한국축산식품학회지
Korean J. Food Sci. Ani. Resour.
Vol. 28, No. 4, pp. 431~436(2008)

Neuroprotective Effects and Physicochemical Characteristics of Milk Fortified with Fibroin BF-7

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BF-7 강화 우유의 뇌기능보호 효과 및 물리화학적 특성

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Abstract

The impact of storage on the neuroprotective effects against Aβ–induced cell death and physicochemical characteristics of milk fortified with BF-7 were investigated. The BF-7 milk exerted protection of neuronal cells SK-N-SH from amyloid beta (Aβ)-induced neuronal stress. Our results showed that incubation of the cell with pretreated BF-7 milk, significantly attenuated apoptotic stress by Aβ, considered in cell morphology and nucleus shape. The general compositions were maintained consistently in BF-7 fortified milk (BF-7 milk). The BF-7 did not make any disturbance on pH and titratable acidity. The color change was not detected, either. Also, any microorganism had not been detected with more than 7 days storage at 4°C. In sensory evaluation study, the average scores of each sensory attribute were quite similar with plain milk. In conclusion, our results strongly indicate that BF-7 characteristics are quite adequate to be included in milk and BF-7 milk is still working well on neuro-protection, result in enforcing our brain and delaying neurodegeneration.

Key words : BF-7, nutraceutical substance, milk, neuroprotective effect, amyloid β-peptide

Introduction

According to general definition, a functional food is any modified food that may provide a health benefit beyond the nutritional value. These healthy foods include products with reduced fat, sugar or salt, added with vitamins, minerals, phytochemicals, bioactive peptides or ω3 polyunsaturated fatty acids (Manzi *et al.*, 2007). Some examples of functional milk products are lactose-hydrolyzed milk, milk with added probiotics, prebiotics, vitamins, and functional ingredients, probiotic fermented milk, and synbiotic fermented milk (Lee and Lee, 2000; Vinderola, 2008). Omega 3 fatty acids including EPA/DHA have been widely used to make functional milk. DHA has been known to be good for brain function, because DHA is a component of cellular membrane in brain. However, the clinical effects of DHA are not fully identified. Moreover, unsaturated fatty acid like as DHA is easily oxidized by the air and its oxidized form is reported to be toxic. And the oxidized form of DHA was highly neurotoxic (Kruman *et al.*, 1997; Keller *et al.*, 1999). It has been also suggested that oxidized DHA may play a pathological role in CNS disorders such as Alzheimer's disease with significant components of oxidative damage (Reich *et al.*, 2001; Montine *et al.*, 2002; Musiek *et al.*, 2004; Milne *et al.*, 2006).

Alzheimer's disease (AD) is one of the most common causes of dementia affecting elderly people. AD is a progressive neurodegenerative disorder of the brain characterized by the presence of intracellular neurofibrillary tangles and extracellular neuritic plaques in affected regions of the brain (Iqbal *et al.*, 2005; Wei *et al.*, 2008; Wisniewski *et al.*, 2008).

The brain of AD patients is characterized by deposition of amyloid β-peptide (Aβ). It has been shown that soluble, oligomeric or fibrillar forms of Aβ exhibit certain extent of

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neurotoxicity. Amyloid β-peptide (Aβ), a 39–43 amino acid β-sheet peptide, aggregates in the brain to form the major component of characteristic deposits know as neuritic plaques (Blennow *et al.*, 2006; Ji *et al.*, 2006; Shi *et al.*, 2006).

Previous experiments have demonstrated that Aβ-peptides are toxic to cultured neurons. Evidence from *in vivo* experiments also showed that Aβ is one of the pathological factors leading to neuronal loss, tau phosphorylation and activation of microglia. The mechanisms of Aβ to impose toxicity on neurons have been studied extensively. It has been suggested that the activation of caspase, stress kinases, and induction of oxidative stress are involved in the apoptotic processes (Barkats *et al.*, 2000; Folin *et al.*, 2006; Boldogh *et al.*, 2008).

