Curcumin and hesperetin attenuate D-galactose-induced brain senescence \textit{in vitro} and \textit{in vivo}

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ABSTRACT

BACKGROUND/OBJECTIVES: Brain senescence causes cognitive impairment and neurodegeneration. It has also been demonstrated that curcumin (Cur) and hesperetin (Hes), both antioxidant polyphenolic compounds, mediate anti-aging and neuroprotective effects. Therefore, the objective of this study was to investigate whether Cur, Hes, and/or their combination exert anti-aging effects in D-galactose (Dg)-induced aged neuronal cells and rats.

MATERIALS/METHODS: SH-SY5Y cells differentiated in response to retinoic acid were treated with Cur (1 \textmu M), Hes (1 \textmu M), or a combination of both, followed by 300 mM Dg. Neuronal loss was subsequently evaluated by measuring average neurite length and analyzing expression of \textit{\beta}-tubulin III, phosphorylated extracellular signal-regulated kinases, and neurofilament heavy polypeptide. Cellular senescence and related proteins, p16 and p21, were also investigated, including their regulation of antioxidant enzymes.

In vivo, brain aging was induced by injecting 250 mg/kg body weight (b.w.) Dg. The effects of supplementing this model with 50 mg/kg b.w. Cur, 50 mg/kg b.w. Hes, or a combination of both for 3 months were subsequently evaluated. Brain aging was examined with a step-through passive avoidance test and apoptosis markers were analyzed in brain cortex tissues.

RESULTS: Cur, Hes, and their combination improved neuron length and cellular senescence by decreasing the number of \textit{\beta}-gal stained cells, down-regulated expression of p16 and p21, and up-regulated expression of antioxidant enzymes, including superoxide dismutase 1, glutathione peroxidase 1, and catalase. Administration of Cur, Hes, or their combination also tended to ameliorate cognitive impairment and suppress apoptosis in the cerebral cortex by down-regulating Bax and poly (ADP-ribose) polymerase expression and increasing Bcl-2 expression.

CONCLUSIONS: Cur and Hes appear to attenuate Dg-induced brain aging via regulation of antioxidant enzymes and apoptosis. These results suggest that Cur and Hes may mediate neuroprotective effects in the aging process, and further study of these antioxidant polyphenolic compounds is warranted.

Keywords: Curcumin; hesperetin; brain; aging; D-galactose

INTRODUCTION

Aging is characterized by a progressive decline in physiological function and integrity [1]. Similar to most organs which deteriorate and accumulate deleterious changes with aging,
Conflict of Interest
The authors declare no potential conflicts of interests.

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the brain also becomes impaired in regard to its structure, function, and neurocognition with aging [2,3]. An aged brain is characterized by fewer neurons, hippocampus atrophy, and cognitive decline [3]. Correspondingly, cognitive and memory impairments are common clinical characteristics of brain aging, and are also key factors for maintaining independence in daily activities and overall quality of life in older adults [4]. In particular, the cerebral cortex and hippocampus are the most relevant regions of the brain for aging. These regions undergo changes in structure, weight, and thickness [5]. These regions are also responsible for long-term and short-term memory, physical performance, and cognition [5,6]. Both brain volume and weight decrease with aging. In addition, neuronal generation is rapidly diminished [7,8].

D-galactose (Dg) is a reducing sugar which naturally exists in the body. Foods such as dairy products and soy sauce also contain Dg [9]. However, the presence of excess Dg can induce inflammation, oxidative stress, and apoptosis in neuronal cells [10,11]. Thus, chronic or high-dose exposure to Dg either in vitro or in vivo is commonly used to induce artificial senescence and brain damage. Markers of this damage include the senescence-related markers, p16, p21, and p53, as well as senescence-associated β-galactosidase (SA-β-gal) staining [3,12,13]. Furthermore, exposure to Dg can enhance mitochondria dysfunction, inflammation, oxidative stress, and apoptosis to cause memory decline and aging [3,14].

Over the past few decades, it has been demonstrated that plant-based polyphenols can mediate delay in senescence by regulating oxidative stress. For example, anti-aging effects of polyphenols such as anthocyanin, isoflavone, and caffeine have been verified in Dg-induced aged models [15-17]. In Asia, curcumin (Cur) is a polyphenolic non-flavone compound derived from turmeric (Curcuma longa), and it is broadly used as both a food spice and an herbal medicine [18]. Cur has been associated with several biological benefits, including anti-inflammation, anti-cancer, and anti-aging properties [19]. These anti-aging and longevity effects of Cur have been elucidated in various experimental models, including Caenorhabditis elegans, fruit fly, and mouse [20-22]. However, despite these significant beneficial effects, Cur is characterized by low bioavailability and low solubility. Thus, it has been proposed that encapsulation or a combination administration can improve absorption [23-25].

Hesperetin (Hes), a hesperidin aglycone, is a polyphenolic flavanone which can be extracted from citrus fruit [26,27]. It has been reported that Hes mediates antioxidant and neuroprotective effects both in vitro and in vivo [26,28]. However, the ability of Cur and Hes to improve Dg-induced brain aging has not been elucidated. Therefore, the objective of this study was to investigate whether Cur, Hes, and/or their combination exert anti-aging effects in Dg-induced aged neuronal cells and rats.

MATERIALS AND METHODS

Cell culture and reagents
SH-SY5Y (SY5Y) cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in a 1:1 mixture of Minimum Essential Medium and Ham’s F-12 (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1% penicillin (100 U/mL), and streptomycin (100 μg/mL) (Invitrogen, Carlsbad, CA, USA). Cells were maintained at 37°C with 5% CO₂ atmosphere.

