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**Original Research Article**

# Anti-amyloidogenic effect of menaquinone-7 on beta-amyloid production and aggregation

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## Abstract

***Purpose:*** *To investigate the beneficial effects of menaquinone-7 (MK-7), an isoform of vitamin $K_2$, against beta-amyloid (Aβ) production and aggregation in Alzheimer's disease using in vitro assays.*
***Methods:*** *The cytotoxicity of MK-7 was determined by MTT assay. The amount of Aβ produced and secreted into the supernatant by APP-CHO cells treated with MK-7 was evaluated by ELISA. The expression of β-secretases and ADAM10, a representative α-secretase, was determined using western blot analysis. The production of sAPPβ and sAPPα fragments generated by β-secretases and α-secretase, respectively, were also determined by western blot analysis. The effect on Aβ aggregation was assessed using Thioflavin T (Th T) assay.*
***Results:*** *MK-7 (up to 75 nM) significantly decreased Aβ production in APP-CHO cells. This was accompanied by decreased expression of β-secretase and lower production of sAPPβ ($p < 0.05$). However, expression of ADAM10 and production of sAPPα were not significantly affected. In contrast, MK-7 significantly decreased Aβ aggregation in a dose-dependent manner ($p < 0.05$).*
***Conclusion:*** *MK-7 exerts anti-amyloidogenic effects via decreased production and lower aggregation of Aβ into oligomers and fibrils. Therefore, dietary supplementation with MK-7 may be beneficial for the prevention of Alzheimer's disease.*

***Keywords:*** *Menaquinone-7, Beta-amyloid, β-Secretases, Aggregation, Alzheimer's disease, Vitamin $K_2$*



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## INTRODUCTION

Vitamins are a class of organic compounds which function in diverse biological activities and are needed in minute quantities. Often, vitamins are not synthesized in sufficient amounts and must be supplemented through food. Vitamin K functions as a cofactor for enzymes carboxylating vitamin K-dependent proteins [1]. The two major naturally occurring vitamin Ks are vitamin $K_1$ and vitamin $K_2$ [2]. Vitamin $K_1$, also known as phylloquinone, has only 1 type and is produced by plants, whereas vitamin $K_2$, also known as menaquinone (MKn), has 14 forms and synthesized by animals and bacteria [3,4]. Although MK-4 and MK-7 are the most common forms of vitamin $K_2$, MK-7 is more effective because of its longer half-life in human serum [5].

It has been suggested that the reduced intake of vitamin K in diet is associated with a higher incidence of various diseases including osteoporosis [6], osteoarthritis [7], Parkinson's disease [8], and Alzheimer's disease [9]. However, the potential beneficial effects of vitamin $K_2$, particularly MK-7, in Alzheimer's disease have not been reported. Therefore, in this study, the potential beneficial effects of MK-7 on the inhibition of Aβ production and aggregation were examined using *in vitro* assay.

## EXPERIMENTAL

### Chemicals and reagents

Tetrahydrofuran (THF), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and theoflavin T (Th T) were purchased from Sigma Aldrich (St. Louis, MO, USA). The MK-7 standard and MK-7 samples were provided by GF Fermentech (Cheongju, South Korea). Beta-amyloid (Aβ) was purchased from GL Biochem (Shanghai, China) and dimethyl sulfoxide was purchased from Wako Pure Chemical (Osaka, Japan).

### Cell culture and cell viability

Chinese hamster ovarian (CHO) cells stably expressing amyloid precursor proteins (APP) (APP-CHO cells) were grown in RPMI1640 (Welgen, Gyeongsan, South Korea) supplemented with 10 % FBS (Equitech-BIO, Kerrville, TX, USA) including 1 mg/mL of gentamicin (Invitrogen, Waltham, MA, USA) as described previously [10]. The cytotoxicity of MK-7 was measured using the MTT assay as described previously [10].

### Enzyme linked immunosorbent assay (ELISA)

The APP-CHO cells seeded in 96-well plates were treated with MK-7 (25, 50, or 75 nM) for 24 h. The amount of Aβ in the supernatant was measured using the Human Aβ40 ELISA kit (Invitrogen) as per manufacturer's instructions.

