluids.[13,17,77,78] The heterogeneity of Aβ is enhanced by other enzymes, such as aminopeptidases, glutaminyl cyclase, isomerases, and protein kinases, resulting in a mix of more than 20 Aβ peptides that all participate in putative Aβ functions in the normal brain and in oligomerization and fibrillization in AD.[13,17,77,78] Aβ40 is continuously and abundantly produced in both the normal and AD brain, whereas other Aβ peptides are formed at lower levels. Aβ40 and Aβ42 are present in plaques, and increased Aβ40 and/or Aβ42 levels have been implicated in the pathogenesis of AD.[1,15,21,79] Aβ can self-assemble into fibrils, protofibrils, and soluble oligomeric structures. Amyloid plaques might exist in equilibrium with oligomeric forms of Aβ, consisting of cross-β-sheet units of Aβ peptides that are arranged to form amyloid fibrils. The monomeric form of Aβ has no cytotoxicity toward neurons, whereas Aβ oligomers are neurotoxic, resulting in cellular dysfunction and cell death.[77–80] Aβ oligomers may be metastable intermediates in the pathway to an insoluble, cross-β-sheet-based fiber. Aβ monomers, oligomers, and fibrils exist in a complex equilibrium, sensitive to numerous external factors. Loss of function and decrease in the number of neurons eventually impair cognitive function and lead to AD.[60] There is evidence that Aβ oligomers are important neurotoxic forms, which are composed of two to 32 monomers.[77–80] The neurotoxicity of Aβ oligomers is associated with the loss of synapses, a process that leads to hippocampal long-term potentiation decrease. This impairing effect of Aβ oligomers could underlie the cognitive impairment associated with AD.[81,82] In the present study, we found that treatment of N2a/APP cells with Coff or Caff plus Mel increased the extracellular levels of the monomeric form of Aβ40/42 but decreased the extracellular level of Aβ oligomers, suggesting that the combination therapy suppressed Aβ oligomerization and thus increased the level of nontoxic monomers of Aβ peptides. Coff or Caff plus Mel combinations appeared to affect the equilibrium between Aβ monomers and oligomers, favoring the existence of Aβ monomers.

Intervention in the Aβ aggregation process has been considered as a practical approach to stop or slow the progression of AD. It has been demonstrated that Mel directly interacts with Aβ, and prevents its aggregation and inhibits the progressive formation of β sheet and/or amyloid fibrils.[83,84] Mel may accelerate the conversion of β sheets into random coils by disrupting the imidazole–carboxylate salt bridges, which prevent Aβ fibrillogenesis and aggregation. It therefore blocks the formation of the secondary β sheet conformation. Moreover, Mel facilitates the clearance of Aβ peptides via increasing proteolytic degradation. On the other hand, it has been shown that Coff and Caff can attenuate cognitive impairment and improve memory function, in AD and in other chronic neurodegenerative disorders, through the regulatory effect on mitochondrial function and oxidative stress.[85]

Previous studies on Coff, Caff, and Mel have shown beneficial effects in AD.[86,87] However, there was no report on the combined use of Coff/Caff with Mel with chronological considerations. In our study, we treated A2a/APP cells with Coff/Caff in the day, followed with Mel in the night, which is consistent with rhythm of Mel secretion in human pineal gland. We therefore speculated that N2a/APP cells may have their own circadian rhythms, leading to different effects on cells treated with the same drug at the same concentration with different chronological regimens. To test this speculation, we treated N2a/APP cells with three different chronological regimens and found that the antiamyloidogenic effects of the Coff/Caff plus Mel was regimen-dependent, suggesting the importance of chronotherapy in AD treatment. A most remarkable and synergistic/additive effect on reduced extracellular Aβ40/42 level was observed in N2a/APP cells treated with regimen 1, where cells were treated with Caff/Coff in day time, followed by Mel in night time, whereas this effect was smaller or disappeared when regimens 2 and 3 were used. These findings support the pathophysiological and therapeutic implications of circadian rhythms in AD. Previous studies have shown that Caff/Coff administration in day time benefited AD or dementia in mice or humans.[88–90] It has also been shown that there is a circadian clock in N2a cells

and that treatments were only effective at specific circadian times.[91] Abnormal and disturbed circadian rhythms are common in AD.[30,46] Chronotherapeutic approaches aimed at bolstering weakened circadian rhythms in AD produce beneficial outcomes. A double-blind study of Mel in AD patients revealed improvements in cognition as well as decreasing nocturnal activity and increased nocturnal sleep.[92] Further studies are needed to optimize the chronotherapy for AD using Coff/Caff and Mel.