Although many studies have been directed toward AD treatment, there is still no promising intervention for curing the disease. Neuroprotection is the attempt to preserve normal cellular interaction in the brain and minimize loss of neuronal functions in pathological conditions. Currently, much attention has been focused on the potential of using natural meterials as neuroprotective agents (Heo and Lee, 2006; Ban *et al.*, 2007; Ho *et al.*, 2007). The BF-7 was effective to block ceramide induced neuronal damage and to protect roles against Aβ toxicity (Chae *et al.*, 2004). In addition, it has enhanced memory and cognitive function (Chae *et al.*, 2004; Lee *et al.*, 2004). It has been suggested that BF-7 enhanced brain function of normal and demented persons in both learning and memory and protected neuronal cell SK-N-SH against reactive oxygen species (Lee *et al.*, 2004; Kim *et al.*, 2005). Fortunately, fibroin BF-7 was certificated safety and effectiveness by Korea Food & Drug Administration (KFDA).

Therefore, the objective of this study was to examine the neuroprotective effect of milk supplemented with BF-7 against Aβ-induced cell death and to investigate whether BF-7 was suitable as an nutraceutical additive to the milk.

Materials and Methods

Sample preparation

All UHT-treated milk samples and BF-7 were provided by RDA, Korea. BF-7 was melted at doses of 5, 10, and 20 mg per 200 mL of milk. For storage tests, the samples were placed in a 4°C refrigerator for up to 7 days. The day after the designated expiration date was fixed as 0 day of storage, and samples were taken at 1 or 2 day intervals for analytical and microbial measurements.

Cell culture

SK-N-SH human neuroblastoma cells were maintained at 37°C in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, CA, USA) in a humidified 95% air, 5% CO_2 incubator. The medium was changed to DMEM containing 2% fetal bovine serum for 1 hr before $A\beta_{1-42}$ (10 mM) treatment.

Pharmacological treatment

Beta amyloid peptide 1-42 ($A\beta_{1-42}$) was obtained from Biosource (CA, USA) and dissolved in water. A dose of 10 mM $A\beta_{1-42}$ was used and was aged at 4°C overnight before use. BF-7 was melted at a dose of 10 mg per 200 mL of milk.

Hoechst 33258 staining

SK-N-SH cells were fixed with 4% paraformaldehyde for 20 min and then stained with 8 g/mL of Hoechst dye 33258 (Sigma, St Louis, MO, USA) for 5 min. They were washed twice with phosphate-buffered saline and then observed using an IX70 microscope (Olympus, Tokyo, Japan) equipped with attachments for fluorescence microscopy. Dead cells and apoptotic bodies were characterized by condensed or fragmented nuclei.

General composition analysis

Protein, fat, lactose, and total solid contents were measured using a MilkoScan FT120 (Foss Electric, Hillerod, Denmark).

pH, titratable acidity, and color evaluation

The pH of samples was determined with a pH meter (Model 340, Mettler-Toledo GmbH, Schwerzenbach, Switzerland). The pH values of milk product samples were measured in 30 mL sample. The titratable acidity was determined according to the Association of Official Analytical Chemists (AOAC) method. It was determined using 0.1 M NaOH to the end point of pH 8.1 and expressed as grams of citric acid per liter

The instrumental color analysis of milk products was performed. Milk products were measured (30 mL) into petri dishes (90×15 mm) for color analysis. Color measurements were taken with a colorimeter (Chroma meter CR-210, Minolta, Japan; illuminate C, calibrated with white standard plate L = 97.83, a = -0.43, b = +1.98), consisted of an 8 mm diameter measuring area and a 50 mm diameter illumination area. Color values (CIE L, a, and b) were measured on the surface of samples and results were taken in triplicate for each sample.

Microbiological analysis

Viable cells were counted using the pour plate method with plate count agar (PCA). The plates were incubated at 30°C for 3 days.

Sensory evaluation

Sensory evaluation of milk product samples was carried out at 1, and 7 days using a five-point hedonic scale ranging from "dislike extremely" to "like extremely". Ten panelists (5 females, 5 males) were selected based on interest, availability and performance in the screening tests conducted with dairy products, and those who confirmed consuming milk at least once a week were chosen for the present study.

Statistical analysis

All experiments were performed at least three times under each experimental condition and mean values were reported. An analysis of variance were performed on all the variables measured using the General Linear Model (GLM) procedure of the SAS statistical package (SAS Institute, Inc., 1999). The Duncan's multiple range test ($p<0.05$) was used to determine differences between treatment means.

Results and Discussion

BF-7 milk significantly attenuated neuronal cell death induced by $A\beta_{1-42}$

To investigate the effect of BF-7 milk on neuronal cells, $A\beta_{1-42}$, a neuro-stress was applied to SK-N-SH cells, a human neuroblastoma. Accumulation of $A\beta_{1-42}$ in the brain is a hallmark of Alzheimer's disease, followed by inducing massive neuronal cell death. Ten mg BF-7 melted in 200 mL milk was incubated at 4°C for 0 day and 7 days.