### Western blot analysis

For whole cell lysates, the cells were extracted with RIPA buffer. The protein concentration in the cell lysates and supernatants was determined using the Bradford Assay Kit (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins for each cell lysate and supernatant sample (30 µg) were separated on 7.5 % polyacrylamide gels using denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrotransferred onto an Immobilon-P PVDF membrane (Millipore, Burlington, MA, USA) for 30 min using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). After blocking the membrane with 5 % nonfat dry milk, the membrane was incubated with primary antibody overnight at 4 °C, and then with secondary antibody (1:2000; Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 2 h. The following antibodies were used: anti-sAPPβ (1:1000; IBL-America, Minneapolis, MN, USA), anti-sAPPα (1:1,000; IBL-America, USA), anti-β-secretase (1:1000; Abcam, MA, USA), anti-ADAM10 (1:1000; Abcam, Cambridge, UK), and anti-α-tubulin (1:20,000; Sigma Aldrich). The proteins were detected by chemiluminescence using a ChemiDoc system (BioRad). Densitometric values were normalized using α-tubulin as an internal control. Western blot analysis showed that the curve was linear in the range used for each antibody.

### Th T assay for the determination of Aβ aggregation

Beta-amyloid (1-42) solution (1 mM) was diluted in distilled water (DW) and transferred to a black 96-well plate and DW was used for blank. MK-7 solution or DW was added to the Aβ solution. The plates were sealed and incubated for 24 h at 37 °C. After incubation, Th T solution (thioflavin T with 100 mM glycine, pH 8.5) was added to a final concentration of 3 µM. The plate was incubated at 37 °C for 20 min. Fluorescence was measured at 442 nm excitation and 485 nm emission wavelengths.

### Statistical analysis

All data in the text and figures are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test (SPSS version 17.0, IBM, Armonk, NY, USA) was used to determine statistical significance for two or more group comparisons. Differences were considered statistically significant at $p < 0.05$.

## RESULTS

### Inhibitory effect of MK-7 on production of Aβ

The cytotoxicity of MK-7 determined using MTT assay showed that MK-7 (up to 75 nM) did not have any significant effect on the cell viability of APP-CHO ($p < 0.05$, Figure 1 A). To elucidate the effect of MK-7 on the production of Aβ, the amount of Aβ released into the supernatant from APP-CHO cells was determined using ELISA. As shown in Figure 1 B. MK-7 reduced Aβ

production in a dose-dependent manner. In particular, treatment of APP-CHO cells with 75 nM MK-7 significantly decreased Aβ production to 75 % when compared to the THF-treated control group ($p < 0.05$).

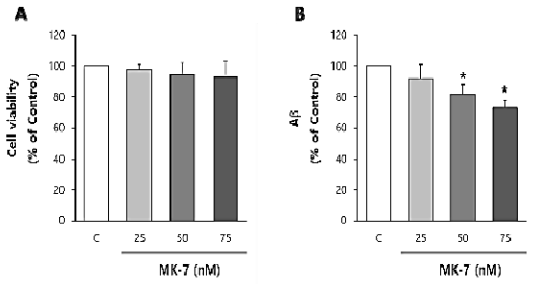


**Figure 1:** Effect of MK-7 on the cell viability of APP-CHO cells and the production of Aβ from APP-CHO cells. (A) The possible cytotoxicity of MK-7 measured using MTT assay. (B) The amount of Aβ released into the supernatant determined using ELISA. *$p < 0.05$ vs the THF-treated control groups

## Inhibitory effect of MK-7 on sAPPβ and β-secretase production

As shown in Figure 2 A, the treatment of APP-CHO cells with MK-7 decreased the production of sAPPβ in a dose-dependent manner. MK-7 at 50 and 75 nM significantly decreased sAPPβ production ($p < 0.05$). In particular, 75 nM MK-7 treatment decreased the amount of sAPPβ produced to 60 % of the THF-treated control groups ($p < 0.05$).

β-Secretase is an enzyme involved in the cleavage of APP to sAPPβ and the production of Aβ. As shown in Figure 2 B, MK-7 decreased β-secretase expression in APP-CHO cells in a dose-dependent manner. Treatment of APP-CHO cells with 75 nM MK-7 significantly reduced β-secretase levels to 60 % of the THF-treated control group. The decrease in levels of sAPPβ and β-secretase corresponded to the decreased production of Aβ (Figure 1 A) ($p < 0.05$).