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All-trans-retinoic acid (RA), Cur, Hes, and Dg were purchased from Sigma-Aldrich (St. Louis, MO, USA). RA, Cur, and Hes were dissolved in dimethyl sulfoxide (DMSO). SY5Y cells were treated with RA (10 μM) for 6 days to induce differentiation. Media containing 5% FBS was subsequently used to minimize a serum effect. Due to the light sensitivity of the compounds used, all cell culture experiments were performed under dim light.

**Cell proliferation assays**

Cell proliferation was evaluated in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays. Briefly, SY5Y cells were seeded in 96-well plates (6 × 10⁴ cells/well) for 24 h and then were differentiated with RA. The cells were then pretreated with various doses of Cur and Hes for 24 h before treatment with 300 mM Dg for 3 days. Treated cells were subsequently incubated in medium containing MTT solution for 3 h at 37°C. After the medium was removed, 150 μL DMSO was added. Optical density was measured for each well at 560 nm in a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

**Morphological changes in differentiated SY5Y cells**

To evaluate neurite outgrowth after neuronal cell differentiation, a low density of SY5Y cells were seeded on 6-well plates and differentiated with RA for 6 days. The cells were subsequently treated with Cur, Hes, or their combination for 24 h, followed by Dg to induce cellular senescence. After 3 days, cell images were obtained at 100× magnification (Olympus, Tokyo, Japan). Neurite length was measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

**Assessment of cellular senescence**

SA-β-gal assays were performed as previously described [12]. Briefly, differentiated SH-SY5Y cells were pre-treated with 1 μM Cur, 1 μM Hes, or their combination for 24 h, Dg (300 mM) was subsequently added. After 3 days, cells were washed with phosphate buffered saline and fixed with formaldehyde for 15 min. After incubation with SA-β-gal staining solution (Cell Signaling Technology, Danvers, MA, USA) for 24 h at 37°C, stained cells were imaged at 100× magnification (Olympus). The numbers of positively stained cells were counted and the percentage of positive cells was calculated.

**Protein extraction and Western blot assays**

Western blot assays were performed as previously described [12]. Briefly, cell cytosol or frozen brain tissue proteins were extracted with protein extraction solution (PRO-PREP; iNtRON Biotechnology, Seongnam, Korea). These extracts were then separated in sodium dodecyl sulfate polyacrylamide gels and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). After the membranes were blocked with 5% bovine serum albumin or non-fat dry milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBS-T) at room temperature (RT) for 1 h, they were incubated with primary antibodies diluted in blocking solution at 4°C overnight. The membranes were subsequently washed with TBS-T several times and then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies at RT. After 1 h, the membranes were incubated with enhanced chemiluminescence solution (Visual Protein, Taipei, Taiwan) to detect and visualize bound antibodies with X-ray film (Agfa, Mortsel, Belgium). The primary antibodies were used for Western blot as follows: β-tubulin III, neurofilament heavy polypeptide (NEFH), and α-tubulin from Sigma Aldrich; superoxide dismutase (SOD) 1, catalase, Bax, and Bcl-2 from Santa Cruz Biotechnology (Dallas, TX, USA); glutathione peroxidase (Gpx) 1 from Abfrontier (Seoul, Korea); phosphorylated extracellular signal-regulated kinases (ERK) and...
poly (ADP-ribose) polymerase (PARP) from Cell Signaling Technology; p16, p21, and β-actin from Abcam (Cambridge, MA, USA).

**Animal treatment**
Male Wistar rats (10 weeks old, 290–310 g) were purchased from Central Lab Animal Inc. (Seoul, Korea). All of the animals were maintained under controlled temperature (22 ± 2°C) and humidity (50 ± 5%) conditions with a 12 h/12 h light/dark cycle. All of the animals received an AIN-93G diet (Raon Bio, Seoul, Korea) and sterile water *ad libitum* during the experimental period. After a 7 days adaptation period, the animals were randomly divided into 5 groups as follows: control (Ctrl) group (n = 10), 250 mg/kg body weight (b.w.) Dg-induced aging (n = 10), 250 mg/kg b.w. Dg with 50 mg/kg Cur (n = 10), 250 mg/kg b.w. Dg with 50 mg/kg b.w. Hes (n = 9), and 250 mg/kg b.w. Dg in combination with 50 mg/kg b.w. Cur and 50 mg/kg b.w. Hes (Combi, n = 10). The Ctrl and Dg groups received 1.5% carboxyl methycellulose (CMC, Sigma Aldrich) orally as a vehicle. Animals in the Cur, Hes, and Combi groups were administered each compound mixed with 1.5% CMC orally. Aging was induced by subcutaneous injection of 250 mg/kg b.w. Dg daily for 3 months. Control animals were injected with 0.9% saline. Body weight and food intake were recorded twice a week and supplements were freshly prepared based on body weight prior to each administration.

**Passive-avoidance test**
Passive avoidance tests were performed by using a shuttle box apparatus (Jeong do BNP, Seoul, Korea). This apparatus is divided into 2 parts: an illuminated room and a dark room which are separated by a guillotine door. The behavior test was performed as previously described with modification [29-31]. Before the rats were trained, each of the animals was put into the apparatus and allowed to move freely between the 2 rooms for 5–10 min. This was conducted for the last 2 days of the treatment in order to adapt the animals to the apparatus. On the following day, an acquisition trial was performed. Briefly, each rat was gently held with their body placed on the illuminated room, not faced the guillotine door. When the rats turned their body to the door, the door was opened to let the animals enter the dark room. The initial latency time of entrance of the animals to the dark room from the time of the door opened was recorded. Once the entire body and tail of the animal entered the dark room, the door was closed and an electronic shock (0.6 mA, 5 sec) was delivered when all paws were on the grid. The animals were subsequently returned to the illuminated room. Twenty-four h later, the animals were placed in the illuminated room and latency was recorded to check retention time. Each rat was given a latency time up to 3 min.

