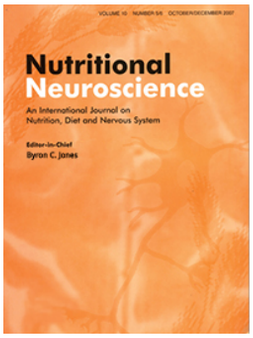

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Nutritional Neuroscience

An International Journal on Nutrition, Diet and Nervous System

ISSN: 1028-415X (Print) 1476-8305 (Online) Journal homepage: https://www.tandfonline.com/loi/ynns20

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**To cite this article:** Masahiro Nagai, Madoka Kubo, Rina Ando, Masayuki Ikeda, Hiroshi Iwamoto, Yasuhiro Takeda & Masahiro Nomoto (2020): Comparative examination of levodopa pharmacokinetics during simultaneous administration with lactoferrin in healthy subjects and the relationship between lipids and COMT inhibitory activity *in vitro*, Nutritional Neuroscience, DOI: 10.1080/1028415X.2020.1760532

**To link to this article:** https://doi.org/10.1080/1028415X.2020.1760532

NUTRITIONAL NEUROSCIENCE
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# Comparative examination of levodopa pharmacokinetics during simultaneous administration with lactoferrin in healthy subjects and the relationship between lipids and COMT inhibitory activity *in vitro*

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**ABSTRACT**

**Background:** Lactoferrin (bLF) is an iron-binding multifunctional protein that is abundant in milk. In mice, it inhibits catechol-*O*-methyltransferase (COMT) activity and increases blood levodopa levels. However, the clinical effects are unknown.

**Objective:** The objective of this study was to determine the effect of bLF on the kinetics of levodopa in blood.

**Design:** The effects of the concomitant administration of a combined formulation of levodopa and an aromatic amino acid decarboxylase inhibitor and bLF on the concentration of levodopa in blood and its metabolism were assessed in eight healthy subjects. In addition, we analyzed the association with clinical factors and evaluated whether clinical factors affected the COMT inhibitory activity of bLF *in vitro*.

**Results:** Although not statistically significant, the peak plasma concentration ($C_{max}$) of levodopa increased by 18.5%. From the results of the stratified analysis of total cholesterol, a relationship with $\Delta C_{max}$ was predicted. Therefore, bLF was reacted with cholesterol in the presence of lecithin and sodium deoxycholate *in vitro* to evaluate COMT inhibitory activity, and an increase in inhibitory activity was observed. By contrast, the ester compound cholesteryl oleate had no effect. The inhibitory activity of free fatty acids, which are known to interact with bLF, was also enhanced.

**Conclusion:** The COMT inhibitory activity of bLF is not effective in elevating blood levodopa levels. However, in humans with high lipid levels, such as cholesterol, interactions may enhance the inhibitory effect, resulting in the enhanced absorption of levodopa.

Trial registration: ID, UMIN000026787, registered 30 March 2017; URL, https://upload.umin.ac.jp/cgi-open-bin/ctr_e/ctr_view.cgi?recptno=R000030749

**KEYWORDS**
Lactoferrin; levodopa; Parkinson's disease; catechol-*O*-methyltransferase; cholesterol; free fatty acid; inhibitor; pharmacokinetics

**Trial registration:** UMIN Japan identifier: UMIN000026787

**Abbreviations:** COMT, catechol-*O*-methyltransferase; DCI, decarboxylase inhibitor; bLF, lactoferrin; PD, Parkinson's disease; 3-OMD, 3-*O*-methyldopa; DOPAC, 3,4-Dihydroxyphenylacetic acid; HVA, homovanillic acid; $C_{max}$, peak plasma concentration; AUC, area under the curve; $T_{max}$, time of the maximum plasma concentration; $t_{1/2}$, elimination half-life; PBS, phosphate buffered saline.

## Introduction

Lactoferrin (bLF) is an 80 kDa iron-binding protein that is abundant in the milk of several mammals and has a variety of effects [1]. For example, it has an immune activity, exhibits antioxidant activity by removing free iron [2], improves intestinal bacterial flora, and inhibits the activation of hepatitis C and other viruses. bLF is isolated from milk and is used as a food additive in infant milk, yogurt, and supplements [3].

Catechol-*O*-methyltransferase (COMT) is an enzyme that metabolizes biologically active substances, including catechols and methylated hydroxyl groups using *S*-adenosylmethionine as a methyl donor. It is widely distributed in living tissues and metabolizes biologically active substances, including the neurotransmitters dopamine and noradrenaline [4]. COMT is expressed as a membrane-bound and soluble isoform, and neurotransmitters are inactivated at neuronal synapses by these enzymes. COMT inhibitors, such as entacapone, have been studied for a long time and are used for the treatment of Parkinson's disease (PD) [5]. As natural derivatives, epigallocatechin gallate and quercetin are known to exhibit COMT

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Supplemental data for this article can be accessed https://doi.org/10.1080/1028415X.2020.1760532