## Effect of MK-7 on sAPPα and ADAM10 production

The amount of sAPPα, a fragment of APP produced by α-secretase, secreted by APP-CHO cells into the supernatant is shown in Figure 3 A. The amount of sAPPα secreted by APP-CHO cells was not altered by treatment with MK-7 up to 50 nM. On the other hand, treatment with 75 nM of MK-7 increased the production of sAPPα compared to the THF-treated control group, however, this was not statistically significant ($p < 0.05$).

In addition, the effect of MK-7 on the level of ADAM10, a representative α-secretase, was also determined (Figure 3 B). Treatment with 25 nM MK-7 did not alter ADAM10 expression, whereas 50 and 75 nM of MK-7 increased the expression of ADAM10, but the observed changes were not statistically significant ($p < 0.05$).

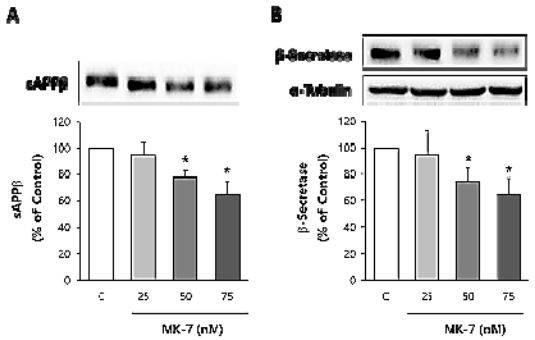


**Figure 2:** Effect of MK-7 on the production of sAPPβ and β-secretases in APP-CHO cells. (A) The level of sAPPβ produced from APP-CHO cells after treatment with MK-7 for 24 h. (B) The level of β-secretases on APP-CHO cells after treatment with MK-7 for 24 h. The graph depicts the changes in the levels of sAPPβ and β-secretases. *$P < 0.05$ vs the THF-treated control groups

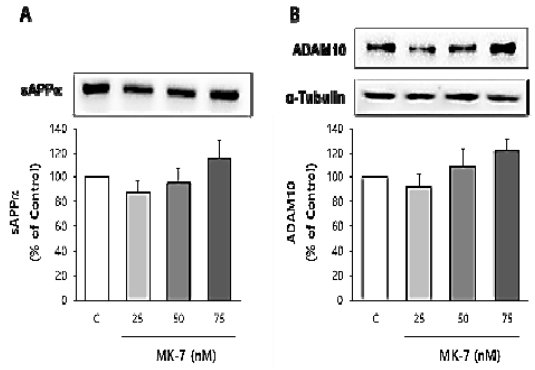


**Figure 3**: Effect of MK-7 on the production of sAPPα and the expression of ADAM10 in APP-CHO cells. (A) APP-CHO cells were treated with MK-7 and the production of sAPPα. (B) The effect of MK-7 treatment on the expression of ADAM10. The graph depict changes in the levels of sAPPα and ADAM10. *$P < 0.05$ vs THF-treated control groups

## Inhibitory effect of MK-7 on Aβ aggregation

The aggregation of Aβ from monomers to oligomers and fibrils is known to induce the neurotoxicity and subsequently cause neuronal cell death. Incubation of Aβ solution with MK-7 decreased Aβ aggregation in a dose-dependent manner (Figure 4). In particular, 75 nM of MK-7 significantly decreased Aβ aggregation to 80 % of the THF-treated control group ($p < 0.05$).