**Statistical analysis**
GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used to perform statistical analyses. One-way analysis of variance with Newman-Keuls multiple comparison test was used to identify significant differences between groups. Data are presented as the mean ± standard error of the mean. *P*-values < 0.05 were considered statistically significant.

**RESULTS**

**Effects of Cur, Hes, and their combination on cell proliferation**
To identify an optimal dose of Dg which affects proliferation of SY5Y cells, MTT assays were performed. Following treatment with 300 mM D-gal and 400 mM D-gal, cell proliferation decreased by 53% and 33%, respectively, compared to the Ctrl group (Fig. 1A). Thus, in
subsequent experiments, 300 mM D-gal was used to induce aging. MTT assays with SY5Y cells were also performed to determine appropriate doses of Cur and Hes. Treatment with 0.3 μM and 1 μM Cur increased cell viability by 19% and 22%, respectively, compared with the D-gal group (Fig. 1B). In contrast, cell proliferation was largely unchanged following treatment with Hes (Fig. 1C). Thus, a dose of 1 μM was used for both Cur and Hes in subsequent experiments.

Effects of Cur, Hes, and their combination on D-gal-induced neuronal degeneration

To investigate changes in neurite growth induced by D-gal in SY5Y cells, average neurite length was analyzed (Fig. 2A). Treatment with D-gal significantly reduced the average neurite length compared to the Ctrl group (P < 0.001). Meanwhile, treatment with Cur, Hes, or their combination significantly rescued the average neurite length by 56%, 46%, and 69%, respectively, compared to the D-gal group (P < 0.001 for all).

Next, we examined neuronal cell differentiation and neuro-axonal damage-associated markers, β-tubulin III, p-ERK, and NEFH (Fig. 2B and C). D-gal treatment significantly down-regulated expression of all 3 markers compared to the Ctrl group (P < 0.001 for β-tubulin III and p-ERK, P < 0.01 for NEFH). In contrast, Cur treatment restored expression of p-ERK and NEFH, while Hes increased the expression of β-tubulin III and p-ERK. A combination treatment with both Cur and Hes rescued the expression of all 3 markers.

Effects of Cur, Hes, and their combination on D-gal-induced cellular senescence

To analyze cellular senescence induced by D-gal in SY5Y cells, SA-β-gal staining assays were performed (Fig. 3A). In these assays, D-gal treatment significantly increased the number of SA-β-gal positive cells compared with the Ctrl group (P < 0.001). In contrast, treatment with Cur, Hes, or their combination markedly reduced the number of SA-β-gal positive cells by 79%, 82%, and 88%, respectively, compared with the D-gal treatment group (P < 0.001 for all).

Expression of the cellular senescence-associated markers, p16 and p21, were also investigated by Western blot (Fig. 3B and C). Consistent with the SA-β-gal staining results, D-gal treatment appeared to significantly up-regulate expression of p16 compared with the Ctrl group (P < 0.001). Meanwhile, treatment with Cur, Hes, or their combination significantly...
attenuated the levels of p16 by 52%, 55%, and 51%, respectively, compared with the D-gal treatment group (P < 0.01 for all). Similarly, expression of p21 significantly increased in the D-gal treatment group compared to the Ctrl group, while expression of p21 was reduced in the Cur, Hes, and Combi groups (P < 0.01 for all).

**Effects of Cur, Hes, and their combination on D-gal-induced oxidative enzyme expression**

Since oxidative stress is a cause of aging and the mechanism of Dg, expressions levels of the antioxidant enzymes, SOD1, Gpx1, and catalase, were examined (Fig. 4A and B). The D-gal treatment group exhibited significantly lower expression of SOD1, Gpx1, and catalase compared to the Ctrl group (P < 0.001 for SOD1 and Gpx1, P < 0.05 for catalase). Meanwhile, Cur treatment recovered the expression of SOD1 and Gpx1 to control levels, while Hes attenuated the suppression of SOD1 and Gpx1. Treatment with both Cur and Hes recovered expression of all 3 antioxidant enzymes to control levels.
Curcumin, hesperetin and brain senescence

Fig. 3. Effect of Cur (1 μM) and Hes (1 μM) on cellular senescence in Dg-induced aged SH-SY5Y cells. (A) Representative images (100× magnification) are shown. Cellular senescence was examined by SA-β-gal staining. (a) Ctrl; (b) D-gal; (c) Cur; (d) Hes; (e) Combi; (f) Quantification of β-galactosidase stained cells. (B, C) The protein expressions of cellular senescence markers, p16 and p21 were analyzed by Western blot assay. α-tubulin was used as a loading control. (B) Representative blots, (C) quantified proteins level data are represented as the mean ± SEM. Letters label the bars which represent significant differences (P < 0.05).
Ctrl, control; Dg, D-galactose; Cur, curcumin; Hes, hesperetin; Combi, curcumin + hesperetin; SEM, standard error of the mean.

Fig. 4. Effect of Cur (1 μM) and Hes (1 μM) on the expression of anti-oxidant enzymes in Dg-induced aged SH-SY5Y cells. The protein expressions of anti-oxidant enzymes, SOD1, Gpx1, and catalase were analyzed by Western blot assay. α-tubulin was used as a loading control. (A) Representative blots, (B) quantified proteins level. Data are represented as the mean ± SEM. Letters label the bars which represent significant differences (P < 0.05).
Ctrl, control; Dg, D-galactose; Cur, curcumin; Hes, hesperetin; Combi, curcumin + hesperetin; SOD1, superoxide dismutase 1; Gpx1, glutathione peroxidase 1; SEM, standard error of the mean.
Effects of Cur, Hes, and their combination on passive avoidance tests in D-gal-induced aged rats

During training, there were no significant differences in passive avoidance latency among the 5 experimental groups. However, the D-gal group tended to have a shorter latency time compared with the Ctrl group during the 24-h retention trial test, although the results were not significant (Fig. 5A). Hes supplementation blocked the decrease in latency time compared with the D-gal group (P < 0.05), whereas Cur and the combination treatment tended to recover the latency time, albeit not significantly.

