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Research Paper

Preparation of ONO-1301-loaded poly(lactide-co-glycolide) microspheres and their effect on nerve conduction velocity

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Abstract

Objectives The aim of this study was to prepare poly(lactide-co-glycolide) (PLGA) microspheres containing ONO-1301, a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity, with 10% of drug released in the initial burst and a sustained-release period of about 3 weeks after administration. The effect of PLGA type (molecular weight and the lactide/glycolide (L/G) ratio in PLGA), the preparative conditions and the particle size on the in-vitro release profile were examined. The effect of optimized ONO-1301-loaded PLGA microspheres on delayed nerve condition velocity (NCV) was investigated in streptozotocin (STZ) induced diabetic rats.

Methods ONO-1301 PLGA microspheres were produced by the oil-in-water emulsion/solvent evaporation method. Drug release from the prepared microspheres was monitored in phosphate buffer solution at 37°C for 4 weeks by high-performance liquid chromatography. The in-vivo study was performed in STZ-induced diabetic rats treated with optimized ONO-1301 PLGA microspheres (10 mg/kg by intramuscular or subcutaneous injection every 3 weeks). NCV was measured in the thigh 4, 8 and 12 weeks after induction.

Key findings The molecular weights of PLGA, the L/G ratio in PLGA and the particle diameter all affected the length of the sustained release period. Drug release from microspheres containing PLGA 5050 (MW 50 000, L/G 50/50), with an average diameter of about 30 μm, could be sustained for 3 weeks *in vitro*. In the in-vivo study, delayed NCV was significantly increased by treatment with these ONO-1301 PLGA microspheres once every 3 weeks, in comparison with vehicle only.

Conclusion Local intramuscular injection of sustained-release ONO-1301 PLGA microspheres improved delayed NCV in STZ-induced diabetic rats.

Keywords nerve condition velocity; ONO-1301; PGI_2 agonist; PLGA microspheres; sustained release

Introduction

ONO-1301 is a novel long-acting prostacyclin agonist with thromboxane synthase inhibitory activity. Unlike prostacyclin, ONO-1301 does not possess a five-membered ring or allylic alcohol in its molecular structure, making it more biologically and chemically stable. Its inhibitory effect on thromboxane synthetase is mediated by binding of thromboxane synthase to the 3-pyridin moiety and a carboxylic acid group in ONO-1301.[1] A prostaglandin analogue, beraprost sodium, is known to significantly increase tail nerve conduction velocity (NCV) in rats with streptozotocin (STZ) induced diabetes.[2] These findings suggest that ONO-1301 may be useful for prevention of neural dysfunction in peripheral nerves. However, the effect of ONO-1301 on delayed NCV induced by diabetes has not been clarified *in vivo*, and although the plasma half-life of ONO-1301 is longer than that of other prostacyclin analogues, ONO-1301 still needs to be administered subcutaneously twice a day to achieve a significant improvement in peripheral neuropathy.

Vascular disease and peripheral neuropathy are frequently observed in diabetes mellitus patients. For diabetic patients with peripheral neuropathy, development of a long-acting, sustained-release prostacyclin analogue would be beneficial in providing a stable improvement of peripheral neuropathy and therefore quality of life. In order to achieve this, a novel sustained-release prostacyclin analogue, ONO-1301, polymerized with poly(D,L-lactic-co-glycolic acid) (PLGA) was prepared. PLGA microspheres are biodegradable and

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biocompatible, and have been used as a controlled delivery system for proteins and drugs.[3–8] The release of drug from PLGA microspheres occurs through degradation of the polymer matrix.

The purpose of this study was to investigate the preparative methods for ONO-1301 PLGA microspheres necessary to achieve sustained drug release for 3 weeks and 10% of drug in the burst release *in vitro*. The effect of PLGA type (molecular weight and the lactide/glycolide (L/G) ratio in PLGA) and preparative conditions on particle size were examined in order to optimize the ONO-1301 PLGA microspheres so as to maintain drug release for 3 weeks. The efficacy of optimized ONO-1301 PLGA microspheres on delayed NCV in STZ-induced diabetic rats was investigated. The plasma ONO-1301 concentration was also measured in rats treated orally with ONO-1301 and by intramuscular injection of ONO-1301 PLGA microspheres to investigate the pharmacokinetics of ONO-1301 *in vivo*.

Materials and Methods

Materials

ONO-1301 was generously supplied by ONO Pharmaceutical Co., Ltd., Osaka, Japan. Different types of PLGA (average molecular weight: 10 000, co-polymer ratio of D,L-lactide to glycolide: 50/50, PLGA 5010; average molecular weight: 20 000, co-polymer ratio of D,L-lactide to glycolide: 50/50, PLGA 5020; average molecular weight: 50 000, co-polymer ratio of D,L-lactide to glycolide: 50/50, PLGA 5050; average molecular weight: 20 000, co-polymer ratio of D,L-lactide to glycolide: 100/0, PLGA 0020; and average molecular weight: 20 000, co-polymer ratio of D,L-lactide to glycolide: 75/25, PLGA 7520; all from Wako Pure Chemical Industries Ltd., Osaka, Japan) were used as substrate in the microspheres. Polyvinyl alcohol and Tween 80 (Nacalai Tesque Ltd., Kyoto, Japan) were used as dispersants in the production of PLGA microspheres. D-Mannitol (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as addition agent in the freeze-drying process. High reagent grade acetone, acetonitrile, methanol and ethanol were used as good solvents for PLGA, and Japanese Pharmacopoeia grade purified water was used as the poor solvent.