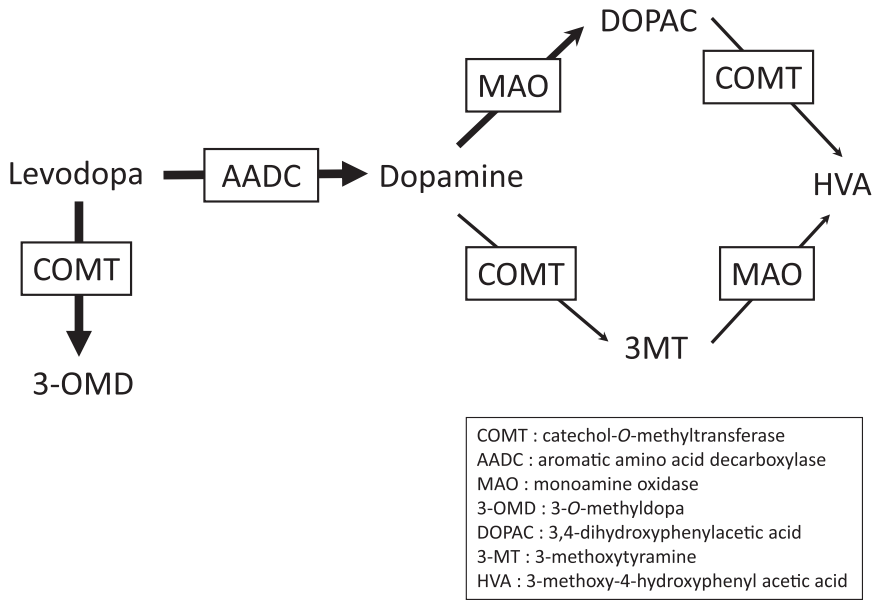


**Figure 1.** Metabolic pathway of levodopa.

inhibitory effects [6,7]. COMT inhibitors inhibit the metabolism of levodopa in the treatment of PD and are used in combination with carbidopa and benserazide aromatic amino acid decarboxylase inhibitors (DCI) to enhance the brain entry (Figure 1).

PD is a neurodegenerative disorder that is characterized by the degeneration and death of dopaminergic neurons of the substantia nigra in the midbrain. Insufficient dopamine in the brain leads to the development of neurological symptoms, such as bradykinesia, muscle rigidity, and tremors. The gold standard PD therapy is the oral administration of the dopamine precursor levodopa. After absorption within the duodenum, levodopa passes through the blood–brain barrier and reaches the central nervous system. Levodopa is metabolized into dopamine by the aromatic L-amino acid decarboxylase in the striatum. The absorption of levodopa in the digestive tract is unstable, and its half-life is short (approximately 1 h). Therefore, levodopa concentrations in the blood easily fluctuate, and long-term use causes a diurnal variation of symptoms known as the *wearing-off phenomenon* [8]. As a countermeasure, levodopa or COMT inhibitors are frequently administered [9].

Recently, bLF has been shown to inhibit COMT *in vitro* and *ex vivo* [10,11]. The mode of inhibition is attributed to the binding of bLF to COMT, and the two disulfide-linked regions that are approximately 45 amino acids at the N-terminus of bLF are thought to be involved in this activity. The exposure of this region is important for the inhibitory activity, which is enhanced by denaturation, such as with acid treatment. The inhibitory effect has also been found not to compete with the substrate or methyl donor *S*-adenosylmethionine.

As bLF inhibits COMT, the effects of bLF on levodopa metabolism were investigated in mice. The administration of bLF and levodopa had no significant effects. However, when combined with DCI, which inhibits one of the metabolic pathways of levodopa, the administration of bLF increased blood levodopa levels [11]. In this study, a crossover study in healthy male subjects was conducted to investigate the effects of bLF on blood levodopa concentrations and determine its safety in humans.

When bLF is orally ingested, changes in pH due to gastric acid and degeneration due to digestive enzymes pepsin and trypsin are expected in the gastrointestinal tract, suggesting that the health and nutritional status of subjects influences the effects of bLF. Therefore, the association with various clinical factors, including blood composition, was also evaluated.

## Methods

### *Materials*

bLF produced by Morinaga Milk Industry Co., Ltd. (Tokyo, Japan) was used. COMT enzymes were purchased from ATGen Ltd. (Seongman, South Korea). Cholesterol, lecithin, and sodium deoxycholate were produced by Fujifilm Wako Pure Chemicals (Tokyo, Japan), and cholesteryl oleate was produced by Nacalai Tesque Inc. (Kyoto, Japan). Other reagents used were from Sigma-Aldrich Corporation unless otherwise specified.

### Subjects

This study included eight healthy male adults aged 20 years or older who did not meet the following exclusion criteria: 1) individuals with hypersensitivity, such as allergies to dairy products; 2) those with diseases currently requiring regular outpatient visits; 3) those with serious medical histories, such as heart disease, renal disease, hepatic disease, and cancer; and 4) others who were found ineligible for the study by the study physician.

This study was conducted in compliance with the ethical principles of the Declaration of Helsinki. The study was conducted after being approved by the Institutional Review Board of Ehime University Hospital and registered in the UMIN (UMIN000026787). The subjects received written informed consent forms in advance and provided consent.

### Study design

A two-stage crossover open-label study was conducted in the Phase I unit of Ehime University Hospital. The subjects were alternately allocated to two groups in order of body weight (Figure 2). The washout time was determined from the time that the concentration of 3-*O*-methyldopa (3-OMD) returned to the initial level. bLF taken concurrently with levodopa preparations was supplied by Morinaga Milk Industry Co., Ltd. (Tokyo, Japan). The levodopa/benserazide (100 mg/25 mg per tablet) used was Ecdoparl® (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan). Benserazide is a DCI. The subjects fasted after dinner the previous day and orally took one tablet of Ecdoparl® and bLF powder (1.0 g) dissolved in 100 mL of water. If bLF was not administered, then one tablet of Ecdoparl® was taken orally with 100 mL of water. Blood was collected prior to ingestion and 15, 30, and 45 min and 1, 1.25, 1.5, 2, 2.5, 3, 3.5, and 4 h after ingestion. On the day of the study, the subjects fasted until the end of blood collection. The subjects were allowed to drink water freely. During the study period, the subjects were prohibited from using any drugs other than those routinely used at the time of registration. In addition, the subjects were interviewed regarding their subjective and objective symptoms, examined, and observed. We did not conduct daily dietary control or dietary surveys. The primary endpoints were levodopa area under the curve (AUC), $C_{max}$, time of the maximum plasma concentration ($T_{max}$), elimination half-life ($t_{1/2}$), and blood concentrations at each blood collection time point. Secondary endpoints included AUC, $C_{max}$, $T_{max}$, and $t_{1/2}$, and blood concentrations of 3-OMD, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) at different collection time points.