Effects of Cur, Hes, and their combination on apoptosis-related markers in D-gal-induced aged rats

To investigate whether Cur, Hes, or their combination can ameliorate apoptosis in the brain of D-gal treated rats, expression levels of Bax, Bcl-2, and PARP were analyzed in brain cortex tissues (Fig. 5B and C). The expression level of Bax induced by D-gal treatment was recovered to control levels by Hes and the combination treatment. Expression of Bcl-2 was up-regulated by Hes treatment and expression of PARP was inhibited by both Hes and the combination treatment.

DISCUSSION

In the present study, treatment with Cur, Hes, or their combination exerted an anti-brain aging effect by regulating neuron loss, antioxidant enzyme expression, and apoptosis in a D-gal-induced brain aging model both *in vitro* and *in vivo*. In particular, all 3 treatments improved neuron loss and degeneration, while also inhibited cellular senescence and oxidative stress by...
upregulating antioxidant enzyme expression in RA-induced SY5Y cells. Correspondingly, our animal study indicated that administration of Cur, Hes, or their combination also protected cognitive impairment and suppressed neuronal apoptosis in the cerebral cortex of Dg-induced brain aged rats.

Dg is widely used to mimic natural aging. Dg is metabolized to glucose and hydrogen peroxide and it turns on the apoptosis pathway when it is chronically or excessively administered. The latter is activated by an accumulation of superoxide anions and free radicals which lead to oxidative stress and aging [3,32]. We observed in the present study that treatment with 300 mM Dg for 48 h induced cellular senescence in SY5Y cells. Previously, it was reported that Dg treatment efficiently induced senescence not only in animal models, but also in in vitro cell models [12,33,34]. For example, Wang et al. [35] and Rahimi et al. [36] reported that 200–400 mM Dg could cause senescence in RA-induced differentiated SY5Y cells as a result of increased oxidative stress and apoptosis. These results are consistent with those of the present study.

Both dementia and mild Alzheimer disease can be induced by Dg administration. This is a widely employed brain aging model since it exhibits characteristics which are similar to those of aging in the human brain, such as memory decline, motor dysfunction, mitochondria dysfunction, and accumulated oxidative stress [3]. Numerous studies have demonstrated that animal models treated with Dg exhibited declines in spatial learning and memory, decreased antioxidant enzyme activity, and increased apoptosis. As a result, neurodegeneration and brain aging were observed [23,37,38].

Polyphenols possess various biological properties, including anti-cancer, anti-inflammation, antioxidation, and anti-aging properties. For example, resveratrol, which is most abundant in red grapes, has exhibited anti-aging effects by improving age-related memory and mood dysfunction with neurogenesis in older F344 rats [39]. Flavonoid rich orange juice has also been shown to benefit cognitive function when it was consistently consumed by healthy middle-aged men [40]. Cur is a bioactive polyphenol with a long history of use as a therapeutic reagent against inflammation and aging-related disorders [41,42]. Anti-aging effects of Cur have been reported in various cell lines, in an animal model, and in a human intervention trial [43-45]. In addition, Cur exerted a synergistic effect when it was administered with piperine or hesperidin in Dg-induced aged rats [23,46,47].

Emerging evidence suggests that the role of flavonoids in cognition and memory enhancement is mediated by scavenging reactive oxygen species, activating signaling proteins of the survival pathway, and inhibiting of oxidant-induced neuronal apoptosis [27]. It has recently been demonstrated that 2 flavonoids present in citrus fruit, hesperidin (a flavanone glycoside) and Hes (an aglycone form of hesperidin), exhibit a variety of biological activities, including anti-inflammatory, anti-depressant, and neuroprotective effects [26,48-50]. For example, hesperidin and Hes both induce neuroprotective effects against H2O2-induced cytotoxicity in PC12 cells and amyloid-induced autophagy in Neuro-2A cells [51,52]. However, while both hesperidin and Hes exert similar 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity, Hes has been found to provide better protection from lipid peroxidation in rat brain homogenates and to increase cell viability in primary rat cortical cells [26].

In this study, both Cur and Hes significantly increased neurite length and neuronal marker expression. Neurite outgrowth occurs in the nervous system and also during nerve
regeneration [53]. During the latter, neurites grow and connect to other neurites, thereby establish trans neuronal signaling [53]. However, when neurite length is decreased and fewer neurite are present, synaptic degeneration can occur. This is a common characteristic in dementia and Alzheimer disease [54].

In the present study, Cur, Hes, and their combination significantly reduced the number of SA-β-gal-positive Dg-aged cells. When cells are aged by various factors, the cells accumulate lysosomes, and begin to lose their function. SA-β-gal is a biomarker which is widely used to detect aged cells based on its staining of accumulated lysosome [12,55-57]. A molecular analysis of aged SY5Y cells in the present study showed that Cur, Hes, and their combination were able to down-regulate the senescence-associated proteins, p16 and p21. Moreover, Cur, Hes, and their combination were found to recover antioxidant enzyme expression levels suppressed by Dg. These findings are consistent with those of previous reports which have showed that Cur or other antioxidant polyphenols, such as caffeine and hesperidin, are able to inhibit oxidative stress and apoptosis related proteins, such as Bax, Bcl-2, and caspase 3 [46,58]. Moreover, although hesperidin and Hes are detected at very low concentrations in the brain, they can across the blood brain barrier [59]. An additive effect of Cur and hesperidin in Dg-treated rats has also previously been reported. This combination of supplements improved both cognition and mitochondrial enzymes [46]. Mitochondria play a vital role in the aging process since oxidative stress mainly occurs in these organelle [46,60].