Treatment of SK-N-SH cells with $A\beta_{1-42}$ alone resulted in an approximately 50% reduction in cell survival within 24 hr, whereas the cells pre-treated with BF-7 milk incubated for each 0 and 7 days, showed a reduction of $A\beta_{1-42}$–mediated cytotoxicity (Fig. 1). As shown in Fig. 1, the majority of SK-N-SH cells had undergone morphological changes such as membrane blebbing and cell shrinkage when treated with $A\beta_{1-42}$ alone. There was an increase in the number of apoptotic cells. Pre-treatment with BF-7 milk almost completely prevented this increase in apoptotic cells when 10 μM $A\beta_{1-42}$ was administered. Also the DNA status detected by Hoechst 33258 dye staining was evaluated. The nucleus in stressed cell are condensed and fragmented. Fig 2A, 2A0 and 2A7 showed the normal healthy nucleus, while Fig 2C, 2C0 and 2C7 represented the damaged nucleus undergoing condensation and fragmentation. Interestingly, 2 μL of BF-7 milk pretreated cells had almost similar state with normal healthy nucleus even the $A\beta_{1-42}$ was still in the culture.

General composition analysis

Fat, protein, lactose, and total solid contents of the milk were 3.71-3.79, 2.81-2.86, 4.57-4.62 and 11.97-12.10%, respectively, without any difference among BF-7 contents

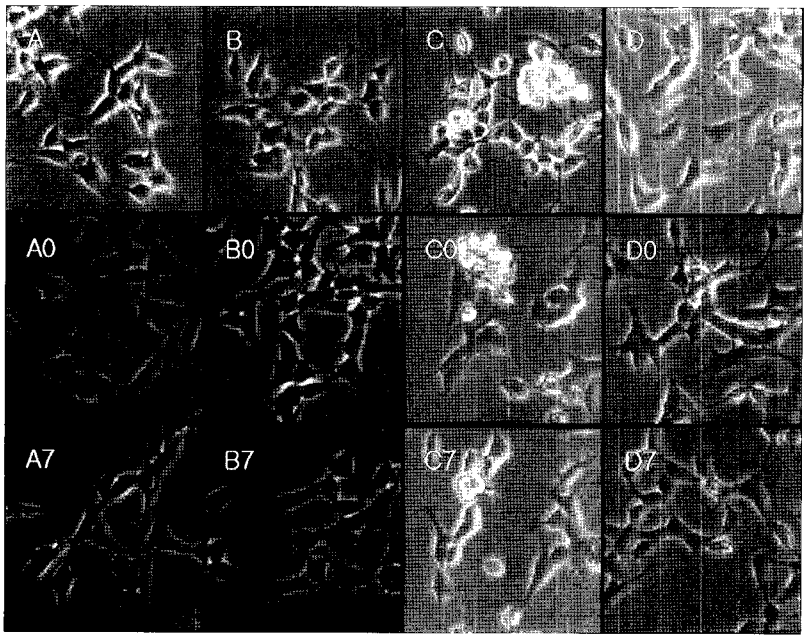

Fig. 1. Morphological assessment of Aβ-induced apoptosis in human neuroblastoma SK-N-SH cells. A, PBS treatment; B, BF-7 in PBS treatment; C, Aβ in PBS treatment; D, BF-7 and Aβ in PBS treatment; A0, milk treatment; B0, BF-7 in milk treatment; C0, Aβ in milk treatment; D0, BF-7 and Aβ in milk treatment; A7, milk treatment after 7 days; B7, BF-7 in milk treatment after 7 days; C7, Aβ in milk treatment after 7 days; D7, BF-7 and Aβ in milk treatment after 7 days.