### Measurement of drug concentrations in blood

The concentrations of levodopa, 3-OMD, DOPAC, and HVA in the blood were measured by the following methods. Plasma was collected after the centrifugation (1,500 × g, 10 min, 4 °C) of blood obtained using an EDTA-2Na blood collection tube. For 100 µL of plasma, 500 µL of perchloric acid and 5 µL of internal standard solution were added, stirred for 30 s, and incubated on ice for 30 min. The supernatant was then centrifuged (20,000 × g, 15 min, 4 °C) and filtered (0.45 µm) for high-performance liquid chromatography (HPLC). HTEC500 (Eicom Corporation) HPLC machines were used in the analyses. The column used for the measurements was EICOMPAK SC-5ODS (i.d. 2.1 × 150 mm, Eicom Corporation), 0.1% phosphate buffer (pH 2. 7) containing 12% methanol and 224 mg/L octyl sulfate sodium salt were used in the mobile phase, the column oven temperature was 30 °C, the flow rate was 0.23 mL/min, and the voltage of the electrochemical detection was 750 mV.

### Evaluation by a stratified analysis

To investigate the relationships between clinical factors and the amount of changes, differences in $AUC_{(0–240\ min)}$, $C_{max}$, and $T_{max}$ for levodopa were calculated as the amount of changes Δ (values at the time of bLF ingestion − values at the time of ingestion with water). The subjects were divided into two groups of four subjects each in accordance with their clinical factors, and the relationships between $\Delta AUC_{(0–240\ min)}$, $\Delta C_{max}$, and $\Delta T_{max}$ were evaluated using permutation

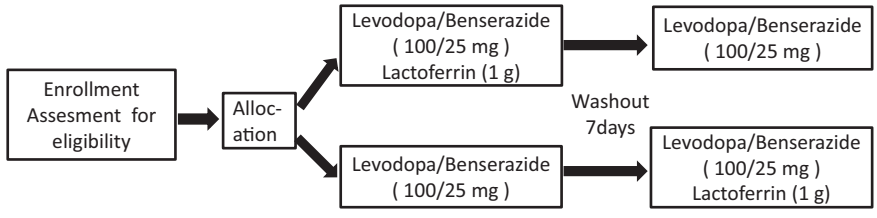


**Figure 2.** Study design.

tests. The clinical factors used in the health checkups routinely conducted at our hospital were used. The test for clinical factors was conducted before the study for the subjects who provided consent.

### Reaction of bLF with cholesterol

Preparation of cholesterol solutions: Cholesterol was dissolved in ethanol at 25 mg/mL and diluted with ethanol. These diluted solutions (25 µL) were mixed with 100 µL of phosphate-buffered saline (PBS) containing lecithin (20 mg/mL) and sodium deoxycholate (10 mg/mL). The mixtures were then reacted at 37 °C for 10 min to obtain cholesterol solutions (0, 1.25, 2.5, and 5 mg/mL).

Time course experiment: 20 µL of cholesterol solution (0 and 5 mg/mL) and bLF (final concentration 10 mg/mL) were mixed in 100 µL of PBS and reacted at 37 °C for 5, 20, or 60 min.

bLF concentration dependency: bLF (0, 1.3–10 mg/mL) was reacted with cholesterol solutions (0 or 1 mg/mL final concentration) for 20 min.

Cholesterol concentration dependency: bLF (final concentration 0 and 10 mg/mL) was reacted with cholesterol solutions (final concentration: 0, 0.25, 0.5, and 1 mg/mL) for 20 min.

Determination of the inhibition constant ($K_i$): Cholesterol (final concentration 1 mg/mL) and bLF (final concentration 10 mg/mL) were reacted at 37 °C for 20 min and then diluted with PBS to measure the COMT inhibitory activity of bLF. Because bLF does not compete with COMT substrates [10], the result was shown using a Dixon plot. bLF was calculated as 80 kDa.

COMT activity was measured as previously reported by Ikeda et al., with a few modifications [10]. Specifically, bLF that was reacted with cholesterol was added to the reaction solution, and the reaction was performed at a final volume of 25 µL. Subsequently, 12.5 µL of 1 M hydrochloric acid was added to stop the reaction, the solution was centrifuged at 17,800 × g for 10 min at room temperature, and 20 µL of the supernatant was analyzed by HPLC. The analytical methods used were previously reported by Shen et al. [12]. The HPLC columns used were XTERRA RP18 3.5 µm 4.5 × 100 mm (Waters Corporation). Measurements were obtained at an absorption signal of 280 nm, and enzymatic activity was calculated as the sum of vanillic acid and isovanillic acid and two methoxylated substances arising from 3,4-dihydroxybenzoic acid substrates.

### Reaction of bLF with fatty acids

bLF at a final concentration of 10 mg/mL was reacted with 0, 0.0074, 0.022, 0.067, and 0.2 mg/mL of sodium oleate, sodium palmitate, or sodium linoleate in PBS at 45 °C for 20 min. Subsequently, 2.5 µL of this solution was added to 25 µL (final volume) of the COMT activity measurement reaction solution to measure the COMT inhibitory activity of bLF. For sodium palmitate, a 50 mg/mL aqueous solution was dissolved at 90 °C and diluted to 2 mg/mL. In a comparative study of responses at 37 and 45 °C, 10 mg/mL of bLF and 0.2 mg/mL of sodium oleate were incubated in PBS at 37 °C or 45 °C for 20 min, after which COMT inhibitory activity was measured. The reaction was performed twice ($n = 2$). COMT activity was measured by separating substrates labeled using the method reported by Ikeda et al. with a toluene:isoamyl alcohol ratio of 7:3 [10].