However, the additive effect was not observed in the present study. Although we found that Cur and Hes individually rescued Dg-induced brain aging in this study in vitro, combination treatment of Cur and Hes alleviate the brain aging only a few markers. Both polyphenols are rapidly metabolized and excreted in urine or feces [61,62]. The absorption of Cur is regulated by the activity of transporter proteins in the luminal membrane of enterocytes. Absorbed Cur is metabolized to a less active form, Cur glucuronides, in the liver. Approximately, 1–2 h after oral ingestion, the plasma level of Cur reaches its highest peak [63]. However, Hes, a glycoside form of hesperidin, is produced by hydrolyzation of hesperidin by colon microflora and a more active form than hesperidin. Hes is detectable 20 min after oral administration of hesperidin, and it reaches its highest peak after 4 h [62]. Since both compounds have different peak times in plasma, they may mediate anti-oxidation effects at different time points. Therefore, if Cur and Hes are administered at regular intervals, additive effects of these 2 polyphenols may be observed. It remains for this hypothesis to be investigated in future studies.

We selected oral supplementation doses based on previous studies [23,46,64] and our preliminary study (unpublished data). Banji et al. [46] reported improved cognitive ability following treatment with Cur (50, 100 mg/kg) and hesperidin (10, 25 mg/kg) in Dg injected rats. Furthermore, in a Morris water maze experiment conducted with D-gal-induced aged rats treated with Cur and hesperidin individually and in combination, a significantly higher number of platform crossings were observed.

Administration of Cur (50, 100 mg/kg b.w.) and Hes (25, 50, 100 mg/kg b.w.) individually, or in combination, were performed in our preliminary study (unpublished data). All of the dosages of Cur and Hes exhibited anti-aging effects. In particular, 50 mg/kg b.w. Cur and both 25 and 50 mg/kg b.w. Hes were associated with improved memorial retention in a passive avoidance test. Based on these results, 50 mg/kg b.w. Cur and 50 mg/kg b.w. Hes were selected as the doses used for oral supplementation in the present study.
Human doses can be extrapolated from animal dosages from calculations of body surface area [65]. By applying the formula, the 50 mg/kg (rat dose) is multiplied by the $K_m$ factor (6) for rat and then divided by the $K_m$ factor (37) for humans. A 486 mg dose of Cur and/or Hes would be administered to a human weighing 60 kg. It has been demonstrated that up to 8 g/day is a pharmacologically safe dosage range for Cur [66]. The highest intake of Cur to date has been reported in India (100 mg/day) [67]. While the dosage used in the present study is somewhat higher than the dose normally consumed by humans, it still remains less than toxic dose. Moreover, since the bioavailability of Cur between humans and rats differs, the dose administered represents a physiological dose for humans. Glycoside forms are abundant in citrus fruit and juices [68]. Daily intake of a diet rich in vegetables and citrus provides 132 mg/day Hes [69]. Based on these studies, the dose of Hes used in this study represents a physiological dose for humans.

It is well-known that Cur exhibits low bioavailability. Correspondingly, ingestion of single doses of Cur (4, 6, and 8 g) resulted in peak bioavailability 1 to 2 h after ingestion. In addition, average serum concentrations were $0.51 \pm 0.11 \mu M$, $0.63 \pm 0.06 \mu M$, and $1.77 \pm 1.87 \mu M$, respectively. Plasma levels of Cur were also found to increase with long-term administration of Cur over 3 months in humans [70]. Furthermore, no toxicity was associated with the doses of Cur ingested (up to 8 g/day) [66]. Based on these results, in vitro experimental dose of 1 $\mu M$ is predicted to represent an oral dose of approximately 7 g in humans. While this small amount of Cur remains in the body, Cur can cross the blood brain barrier and may directly affect brain function and aging [71]. There was a lack of evidence regarding the plasma concentration of Hes. Therefore, we are not able to calculate the oral supplementation dose of Hes from the in vitro results.

Based on the present results, we hypothesize that Cur and Hes are able to minimize Dg-induced neuronal aging by regulating antioxidation enzymes and apoptosis-related proteins. However, additional factors contribute to brain aging. These factors may include inflammatory disorders, misfolded proteins, and accumulation of amyloid $\beta$ plaque. Therefore, it will be important to investigate inflammatory alterations in this model in future studies. In addition, since memory decline which accompanies dementia and Alzheimer disease is governed by both the cerebral cortex and the hippocampus, alterations in the hippocampus should also be investigated.

REFERENCES

1. López-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell 2013;153:1194-217.
2. Peters R. Ageing and the brain. Postgrad Med J 2006;82:84-8.
3. Shwe T, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. Role of D-galactose-induced brain aging and its potential used for therapeutic interventions. Exp Gerontol 2018;101:13-36.
4. Stites SD, Harkins K, Rubright JD, Karlawish J. Relationships between cognitive complaints and quality of life in older adults with mild cognitive impairment, mild Alzheimer disease dementia, and normal cognition. Alzheimer Dis Assoc Disord 2018;32:276-83.
5. Fjell AM, McEvoy L, Holland D, Dale AM, Walhovd KB; Alzheimer's Disease Neuroimaging Initiative. What is normal in normal aging? Effects of aging, amyloid and Alzheimer’s disease on the cerebral cortex and the hippocampus. Prog Neurobiol 2014;117:20-40.