Preparation of ONO-1301 PLGA microspheres

ONO-1301 PLGA microspheres were produced by the oil-in-water emulsion/solvent evaporation method. The preparative procedures for microspheres under basic conditions were as follows: polyvinyl alcohol aqueous solution (poor solvent of PLGA) was put into a glass vessel. An ethanol/dichloromethane solution containing PLGA and ONO-1301 was dropped into the poor solvent while stirring to form an oil-in-water emulsion. Dichloromethane was then evaporated off by stirring at room temperature. After centrifugation and washing, ONO-1301 PLGA microspheres were isolated by lyophilization.

Particle size measurement

The volume diameters of the wet particles sampled before lyophilization were measured using a Multisizer3 Coulter Counter (Beckman Coulter, Inc., Tokyo, Japan). The volume diameters of the wet particles were considered as geometric diameters. When the particle size was measured after lyophilization, the dry particles were resuspended in buffer using a vortex mixer and analysed with the particle size analyser.

Encapsulation efficiency

Acetonitrile containing *n*-propyl 4-hydrobenzoate served as an internal control to determine the encapsulation efficiency, and the solution was homogenized by a sonicator. The concentration of ONO-1301 in this solution was analysed by high-performance liquid chromatography. The encapsulation efficiency was calculated as follows:[9] encapsulation efficiency (%) = (measure value/theoretical value) × 100.

In-vitro drug release from ONO-1301 PLGA microspheres

ONO-1301 PLGA microspheres were suspended in phosphate-buffered saline (0.067 mol/l salt concentration, pH 6.8) containing 0.2% Tween-80 and the concentration of ONO-1301 adjusted to 100 μg/ml. Aliquots of 1 ml were incubated at 37°C. At various time intervals, the liquid phase from one of the aliquots was discarded, the pellet dissolved in acetonitrile, and the ONO-1301 remaining analysed by high-performance liquid chromatography.

Animals and treatments

A total of 40 female Sprague-Dawley rats (Japan SLC, Inc., Hamamatsu, Japan; 171.4–205.4 g at the start of the experiment) were used in the study. Animals were housed in a limited-access animal facility where temperature and relative humidity were set at 22 ± 2°C and 55 ± 10%, respectively. Artificial lighting provided a 24-h cycle of 12-h light/dark (lights on: 0700–1900 hours) with food (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water available *ad libitum*. All experimental procedures regarding animal care and use were performed in accordance with the regulations established by the Experimental Animal Care and Use Committee of Mukogawa Women's University. All experiments conformed to the guidelines on the ethical use of animals of the Japanese Government Notification, and all efforts were made to minimize both the number of animals used for experiments and their suffering. Rats were rendered diabetic with an intraperitoneal injection of streptozotocin (STZ; 40 mg/kg, Sigma-Aldrich, Inc., Tokyo, Japan) dissolved in 0.1 M citrate buffer (pH 4.5). At 2 weeks after administration with STZ, animals with plasma glucose concentrations above 300 mg/dl were considered diabetic and were included in the study. Vehicle-treated rats served as controls. Rats were classified into five groups ($n = 8$ animals/group) as follows: (1) control group, treated by intramuscular injection of 0.2 w/v% Tween 80 saline every 3 weeks; (2) vehicle group, treated by intramuscular injection of PLGA microspheres/0.2 w/v% Tween 80 saline every 3 weeks; (3) ONO-1301-treated group, treated orally with ONO-1301, 3 mg/kg, twice a day; (4) ONO-1301 PLGA microsphere-treated group, treated by intramuscular injection, 10 mg/kg, every 3 weeks; and (5) ONO-1301 PLGA microsphere-treated group, treated by subcutaneous injection,

10 mg/kg, every 3 weeks. The intramuscular and subcutaneous injections were given in the lower left limb. The rats were observed for 12 weeks. The ONO-1301 PLGA microspheres used in the in-vivo study contained PLGA 5050 prepared as microsphere batch No. 1 scaled up, the drug release was confirmed to be 3 weeks *in vitro*.

Neurophysiological assessment

The NCV was determined in the thigh nerve of each animal as previously described[10–16] on the day of diabetes induction and 4, 8 and 12 weeks after induction. In brief, the NCV in the thigh nerve was assessed by placing recording ring electrodes distally in the thigh, while the stimulating ring electrodes were placed 5 and 10 cm proximally with respect to the recording point. The latency of the potentials recorded at the two sites after nerve stimulation was determined (peak-to-peak) and the NCV calculated accordingly. All neurophysiological determinations were performed under standard conditions in a temperature-controlled room.