### Statistical analysis

Statistical analyses were performed using JAMP™ version 13 (SAS Institute Inc., Cavy, NC). The summary statistics of $AUC_{(0–240\ min)}$, $C_{max}$, $T_{max}$ and $t_{1/2}$ were calculated from blood concentrations of the treatment groups, and the corresponding paired t-tests were performed. The two-sided significance level was set at 5%. $t_{1/2}$ was calculated from measurements at four consecutive time points after the time of $C_{max}$. For the evaluation of stratified analyses, permutation tests were performed. *In vitro* cholesterol experiments were assessed using unpaired t-tests.

## Results

### Clinical study

The primary endpoint of levodopa over time is shown in Figure 3. $AUC_{(0–240\ min)}$, $C_{max}$, $T_{max}$, $t_{1/2}$, and blood concentrations at different collection time points were assessed using paired t-tests. Although not statistically significant, $AUC_{(0–240\ min)}$ increased by 1.8% ($P = 0.87$), $C_{max}$ increased by 18.5% ($P = 0.21$), $T_{max}$ decreased by 7.8% ($P = 0.60$), and $t_{1/2}$ decreased by 18.9% ($P = 0.055$) (Table 1). The clinical factors of the eight subjects are shown in the Supplementary file 1.

$AUC_{(0–240\ min)}$, $C_{max}$, and $T_{max}$ of 3-OMD did not show a statistically significant difference. The analysis for $t_{1/2}$ was not performed as there was no decrease in blood concentration. In addition, no statistically significant differences were found in $AUC_{(0–240\ min)}$, $C_{max}$, or $T_{max}$ of DOPAC and HVA. The $t_{1/2}$ analysis was not performed due to the large variability in the time course of individuals (Table 1). No adverse events were observed throughout the study period, and there were no safety issues.

In the stratified analysis, the relationship among $\Delta C_{max}$, $\Delta AUC$, and $\Delta t_{1/2}$ for levodopa was investigated by dividing the subjects into two groups of four subjects

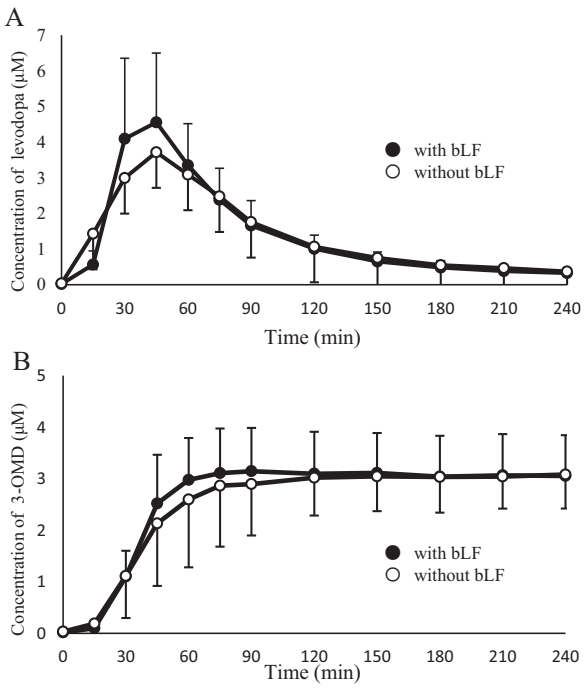


**Figure 3.** Changes in the mean plasma concentrations of levodopa and 3-OMD. A, Levodopa. B, 3-OMD. Values are means ± SD. $n = 8$.

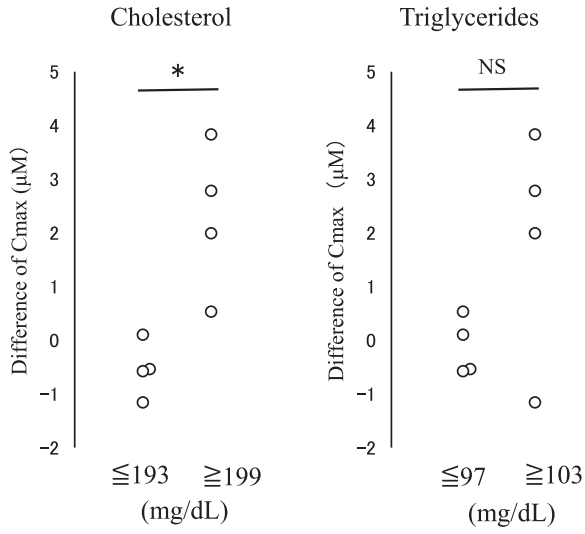


**Figure 4.** Associations of clinical factors and ΔCmax. ΔCmax in the higher-serum-cholesterol group (≧199) increased significantly compared with that in the lower-serum-cholesterol group (≦193). The significant difference in ΔCmax between higher-triglyceride (≧103) and lower-triglyceride (≦97) groups was not observed. * indicates <0.05. NS indicated not significant.

based on high and low values of 26 clinical factors. $T_{max}$ was not different in four out of the eight subjects. Therefore, the differences were calculated for $C_{max}$, AUC, and $t_{1/2}$ (Supplementary file 2). There was a significant difference in $\Delta C_{max}$ of systolic blood pressure and total cholesterol without adjusting multiplicity. In the high-cholesterol group, $C_{max}$ increased with bLF administration. Although a similar result was observed with triglycerides, which is a fat-related item, no significant differences were found (Figure 4). In the group with high systolic blood pressure, $C_{max}$ was lower with bLF administration, and $AUC_{(0–240\ min)}$ increased with bLF administration in the older group. No significant differences in $t_{1/2}$ were found between the groups.