6. Rubin RD, Watson PD, Duff MC, Cohen NJ. The role of the hippocampus in flexible cognition and social behavior. Front Hum Neurosci 2014;8:742.

7. Crews L, Masliah E. Molecular mechanisms of neurodegeneration in Alzheimer’s disease. Hum Mol Genet 2010;19:R12-20.

8. Espuny-Camacho I, Arranz AM, Fiers M, Snellinx A, Ando K, Munck S, Bonnefont J, Lambot L, Corthout N, Omodho L, Eynden EV, Radaelli E, Tresseur I, Wray S, Ebner H, Hardy J, Leroy K, Brion JP, Vanderhaeghen P, De Strooper B. Hallmarks of Alzheimer’s disease in stem-cell-derived human neurons transplanted into mouse brain. Neuron 2017;93:1066-1081.e8.

9. Yokotsuka T. Soy sauce biochemistry. Adv Food Res 1986;30:195-329.

10. Chen P, Chen F, Zhou B. Antioxidative, anti-inflammatory and anti-apoptotic effects of ellagic acid in liver and brain of rats treated by D-galactose. Sci Rep 2018;8:1465.

11. Shan Q, Lu J, Zheng Y, Li J, Zhou Z, Hu B, Zhang Z, Fan S, Mao Z, Wang YJ, Ma D. Purple sweet potato color ameliorates cognition deficits and attenuates oxidative damage and inflammation in aging mouse brain induced by D-galactose. J Biomed Biotechnol 2009;2009:564737.

12. Kim Y, Kim E, Kim Y. L-histidine and L-carnosine accelerate wound healing via regulation of corticosterone and PI3K/Akt phosphorylation in D-galactose-induced aging models in vitro and in vivo. J Funct Foods 2019;58:227-37.

13. Chen X, Li Y, Chen W, Nong Z, Huang J, Chen C. Protective effect of hyperbaric oxygen on cognitive impairment induced by D-galactose in mice. Neurochem Res 2016;41:3032-41.

14. Spencer JPE. Flavonoids: modulators of brain function? Br J Nutr 2008;99:ES60-77.

15. Rehman SU, Shah SA, Ali T, Chung JJ, Kim MO. Anthocyanins reversed D-galactose-induced oxidative stress and neuroinflammation mediated cognitive impairment in adult rats. Mol Neurobiol 2017;54:255-71.

16. Hsieh HM, Wu WM, Hu ML. Soy isoflavones attenuate oxidative stress and improve parameters related to aging and Alzheimer’s disease in C57BL/6j mice treated with D-galactose. Food Chem Toxicol 2009;47:625-32.

17. Ritchie K, Carrière I, de Mendonça A, Portet F, Dartigues JF, Rouaud O, Barberger-Gateau P, Ancelin ML. The neuroprotective effects of caffeine: a prospective population study (the three city study). Neurology 2007;69:536-45.

18. Tsuda T. Curcumin as a functional food-derived factor: degradation products, metabolites, bioactivity, and future perspectives. Food Funct 2018;9:705-14.

19. Shen LR, Parnell LD, Ordovas JM, Lai CQ. Curcumin and aging. Biofactors 2013;39:133-40.

20. Liao VH, Yu CW, Chu YJ, Li WH, Hsieh YC, Wang TT. Curcumin-mediated lifespan extension in Caenorhabditis elegans. Mech Ageing Dev 2011;132:480-7.

21. Lee KS, Lee BS, Semnani S, Avanesian A, Um CY, Jeon HJ, Seong KM, Yu K, Min KJ, Safari M. Curcumin extends life span, improves health span, and modulates the expression of age-associated aging genes in Drosophila melanogaster. Rejuvenation Res 2010;13:567-70.

22. Kitani K, Osawa T, Yokozawa T. The effects of tetrahydrocurcumin and green tea polyphenol on the survival of male C57BL/6 mice. Biogerontology 2007;8:567-73.
23. Banji D, Banji OJF, Dasaroju S, Kranthi KCH. Curcumin and piperine abrogate lipid and protein oxidation induced by D-galactose in rat brain. Brain Res 2013;1515:1-11.

24. Liu Y, Liu D, Zhu L, Gan Q, Le X. Temperature-dependent structure stability and in vitro release of chitosan-coated curcumin liposome. Food Res Int 2015;74:97-105.

25. Abdul Manap AS, Wei Tan AC, Leong WH, Yin Chia AV, Vijayabalan S, Arya A, Wong EH, Rizwan F, Bindal U, Koshy S, Madhavan P. Synergistic effects of curcumin and piperine as potent acetylcholine and amyloidogenic inhibitors with significant neuroprotective activity in SH-SYSY cells via computational molecular modeling and in vitro assay. Front Aging Neurosci 2019;11:206.

26. Cho J. Antioxidant and neuroprotective effects of hesperidin and its aglycone hesperetin. Arch Pharm Res 2006;29:699-706.

27. Spencer JP, Vauzour D, Rendeiro C. Flavonoids and cognition: the molecular mechanisms underlying their behavioural effects. Arch Biochem Biophys 2009;492:1-9.

28. Hirata A, Murakami Y, Shoji M, Kadoma Y, Fujisawa S. Kinetics of radical-scavenging activity of hesperetin and hesperidin and their inhibitory activity on COX-2 expression. Anticancer Res 2005;25:3367-74.

29. Khalili M, Roghani M, Ekhlasi M. The effect of aqueous crocus sativus L. extract on intracerebroventricular streptozotocin-induced cognitive deficits in rat: a behavioral analysis. Iran J Pharm Res 2009;8:185-91.