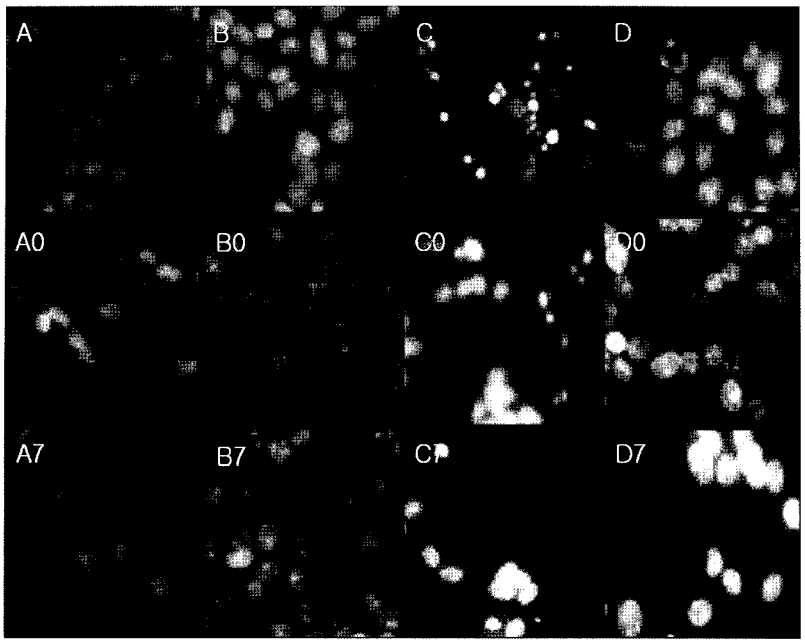

Fig. 2. Hoechst 33258 staining nuclear morphology of Aβ-induced apoptosis in human neuroblastoma SK-N-SH cells. A; PBS treatment, B; BF-7 in PBS treatment, C; Aβ in PBS treatment, D; BF-7 and Aβ in PBS treatment, A0; milk treatment, B0; BF-7 in milk treatment, C0; Aβ in milk treatment, D0; BF-7 and Aβ in milk treatment, A7; milk treatment after 7 days, B7; BF-7 in milk treatment after 7 days, C7; Aβ in milk treatment after 7 days, D7; BF-7 and Aβ in milk treatment after 7 days.

and the storage period at 0, 1, 3, 5, and 7 days (Table 1).

pH and titratable acidity

The pH of the BF-7 treated milks and milk control slowly increased with time in storage but levels of BF-7 in the treated milks did not vary significantly during storage (Table 2). Changes in titratable acidity were not significantly different between samples during storage. Addition of BF-7 into market milk didn't change pH and titratable acidity significantly (Table 3).

Table 1. General composition of BF-7 milks and milk control during storage

Storage period (days)	Component (%)	Total BF-7 content/package			
		Control	5 mg	10 mg	20 mg
0	Fat	3.73	3.76	3.79	3.76
	Protein	2.81	2.82	2.84	2.86
	Lactose	4.58	4.58	4.60	4.62
	Total solid	11.99	12.03	12.10	12.10
1	Fat	3.73	3.73	3.75	3.76
	Protein	2.81	2.81	2.83	2.85
	Lactose	4.57	4.58	4.60	4.61
	Total solid	11.98	11.99	12.05	12.08
3	Fat	3.73	3.74	3.75	3.75
	Protein	2.81	2.83	2.85	2.85
	Lactose	4.58	4.61	4.61	4.62
	Total solid	12.00	12.05	12.08	12.08
5	Fat	3.71	3.74	3.74	3.74
	Protein	2.81	2.84	2.84	2.85
	Lactose	4.58	4.61	4.61	4.61
	Total solid	11.97	12.05	12.06	12.08
7	Fat	3.72	3.74	3.75	3.74
	Protein	2.82	2.84	2.85	2.86
	Lactose	4.59	4.60	4.61	4.61
	Total solid	12.00	12.06	12.07	12.08

All values are mean ± SD of the three replicates.

Table 2. pH values of BF-7 milks and milk control during storage

Storage period (day)	Total BF-7 content/package			
	Control	5 mg	10 mg	20 mg
0	6.69	6.70	6.69	6.69
1	6.73	6.76	6.76	6.77
3	6.76	6.76	6.76	6.76
5	6.81	6.80	6.83	6.83
7	6.86	6.87	6.88	6.89

All values are mean ± SD of the three replicates.

Table 3. Change of titratable acidity of BF-7 milks and milk control during storage

Storage period (day)	Total BF-7 content/package			
	Control	5 mg	10 mg	20 mg
0	1.20	1.20	1.20	1.15
1	1.20	1.20	1.20	1.20
3	1.30	1.25	1.25	1.25
5	1.25	1.30	1.30	1.30
7	1.30	1.25	1.30	1.25

All values are mean ± SD of the three replicates.