## *In vitro* experiments

$\Delta C_{max}$ of levodopa was affected by high and low cholesterol levels (mean ± SD: 212 ± 15.6 mg/dL and 179 ± 9.0 mg/dL, respectively). Therefore, to clarify the relationship between cholesterol and the COMT inhibitory activity of bLF, an *in vitro* test was conducted. To this end, bLF and cholesterol were reacted in the presence of lecithin and sodium deoxycholate. COMT inhibitory activity increased based on the reaction of bLF to cholesterol. By contrast, no effects were observed when the ester was oleic acid ester (Figure 5). This response was dependent on the concentration of cholesterol (Figure 6C), and the effect increased with a prolonged reaction time (Figure 6A), with cholesterol alone not causing an effect (Figure 6B). When complexes were formed and $K_i$ was measured, $K_i$ was 25.2 μM for bLF alone and 18.7 μM for bLF with cholesterol (Figure 6D).

Although bLF is known to react with fatty acids to change their structures, when sodium oleate, sodium linoleate, and sodium palmitate were reacted with lactoferrin at 45 °C in the absence of a surfactant, increasing the sodium fatty acid level resulted in the enhanced COMT inhibitory activity of bLF (Figure 7A). A weaker enhancement of inhibitory activity was also observed with sodium oleate at 37 °C compared with 45 °C (Figure 7B).

**Table 1.** Pharmacokinetic parameters of levodopa and metabolites.

| | *T*max (min) | | *C*max (μM) | | AUC $_{(0–240\ min)}$ (μMmin) | | $t_{1/2}$(min) | |
|---|---|---|---|---|---|---|---|---|
| | bLF | $H_2O$ | bLF | $H_2O$ | bLF | $H_2O$ | bLF | $H_2O$ |
| Levodopa | 45.0 ± 11.3 | 48.8 ± 20.8 | 5.51 ± 1.96 | 4.65 ± 1.81 | 342 ± 104 | 336 ± 130 | 36.4 ± 5.04 | 44.9 ± 11.6 |
| 3-OMD | 144 ± 68.9 | 169 ± 82.8 | 3.31 ± 0.84 | 3.36 ± 0.90 | 633 ± 162 | 609 ± 169 | N.D. | N.D. |
| DOPAC | 45.0 ± 13.9 | 28.1 ± 25.9 | 0.08 ± 0.02 | 0.09 ± 0.04 | 8.26 ± 3.61 | 9.57 ± 3.93 | N.D. | N.D. |
| HVA | 60.0 ± 53.8 | 37.5 ± 21.2 | 0.35 ± 0.27 | 0.37 ± 0.22 | 65.2 ± 52.5 | 69.8 ± 49.1 | N.D. | N.D. |

Mean ± SD
N.D.: not determined

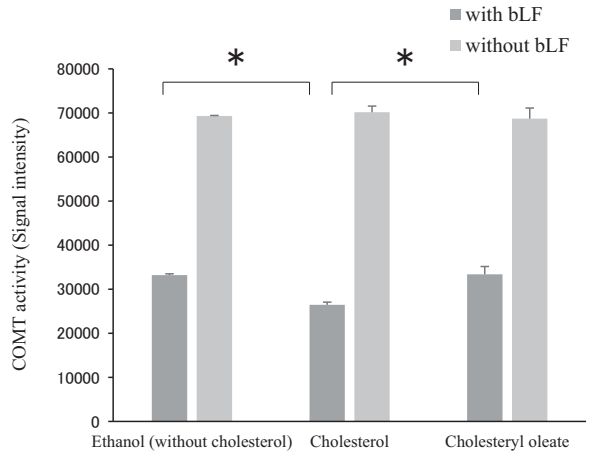


**Figure 5.** Enhancement of the COMT inhibitory activity of bLF by cholesterol. bLF was reacted with cholesterol, cholesteryl oleate, or ethanol for 60 min at 37 °C, and the COMT inhibitory activity was measured. $n = 3$, error bar indicates SD, * indicates $< 0.05$ compared with ethanol and cholesteryl oleate.

## Discussion

bLF inhibits COMT activity *in vitro*; therefore, we conducted a crossover study *in vivo* to determine its impact on the plasma concentrations of levodopa. In past bLF clinical trials, no serious adverse events occurred even after multiple administrations of bLF tablets at 7.2 g/day for 8 weeks [13]. Among the patients who were tube-fed and administered liquid food containing low concentrations of bLF (1 mg/ml) over 12 weeks, no difference was observed in the incidence of adverse events compared with the control group [14]. In the present study, we used single doses of bLF set at 1 g/administration/day to maximize safety. Because no adverse events were observed throughout the study period, this dose was considered safe. There are gender differences in the pharmacokinetics of levodopa. Specifically, AUC and $C_{max}$ are known to be higher in women [15]. As this study was the first to use bLF and levodopa, we included only male subjects.

No significant changes were observed in the pharmacokinetics ($AUC_{(0–240\ min)}$, $C_{max}$, $T_{max}$, and $t_{1/2}$) of levodopa regardless of the concomitant administration of bLF. In addition, no significant changes were observed in the metabolites 3-OMD, DOPAC, and HVA. In mice, the administration of levodopa (approximately 58 mg/kg), carbidopa (approximately 14 mg/kg), and bLF (580 mg/kg) increased levodopa AUC (1.3-fold) and $C_{max}$ (1.2-fold) [11]. The bLF dose in this study was approximately 40-fold higher than that in the present study (14.6 mg/kg). Therefore, large doses may be required to be effective in humans. In this study, benserazide was used as the DCI; however, carbidopa is also an essential DCI formulation. In studies involving healthy individuals and PD patients, benserazide is known to increase $C_{max}$ and AUC compared with carbidopa [16]. In this study, benserazide, which has a stronger aromatic L-amino acid decarboxylase inhibitory effect, was used to evaluate the COMT inhibitory activity of bLF. As carbidopa was used in animal studies, the results may differ depending on the DCI used.