30. Quillfeldt JA. Behavioral methods to study learning and memory in rats. In: Andersen M., Tufik S, editors. Rodent Model as Tools in Ethical Biomedical Research. Cham: Springer; 2016. p.271-311.

31. Rahnama S, Rabiei Z, Alibabaei Z, Mokhtari S, Rafieian-Kopaei M, Deris F. Anti-amnesic activity of Citrus aurantium flowers extract against scopolamine-induced memory impairments in rats. Neurol Sci 2015;36:553-60.

32. Song X, Bao M, Li D, Li YM. Advanced glycation in D-galactose induced mouse aging model. Mech Ageing Dev 1999;108:239-51.

33. Shen Y, Gao H, Shi X, Wang N, Ai D, Li J, Ouyang L, Yang J, Tian Y, Lu J. Glutamine synthetase plays a role in D-galactose-induced astrocyte aging in vitro and in vivo. Exp Gerontol 2014;58:166-73.

34. Lin WL, Wang SM, Ho YJ, Kuo HC, Lee YJ, Tseng TH. Ethyl acetate extract of Wedelia chinensis inhibits tert-butyl hydroperoxide-induced damage in PC12 cells and D-galactose-induced neuronal cell loss in mice. BMC Complement Altern Med 2014;14:491.

35. Wang T, Di G, Yang L, Dun Y, Sun Z, Wan J, Peng B, Liu C, Xiong G, Zhang C, Yuan D. Saponins from Panax japonicus attenuate D-galactose-induced cognitive impairment through its anti-oxidative and anti-apoptotic effects in rats. J Pharm Pharmacol 2015;67:1284-96.

36. Rahimi VB, Askari VR, Mousavi SH. Ellagic acid reveals promising anti-aging effects against D-galactose-induced aging on human neuroblastoma cell line, SH-SYSY: a mechanistic study. Biomed Pharmacother 2018;108:1712-24.

37. Ullah F, Ali T, Ullah N, Kim MO. Caffeine prevents D-galactose-induced cognitive deficits, oxidative stress, neuroinflammation and neurodegeneration in the adult rat brain. Neurochem Int 2015;90:91-102.

38. Gao J, He H, Jiang W, Chang X, Zhu L, Luo F, Zhou R, Ma C, Yan T. Salidroside ameliorates cognitive impairment in a D-galactose-induced rat model of Alzheimer’s disease. Behav Brain Res 2015;293:27-33.

39. Kodali M, Parihar VK, Hattiangady B, Mishra V, Shuai B, Shetty AK. Resveratrol prevents age-related memory and mood dysfunction with increased hippocampal neurogenesis and microvasculature, and reduced glial activation. Sci Rep 2015;5:8075.

https://e-nrp.org

https://doi.org/10.4162/nrp.2020.14.5.438
40. Alharbi MH, Lamport DJ, Dodd GF, Saunders C, Harkness L, Butler LT, Spencer JP. Flavonoid-rich orange juice is associated with acute improvements in cognitive function in healthy middle-aged males. Eur J Nutr 2016;55:2021-9.
  PUBMED | CROSSREF

41. Sikora E, Scapagnini G, Barbagallo M. Curcumin, inflammation, ageing and age-related diseases. Immun Ageing 2010;7:7.
  PUBMED | CROSSREF

42. Jurenka JS. Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research. Altern Med Rev 2009;14:141-53.
  PUBMED

43. Ringman JM, Frautschy SA, Teng E, Begum AN, Bardens J, Beigi M, Gyllys KH, Badmaev V, Heath DD, Apostolova LG, Porter V, Vanek Z, Marshall GA, Hellemann G, Sugar C, Masterman DL, Montine TJ, Cummings JL, Cole GM. Oral curcumin for Alzheimer’s disease: tolerability and efficacy in a 24-week randomized, double blind, placebo-controlled study. Alzheimers Res Ther 2012;4:43.
  PUBMED | CROSSREF

44. Baum L, Lam CWK, Cheung SK, Kwok T, Lui V, Tsoh J, Lam L, Leung V, Hui E, Ng C, Woo J, Chiu HF, Goggins WB, Zee BC, Cheng KF, Fong CY, Wong A, Mok H, Chow MS, Ho PC, Ip SP, Ho CS, Yu XW, Lai CY, Chan MH, Szeto S, Chan IH, Mok V. Six-month randomized, placebo-controlled, double-blind, pilot clinical trial of curcumin in patients with Alzheimer disease. J Clin Psychopharmacol 2008;28:110-3.
  PUBMED | CROSSREF

45. Cui Q, Li X, Zhu H. Curcumin ameliorates dopaminergic neuronal oxidative damage via activation of the Akt/Nrf2 pathway. Mol Med Rep 2016;13:1381-8.
  PUBMED | CROSSREF

46. Banji OJ, Banji D, Ch K. Curcumin and hesperidin improve cognition by suppressing mitochondrial dysfunction and apoptosis induced by D-galactose in rat brain. Food Chem Toxicol 2014;74:51-9.
  PUBMED | CROSSREF

47. Banji OJ, Banji D, Soumya N, Chilipi KK, Kalpana CH, Kranthi Kumar CH, Annamalai AR. Combination of carvacrol with methotrexate suppresses Complete Freund’s Adjuvant induced synovial inflammation with reduced hepatotoxicity in rats. Eur J Pharmacol 2014;723:91-8.
  PUBMED | CROSSREF

48. Rainey-Smith S, Schroetke IW, Bahia P, Fahmi A, Skilton R, Spencer JP, Rice-Evans C, Rattray M, Williams RJ. Neuroprotective effects of hesperetin in mouse primary neurones are independent of CREB activation. Neurosci Lett 2008;458:29-33.
  PUBMED | CROSSREF

49. Banji OJ, Banji D, Soumya N, Chilipi KK, Kalpana CH, Kranthi Kumar CH, Annamalai AR. Combination of carvacrol with methotrexate suppresses Complete Freund’s Adjuvant induced synovial inflammation with reduced hepatotoxicity in rats. Eur J Pharmacol 2014;723:91-8.
  PUBMED | CROSSREF

50.n TIN, Spencer GE. Neurite outgrowth. In: Reference Module in Biomedical Sciences. Amsterdam: Elsevier; 2017.