Oxidative stress and impaired energy metabolism play an important role in the pathogenesis of AD.[1,21,58,59] Cerebral tissues are particularly vulnerable to oxidative damage, due to its high oxygen consumption, low content of antioxidants, and high content of polyunsaturated fatty acids of neuronal membranes. Aβ itself and phosphorylated Tau cause oxidative damage to neurons.[1,21,58,59] Mitochondrial dysfunction in AD could cause an increased generation of free radicals, and damage major cellular macromolecules, including DNA, proteins, and lipids. Dysregulation of Nrf2 plays a role in the development and pathogenesis of AD, and Nrf2 can attenuate Aβ-induced neurotoxicity and Tau phosphorylation.[93–95] The neuroprotective and antiamyloidogenic activities of Coff, Caff, and Mel may be attributed to their antioxidative effects through the involvement of Nrf2 signaling pathway. Previous studies showed that Coff activated the Nrf2-mediated signaling pathway to exert its neuroprotective effect.[96] Nrf2 is a nuclear transcription factor, playing a pivotal role in the regulation of oxidative stress by modulating the transcription of antioxidant response elements.[68] The effect of Coff on Nrf2 activation indicates that Coff may reduce oxidative stress through Nrf2-mediated signaling pathways. In addition, it has been reported that Mel possesses a strong antioxidative effect.[97,98] In the present study, we found that the combination of Coff/Caff plus Mel in regimen 1 significantly reduced the oxidative stress in N2a/APP cells, contributing to the neuroprotective and antiamyloidogenic effect.

It has been reported that inhibition of GSK3 can exhibit profound neuroprotective effects, which have been proposed as promising and practical therapeutic targets for the treatment of AD.[48] GSK3 is regarded as a critical molecular link between senile plaques and neurofibrillary tangles. It can trigger the hyperphosphorylation of Tau, resulting in an exaggerated progressive condition of AD. Moreover, GSK3 can be inhibited by Akt in the Akt/GSK3β/Tau pathway.[99] It has been shown that the regulatory effect of Mel on the Akt/GSK3β/Tau pathway is, at least in part, responsible for is beneficial effect.[100] Wang et al[101] showed the consensus change in the Akt/Gsk3β/Tau pathway in N2a cells. In agreement with the previous studies, our findings showed that phosphorylation levels of Akt, GSK3β, and Tau were decreased in N2a/APP cells treated with the combination of Coff/Caff plus Mel. Notably, our studies provided evidence for the stronger effect of the combination of Caff in day time, followed by Mel in night time on inhibiting Tau hyperphosphorylation. Moreover, the combinations of Coff/Caff plus Mel negatively regulated Wnt/β-catenin signaling, which may contribute to the attenuation in the Akt/GSK3β/Tau signaling in N2a/APP cells. Intriguingly, the combination of Coff/Caff plus Mel showed a promoting effect on the phosphorylation of PI3K and Erk1/2 in N2a/APP cells. PI3K and Erk1/2 play a critical role in the regulation of cell growth, cell migration, cell survival, and cell death. All the regulating effects of Coff/Caff plus Mel on above important signaling molecules may contribute to proproliferative, neuroprotective, and antiamyloidogenic activities in N2a/APP cells.

Inflammation and dysregulated autophagy have a causal role in the pathogenesis of AD.[102–105] However, our data did not support the concept that Coff/Caff plus Mel produced antiamyloidogenic effect through modulation of these two processes in N2a/APP cells. In other studies, Mel has been known to have potent anti-inflammatory effects, via suppression of cytokine and nitric oxide production.[106–108] Caffeine has a potent modulating effect on inflammation, through adenosine receptor and other pathways.[109,110] Both Caff and Mel are potent inducers of autophagy in different animal and disease models.[63,111,112] Therefore, further studies are needed to explore the effects and the underlying mechanisms of Coff, Caff, and Mel on inflammation and autophagy.