A power analysis (comparison of the mean values of the two corresponding groups) was performed at $C_{max}$ and $t_{1/2}$ (Supplementary file 3). In the expectation of the difference between the groups obtained in this study, the results were $n = 37$ at $C_{max}$ and $n = 13$ at $t_{1/2}$ ($\alpha = 0.05$; $\beta = 0.8$).

Monoamine oxidase inhibitors (MAOIs) are prescribed for the treatment of PD. However, foods containing tyramine, such as cheese, may be combined with MAOIs to suppress the degradation of tyramine and cause hypertension [17]. Levodopa has also been reported to interact with drugs and food [18]. For example, when levodopa was administered after ingesting milk or dairy products, such as cheese and butter, $C_{max}$ of levodopa decreased. Neutral amino acids generated from the digestion of protein can compete with levodopa during digestive tract absorption [19]. However, in the present study, the milk-derived protein bLF did not inhibit the absorption of levodopa. Because bLF is the only protein used in this experiment, it is difficult to discuss the superiority of bLF compared with other proteins. In the mouse experiment, levodopa blood levels with bLF were significantly higher than with sodium caseinate. Therefore, it is necessary to conduct a comparative test with other proteins as a control in the future. bLF is an iron-containing protein, and divalent and trivalent irons may bind to levodopa, preventing its uptake into blood [20]. The iron saturation of bLF used in the present study was approximately 10%, and the ingestion of 1 g of bLF means the ingestion of approximately 1.4 mg of iron (19% of the dietary reference intake for Japanese people [30-year-old male]) [21]. A previously conducted human clinical trial examined levodopa absorption inhibition following the administration of 352 mg of iron. The iron intake in the present study was much lower, which may have contributed to the absence of inhibitory effects on levodopa absorption [22]. Alternatively, irons chelated in bLF molecules did not bind well to levodopa because only a small amount of iron is released by digestion in the digestive tract. Concerning the effect of bLF on iron absorption, Koikawa et al. reported that the co-administration of iron material and bLF that was not iron-saturated reduced the decrease in blood iron due to exercise [23].

In the analysis of clinical factors, the subjects were divided into two groups by high and low values, and the factors that showed significance depending on bLF