Table 4. Change of color values of BF-7 milks and milk control during storage

Storage period (days)	Total BF-7 content/package											
	Control			5 mg			10 mg			20 mg		
	L	a	b	L	a	b	L	a	b	L	a	b
0	101.90	-0.45	+2.46	102.62	-0.36	+1.85	101.74	-0.24	+1.86	102.51	-0.20	+1.68
1	95.90	-0.47	+2.31	97.86	-0.32	+1.84	97.91	-0.22	+1.82	97.00	-0.19	+1.78
3	96.69	-0.44	+2.23	97.95	-0.36	+1.94	97.85	-0.39	+1.99	97.78	-0.36	+1.88
5	97.84	-0.45	+1.90	97.89	-0.40	+1.92	97.84	-0.37	+1.90	97.70	-0.38	+1.89
7	97.68	-0.41	+1.99	97.85	-0.40	+1.95	97.97	-0.41	+2.00	95.92	-0.39	+2.49

All values are mean ± SD of the three replicates.

Color evaluation

The L value (lightness) was not significantly different among the BF-7-treated samples, On the other hand, the a values (redness) of BF-7 milk were higher than those of the control, and the b values (yellowness) of BF-7 milk were lower than those of the control, in proportion to the BF-7 concentration, due to value of a and b values of BF-7 powder. But the observed color change were not significantly different between samples during storage (Table 4).

Microbiological analysis

After 7 days storage, BF-7 milks and milk controls possessed no detectible levels of any microorganisms (Table 5). Thermal treatment of milk is an essential operation during the commercial dairy processes in order to provide an acceptable safety and shelf-life. UHT-treated milk maintained sterility after 7 days of storage at 4°C (Imm *et al.*, 2005) and the same effect was observed in BF-7 milk.

Sensory evaluation

Table 6 shows the average score for each sensory attribute and the overall preference of BF-7 milks and milk control after 0 and 7 days of storage at 4°C. Sensory evaluation was not significantly different at any storage point (0 and 7 days) or BF-7 level.

Table 5. Change of microbes of BF-7 milks and milk control during storage

Storage period (day)	Total BF-7 content/package			
	Control	5 mg	10 mg	20 mg
0	ND[1)]	ND	ND	ND
1	ND	ND	ND	ND
3	ND	ND	ND	ND
5	ND	ND	ND	ND
7	ND	ND	ND	ND

All values are mean ± SD of the three replicates.
[1)]ND: Not detected.

Our results strongly implicates that the BF-7 bio-physical characteristics are quite adequate to be included in milk, since in many biophysical study no unfavorable changes in milk was detected. Furthermore, BF-7 milk is working well on neuro-protection, which implicating the BF-7 milk enforcing our brain and delaying neurodegeneration

Acknowledgments

This study was funded by a grant (20080401034034) from

Table 6. Change of sensory scores[1)] of BF-7 milks and milk control during storage

	Storage period (days)	Total BF-7 content / package			
		Control	5 mg	10 mg	20 mg
Odor	0	3.1 ± 0.5	3.3 ± 0.5	3.2 ± 0.4	3.2 ± 0.4
	7	3.6 ± 0.5	3.6 ± 0.5	3.6 ± 0.7	3.3 ± 0.6
Taste	0	3.3 ± 0.8	3.2 ± 0.8	3.3 ± 0.8	3.4 ± 0.7
	7	3.4 ± 0.5	3.2 ± 0.6	3.4 ± 0.8	3.2 ± 0.6
Mouth feel	0	3.0 ± 0.5	3.2 ± 0.4	3.1 ± 0.5	3.1 ± 0.5
	7	3.6 ± 0.5	3.3 ± 0.6	3.5 ± 0.5	3.3 ± 0.8
Color	0	3.4 ± 0.7	3.5 ± 0.7	3.4 ± 0.7	3.5 ± 0.7
	7	3.7 ± 0.6	3.7 ± 0.6	3.7 ± 0.6	3.7 ± 0.6
Total	0	3.2 ± 0.6	3.2 ± 0.6	3.2 ± 0.6	3.4 ± 0.5
	7	3.6 ± 0.5	3.4 ± 0.5	3.5 ± 0.5	3.6 ± 0.5

All values are mean ± SD of the three replicates.
[1)]Sensory scores were assessed on 5 point scale based on 1=extremely bad, 5=extremely good.

the BioGreen 21 Program, Rural Development Administration and the Brain Korea 21 Project, Korea.

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(2008. 8. 13 접수/2008. 9. 10 수정/2008. 9. 12 채택)