In summary, Coff and Caff chronologically enhanced the antiamyloidogenic activity of Mel through suppression of Aβ oligomerization and modulation of the Akt/GSK3β/Tau pathway in N2a/APP cells (Figure 17). We have observed that the antiamyloidogenic effects of Coff/Caff plus Mel were more potent than were Coff, Caff, or Mel alone. Since Caff is mainly metabolized to paraxanthine, theobromine, and theophylline, and Mel is principally metabolized to 6-hydroxymelatonin, which is further conjugated with sulfate, these metabolites may contribute to the antiamyloidogenic action. Further studies are warranted to dissect the role of adenosine and melatonin receptors and other molecules for the neuroprotective and antiamyloidogenic activities of Coff or Caff plus Mel. These data will provide strong rationales for the Phase I trials on the combination of Coff or Caff, plus Mel in AD patients, when chronotherapy is taken into consideration.

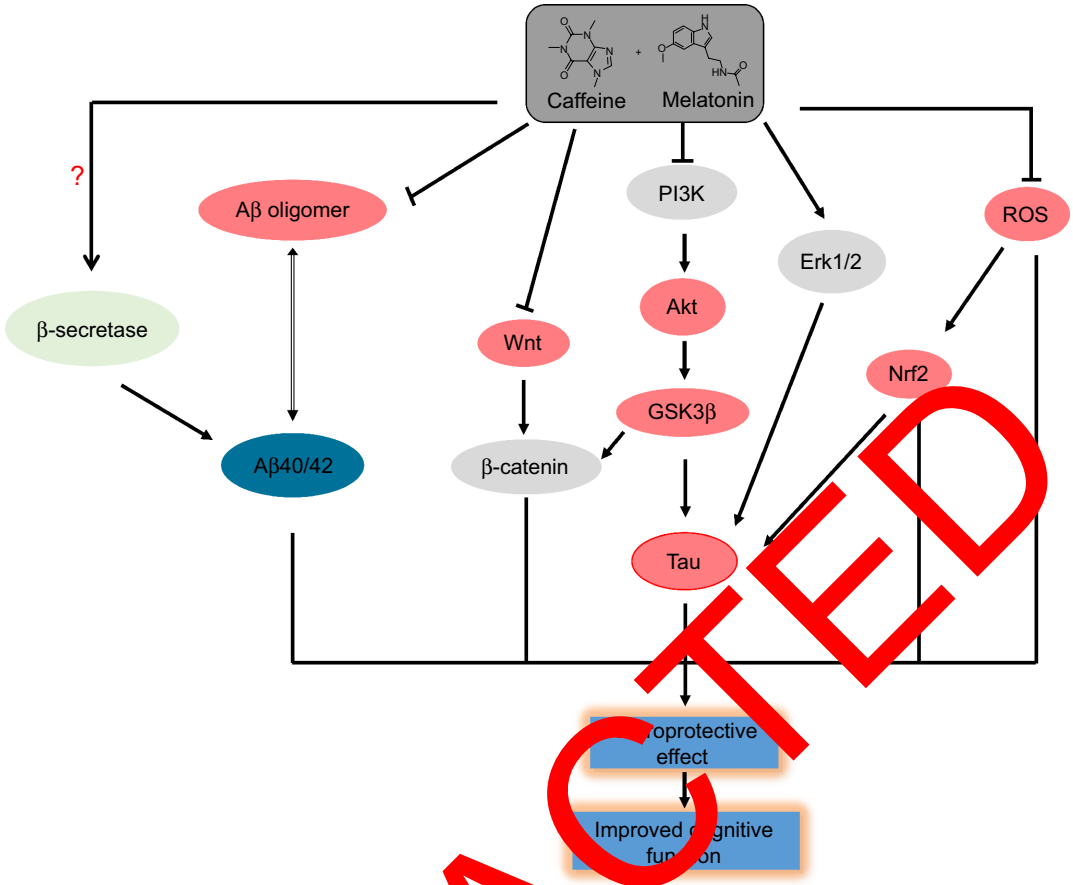

Figure 17 Proposed mechanisms for the neuroprotective effects of Coff/Ca plus Me /APP cells.
Notes: The combination of Coff/Caff plus Mel promotes the phosphorylati of K and Erk1/2 and suppresses the phosphorylation of GSK3β, Akt, and Tau in N2a/APP cells. Moreover, the combinations repress Wnt and RO g, with r ction in the expression level of Wnt3α and Nrf2 and intracellular ROS level. At last, the combinations decreased the extracellular level of Aβ olig ers, whic are neuro xic.
Abbreviations: APP, amyloid precursor protein; Ca caffeine; C , coffee; M melatonin; N2a, Neuro-2a; ROS, reactive oxygen species; GSK3β, glycogen synthase kinase-3β; Aβ, amyloid-β.

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Disclo ure

The au ors repo no conflicts of interest in this work.

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