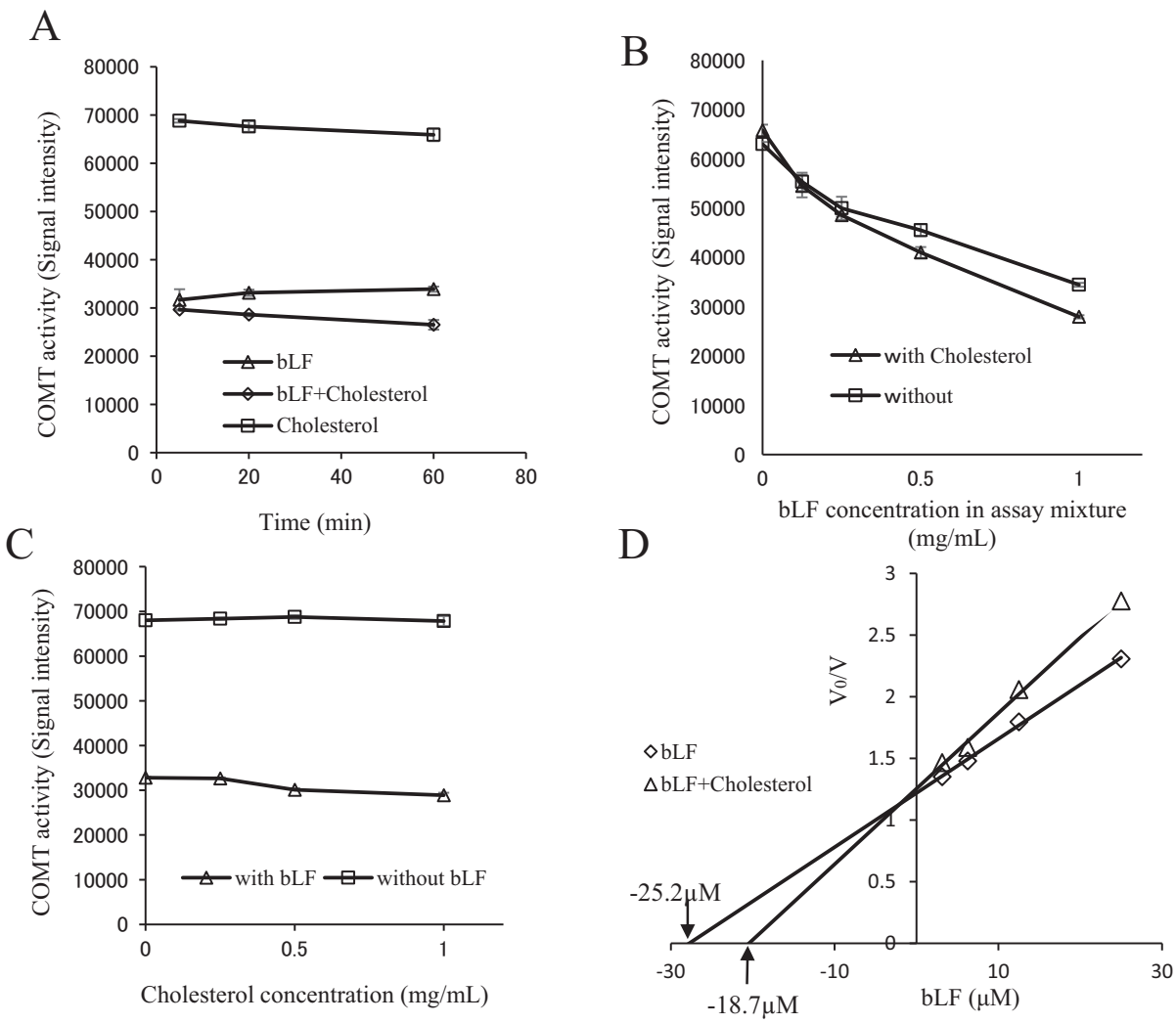


**Figure 6.** Characteristics of the enhancement of the COMT inhibitory activity of bLF by cholesterol. A, Relationship between cholesterol and bLF reaction time and activity. bLF was reacted with cholesterol for 5, 20, or 60 min at 37 °C, and COMT activity was examined. B, Dependency of bLF concentration. bLF (0, 1.3, 2.5, 5, or 10 mg/mL) was reacted at 37 °C for 20 min with 1 mg/mL of cholesterol. Then, COMT inhibitory activities of bLF solutions (1/10 volume of reaction mixture) were measured. C, Enhancement of the inhibitory activity of bLF when cholesterol concentration was changed. Cholesterol was reacted at a final concentration of 0, 0.25, 0.5, or 1 mg/mL with 10 mg/mL bLF at 37 °C for 20 min. D, Titration curves of bLF and cholesterol complex (Dixon plot). The complexes were diluted 2, 4, and 8 times with PBS, and COMT inhibitory activity was measured. The COMT activity measurement was performed twice ($n = 2$), and the variance is indicated by the error bar. The vertical axis is the ratio of $V_0$ (COMT activity without bLF)/V (COMT activity with bLF).

administration were identified. The subjects with high total cholesterol had high $C_{max}$. Thus, we evaluated the effects of cholesterol on the COMT inhibitory activity of bLF *in vitro*, which was enhanced by reacting bLF with cholesterol. This response was dependent on cholesterol concentration. Although not a type of cholesterol, bLF is known to alter conformation by binding to the lipid fatty acid sodium [24]. An inhibitory potentiation effect was also observed when bLF was reacted with fatty acid sodium; however, the effect was not observed with an oleic acid cholesterol ester, in which cholesterol and oleic acid were combined. To increase its solubility and reactivity with bLF, cholesterol is dissolved in lecithin and deoxycholic acid [25]. The enhancement of inhibition is not observed when cholesterol is mixed with water instead of ethanol. These results suggest that solubility or three-dimensional positional relationships are important for the reaction between bLF and cholesterol.

Studies have provided evidence that the onset of PD is related to blood cholesterol, and humans with high blood cholesterol have been reported to develop late-onset PD. Regarding bLF and lipids, Takeuchi et al. have shown that plasma neutral fats and free fatty acid levels can be reduced in mice by bLF [26], and in humans, bLF indicates a decrease in visceral fat area [27]. bLF is also