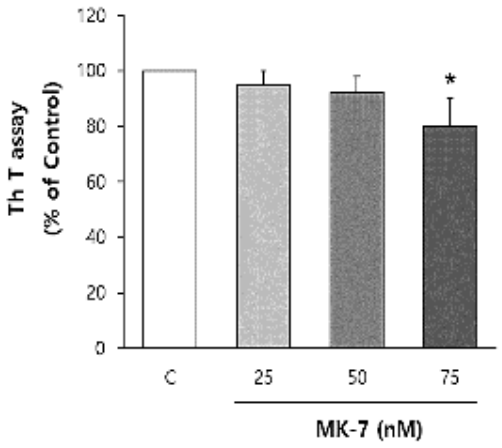


**Figure 4:** Effect of MK-7 on Aβ aggregation. Aβ was incubated with MK-7 for 24 h and the level of Aβ aggregation determined using Th T assay. *$P$ < 0.05 vs the THF-treated Aβ groups

## DISCUSSION

Vitamin K plays a crucial role in blood coagulation and prevents abnormal bleeding. Vitamin K is also known to be beneficial in the prevention of osteoporosis [6], osteoarthritis [7], and neurodegenerative diseases, such as Alzheimer's disease [8] and Parkinson's disease [9]. Lack of vitamin K in diet is associated with age-related cognitive decline in Alzheimer's disease due to the altered sphingolipid metabolism, oxidative stress and inflammation [11]. Previous studies have shown that supplementation with vitamin K in animals improved cognitive function and decreased neuronal degradation [12]. The underlying mechanism for the protective effect of vitamin K against cognitive dysfunction is reported to be through reduction of oxidative stress [13]. Furthermore, vitamin $K_2$ supplementation protected rat pheochromocytoma (PC12) cells from Aβ-induced toxicity via the p38 MAP kinase pathway [14]. Although the beneficial effect of vitamin K against cognitive dysfunction in Alzheimer's disease has been reported, the exact role of MK-7 in the production and aggregation of Aβ has not been studied previously.

The aggregation of Aβ is one of the major causes of Alzheimer's disease. Beta-amyloid is produced by the sequential proteolytic cleavage of APP by β-secretases and γ-secretases [15]. β-secretases cleave APP into sAPPβ and C99 fragments, which are then spontaneously cleaved into Aβ by γ-secretases. Therefore, increased production of sAPPβ is indicative of increased production of Aβ due to the increased activity of β-secretases [16]. On the other hand, APP can be cleaved into sAPPα and P3 by α-secretases, notably ADAM10 [17], which precludes the formation of Aβ. Based on the results of the present study, MK-7 has minor effects on the production of sAPPα and the activity of α-secretases. However, MK-7 significantly decreased the production of Aβ and sAPPβ in a dose-dependent manner. This was accompanied by the decreased activity of β-secretases. These results suggest that MK-7 decrease Aβ production by decreasing β-secretase activity.

Menaquinone-7 decreased Aβ aggregation in a dose-dependent manner. Previously it was reported that vitamin $K_3$, menadione, significantly decreased Aβ aggregation via hydrophobic interactions with amino acids, such as Ser, Glu, Val, Gln, and Phe of Aβ, and hydrogen bonding with Lys of Aβ [18]. Both MK-7 and vitamin $K_3$ have a menadione nucleus, but the difference in structure is that MK-7 has seven isoprene units, while vitamin $K_3$ does not. Therefore, the inhibitory effect of MK-7 on Aβ aggregation could be via hydrophobic interactions or hydrogen bonding of menadione of MK-7 with amino acids of Aβ.

## CONCLUSION

Treatment of APP-CHO cells with MK-7 significantly decreases the production of Aβ. This is accompanied with a decrease in production of sAPPβ and expression of β-secretases, but it does not influence the production of sAPPα and expression of ADAM10. In addition, MK-7 decrease the aggregation of Aβ. These results suggest that MK-7 possesses anti-amyloidogenic effect by decreasing Aβ production via inhibition of β-secretases, and Aβ aggregation. Therefore, dietary supplementation of MK-7 could be beneficial for the prevention of Alzheimer's disease by decreasing Aβ production and reducing Aβ aggregation. However, these beneficial effects against Alzheimer's disease should first be confirmed using animal models.

## DECLARATIONS

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### *Conflict of interest*

No conflict of interest is associated with this work.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chung-Hyun Lee, Eun-Sang Jo, Ji-Yun Yeo, and Seong Jun Choi performed the experiments. So-Young Park and Kwang Woo Hwang designed the study and discussed the data. Kwang Woo Hwang analyzed the data and So-Young Park supervised the experimental work. Kwang Woo Hwang revised the manuscript. All authors read and approved the final manuscript.



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