51. Kashyap G, Bapat D, Das D, Gowaikar R, Amritkar RE, Rangarajan G, Ravindranath V, Ambiga G. Synaptic loss and progress of Alzheimer's disease - a network model. Sci Rep 2019;9:6555.
  PUBMED

52. Zhu J, Mu X, Zeng J, Xu C, Liu J, Zhang M, Li C, Chen J, Li T, Wang Y. Ginsenoside Rg1 prevents cognitive impairment and hippocampus senescence in a rat model of D-galactose-induced aging. PLoS One 2014;9:e101291.
  PUBMED | CROSSREF

53. Elzi DJ, Song M, Shiio Y. Role of galactose in cellular senescence. Exp Gerontol 2016;73:1-4.
  PUBMED | CROSSREF

54. Elzi DJ, Song M, Shiio Y. Role of galactose in cellular senescence. Exp Gerontol 2016;73:1-4.
  PUBMED | CROSSREF

55. Hwang SL, Yen GC. Neuroprotective effects of the citrus flavanones against H2O2-induced cytotoxicity in PC12 cells. J Agric Food Chem 2008;56:859-64.
  PUBMED | CROSSREF

56. Huang SM, Tsai SY, Lin JA, Wu CH, Yen GC. Cytoprotective effects of hesperetin and hesperidin against amyloid β-induced impairment of glucose transport through downregulation of neuronal autophagy. Mol Nutr Food Res 2012;56:601-9.
  PUBMED | CROSSREF

57. Zou J, Mu X, Zeng J, Xu C, Liu J, Zhang M, Li C, Chen J, Li T, Wang Y. Ginsenoside Rg1 prevents cognitive impairment and hippocampus senescence in a rat model of D-galactose-induced aging. PLoS One 2014;9:e101291.
  PUBMED | CROSSREF

58. Elzi DJ, Song M, Shiio Y. Role of galactose in cellular senescence. Exp Gerontol 2016;73:1-4.
  PUBMED | CROSSREF

https://e-nrp.org

https://doi.org/10.4162/nrp.2020.14.5.438
57. Nopparat C, Chantadul V, Permpoonputtana K, Govitrarong P. The anti-inflammatory effect of melatonin in SH-SY5Y neuroblastoma cells exposed to sublethal dose of hydrogen peroxide. Mech Ageing Dev 2017;164:49-60.

58. Justin Thenmozhi A, William Raja TR, Manivasagam T, Janakiraman U, Essa MM. Hesperidin ameliorates cognitive dysfunction, oxidative stress and apoptosis against aluminium chloride induced rat model of Alzheimer’s disease. Nutr Neurosci 2017;20:360-8.

59. Dimpfel W. Different anticonvulsive effects of hesperidin and its aglycone hesperetin on electrical activity in the rat hippocampus in-vitro. J Pharm Pharmacol 2006;58:375-9.

60. Kumar A, Dogra S, Prakash A. Effect of carvedilol on behavioral, mitochondrial dysfunction, and oxidative damage against D-galactose induced senescence in mice. Naunyn Schmiedebergs Arch Pharmacol 2009;380:431-41.

61. Metzler M, Pfeiffer E, Schulz SI, Dempe JS. Curcumin uptake and metabolism. Biofactors 2013;39:14-20.

62. Kanaze FI, Bounartzi MI, Georgarakis M, Niopas I. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. Eur J Pharm Biopharm 2013;82:24-31.

63. Ghosh SS, He H, Wang J, Gehr TW, Ghosh S. Curcumin-mediated regulation of intestinal barrier function: the mechanism underlying its beneficial effects. Tissue Barriers 2018;6:e1425085.

64. Trivedi PP, Tripathi DN, Jena GB. Hesperetin protects testicular toxicity of doxorubicin in rat: role of NFκB, p38 and caspase-3. Food Chem Toxicol 2011;49:838-47.

65. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J 2008;22:659-61.

66. Mishra S, Palanivelu K. The effect of curcumin (turmeric) on Alzheimer’s disease: an overview. Ann Indian Acad Neurol 2008;11:i3-9.

67. Bielak-Zmijewska A, Grabowska W, Ciolkó A, Bojko A, Mosieniak G, Biachoł E, Sikora E. The role of curcumin in the modulation of ageing. Int J Mol Sci 2019;20:1239.

68. Moulpy P, Gaydou EM, Auffray A. Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography. J Chromatogr A 1998;800:171-9.

69. Erlund I, Silaste ML, Alftahan G, Rantala M, Kesäniemi YA, Aro A. Plasma concentrations of the flavonoids hesperetin, naringenin and quercetin in human subjects following their habitual diets, and diets high or low in fruit and vegetables. Eur J Clin Nutr 2002;56:891-8.

70. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko FY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC, Hsieh CY. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 2001;21:2895-900.

71. Garcia-Alloza M, Borrelli LA, Rozkalne A, Hyman BT, Bacskai BJ. Curcumin labels amyloid pathology in vivo, disrupts existing plaques, and partially restores distorted neurites in an Alzheimer mouse model. J Neurochem 2007;102:1095-104.