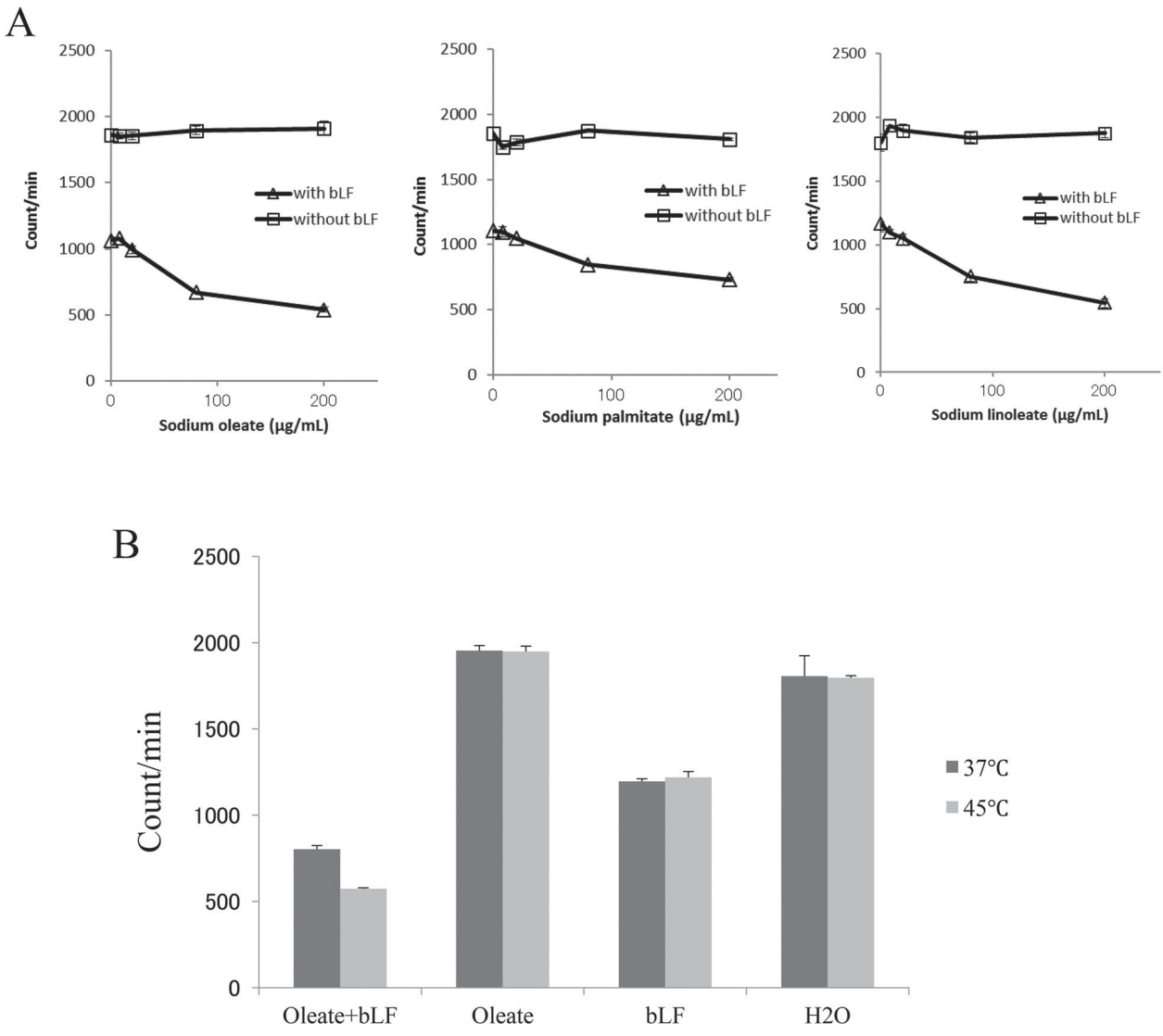


**Figure 7.** COMT inhibitory activity of the reacted products of sodium fatty acid and bLF. A, bLF (15 mg/mL) was reacted with sodium fatty acid (0, 0.0074, 0.022, 0.067, and 0.2 mg/mL) in PBS at 45 °C, and then 2.5 μL of this reaction mixture was used for the COMT assay. B, Comparison of reactions at 37 and 45 °C. 10 mg/mL of bLF and 0.2 mg/mL of sodium oleate were reacted in PBS at 37 °C or 45 °C for 20 min, and then COMT inhibitory activity was measured. The reaction was performed twice ($n = 2$). bLF reacted with sodium fatty acid showed changes in $K_i$ (Supplementary file 5).

known to destabilize the membrane of fatty micelles, and conversely, fatty acid sodium binds to bLF to change its conformation. Because Fang et al. performed reactions with fatty acids and bLF at 45 °C [24], a similar procedure was followed in this study. The inhibitory activity of bLF was enhanced in a concentration-dependent manner. Three major constituent fatty acids *in vivo* were tested and showed inhibitory effects with or without an unsaturated moiety. *In vivo*, considering that the temperature is around 37 °C, a reaction was performed using sodium oleate as an example, and the results showed that the effect was weakened in comparison with at 45 °C, although the potentiation of inhibition was still observed. The COMT inhibitory activity of bLF may be enhanced by fatty acids in clinical studies. Clinical data that did not show significant differences in triglycerides are shown in Figure 4. In the high-triglyceride group, $C_{max}$ increased with bLF administration in all but one subject. The subject showing a large deviation had abnormal triglyceride levels (238 mg/dL). Therefore, a principal component analysis was performed for 25 clinical factors, excluding age (Supplementary file 4). γGTP also showed a large deviation, but its value was normal (24 IU/L). There may be effects of evening meals before the clinical factor test rather than the presence of a chronic illness.

Free fatty acids in the blood are ~0.24 mM in the early morning [28] and ~0.33 mM at 100 μg/mL of sodium

oleate (MW = 304). In this situation, there may be a region that is susceptible to changes in fatty acid concentrations in enhancing the COMT inhibitory activity of bLF by fatty acids (Figure 7A). In addition, the cholesterol concentration is approximately 65 mg/dL because approximately one-third of the total cholesterol laboratory values are present in free form [29]. Assuming that all cholesterols are dissolved in COMT inhibition assays, this level corresponds to a region that enhances the COMT inhibitory activity of bLF (Figure 6). Although a variety of factors may be involved, such as the presence of other binding molecules, high and low concentrations of these two fats may sensitively affect the effects of bLF. Many fats are bound to albumin [30]. In our experiments, the fatty acids used were free of albumin. Therefore, the increase in the COMT inhibitory activity of bLF may be weakened.

In PD patients, bacterial overgrowth in the small intestine and *Helicobacter pylori* have been reported to affect levodopa absorption [31], and elimination has been associated with increased absorption. In addition, the association between fecal flora and PD has been studied [32]. bLF is known to have bacteriostatic activity by removing free iron and antimicrobial activity by peptides that have undergone enzymatic degradation. The spectrum of activity is specific, and differences in the effects on *Escherichia coli* and bifidobacteria are also known [33]. Furthermore, there have been reports of enhancing the elimination of *H. pylori* [34]. The analysis of fecal flora with long-term ingestion has also been performed using genome-wide analysis in recent years, and changes have been shown [35]. With its antibacterial activity, antioxidant activity by removing free iron, and ability to improve intestinal bacterial flora, the long-term intake of bLF may improve activities of daily living and the quality of life in PD patients.

The results of this clinical study did not confirm the $C_{max}$- and AUC-increasing effects on levodopa due to the COMT inhibitory activity of bLF; however, the results of stratified analyses and *in vitro* results in the clinical study indicated that lipids influenced the effect. Although further clinical investigation is necessary, bLF may be a food that assists in the stable supply of drugs to patients with PD taking levodopa.

## Acknowledgments

We thank Kumiko Toshimitsu, Ph. D. from Division of Neutrition, Ehime University Hospital, for her generous support to this study. The authors would like to thank Enago (www.enago.jp) for the English language review.

## Disclosure statement

MI, HI and YT are employed by Morinaga Milk Industry Co., Ltd. MNagai, MK, RA and MNomoto have no conflict of interest.

## Funding

A portion of the funding for this study and the bLF were provided by Morinaga Milk Industry Co., Ltd.

## Contributors

The authors' responsibilities were as follows – MNagai: conducted the experimental work and contributed to the drafting of the manuscript; MK: conducted the experimental work and data management; RA: helped the data analysis and the drafting of the manuscript; MI conducted the experimental work and contributed to the draft of the manuscript; HI contributed to the draft of the manuscript; YT contributed design the study and supervised the experimental work; MNomoto: design the study, the data management, and supervised the experimental work.

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