Assay of plasma levels of ONO-1301

To investigate whether the injection of microspheres produced long-lasting drug release in rats, plasma levels of residual ONO-1301 were measured after a single oral administration of ONO-1301, or single intramuscular and subcutaneous injections of ONO-1301 PLGA microspheres. Blood was withdrawn from the inferior vena cava of eight rats 12 h after they received a single oral administration of 3 mg/kg ONO-1301, and from the inferior vena cava of 10 rats 14 days after they received a single intramuscular or subcutaneous injection of 10 mg/kg ONO-1301 PLGA microspheres. The blood was immediately transferred to chilled glass tubes containing 1 mg/ml disodium ethylenediaminetetraacetic acid and 500 U/ml aprotinin, and centrifuged. The plasma ONO-1301 level was measured by liquid chromatography with tandem mass spectrometry assay. The time of blood collection in each group was set to compare the lowest plasma ONO-1301 level after oral administration of ONO-1301 with the plasma ONO-1301 level during the stable drug release period from ONO-1301 microspheres *in vivo*.

Statistical analysis

All data were expressed as means ± SD. Two-tailed one-way analysis of variance was used for statistical comparison. The Bonferroni/Dunn test was also used for post-hoc analysis. Values of $P < 0.05$ were considered significant.

Results and Discussion

Effect of molecular weight of PLGA on in-vitro drug release

The properties of the ONO-1301 microspheres prepared in this study are shown in Table 1. The comparison of drug release from microsphere formulations using PLGA 5050, PLGA 5020 and PLGA 5010 in phosphate buffer is shown in Figure 1a. There were significant differences between No. 2 and 3 microspheres in Figure 1a ($P < 0.01$ *versus* No.1, analysis of variance).

The drug encapsulation efficiency of prepared microspheres was 74.3% for PLGA 5050, 83.8% for PLGA 5020 and 94.7% for PLGA 5010. The average diameter of the prepared microspheres was 30.3 μm for PLGA 5050, 35.5 μm for PLGA 5020 and 0 31.5 μm for PLGA 501 (Table 1). The burst release was evaluated during the first 24 h; the mean drug residual ratio of prepared microspheres (±SD) using PLGA 5050 was 81.99% (±0.10), for PLGA 5020 was 80.60% (±0.10) and for PLGA 5010 was 51.45% (±0.10). The drug residual ratio of prepared PLGA 5050 microspheres after 1, 2 and 3 weeks *in vitro* was 72.76% (±0.20), 40.37% (±0.10) and 10.94% (±0.10), respectively. For PLGA 5020 microspheres, these figures were 62.06% (±0.20), 26.30% (±0.20), and 9.83% (±0.10), respectively; and for PLGA 5010 microspheres 33.66% (±0.10), 16.83% (±0.10) and 8.67% (±0.10), respectively. The drug release rate increased with a decrease in the molecular weight of the microspheres. These data correspond with those of Heya *et al*.[17] The drug release profiles of prepared microspheres from Day 1 to 21 were fitted to zero- and first-order models, and their correlation coefficients compared (Table 2). We examined the in-vitro drug release for ONO-1301 microsphere batches No. 1 to 3. Yoshida *et al*.[18] evaluated the release kinetics for controlled drug release by zero-order and first-order release kinetics by correlation coefficients. Therefore, in the present study, all ONO-1301 microsphere data were fitted to zero-order and first-order equations. Calculated correlation coefficients of No. 1 and 2 ONO-1301 microspheres based on the zero-order equation (0.970, 0.980) were greater than those calculated based on the first-order equation (0.882, 0.962), which indicated that drug release was constant or suppressed in the drug release in the first phase followed zero-order kinetics.

Table 1 Type of PLGA microspheres, encapsulation efficiency and particle size

Microsphere formulation No.	Type of PLGA	Encapsulation efficiency (%)	Size (μm)
1	PLGA 5050 (MW 50 000, PL/GA = 1)	74.3 ± 1.8	30.3 ± 11.2
2	PLGA 5020 (MW 20 000, PL/GA = 1)	83.8 ± 2.0	35.5 ± 12.5
3	PLGA 5010 (MW 10 000, PL/GA = 1)	94.7 ± 1.1	31.5 ± 12.8
4	PLGA 0020 (MW 20 000, PL = 1)	95.1 ± 1.4	26.7 ± 10.5
5	PLGA 7520 (MW 20 000, PL/GA = 3)	94.1 ± 1.7	26.4 ± 10.4
6	PLGA 5020 (MW 20 000, PL/GA = 1)	94.1 ± 3.2	29.7 ± 10.3
7	PLGA 5050 (MW 50 000, PL/GA = 1)	89.1 ± 0.9	30.9 ± 12.7
8	PLGA 5050 (MW 50 000, PL/GA = 1)	81.4 ± 2.0	16.6 ± 8.1
9	PLGA 5050 (MW 50 000, PL/GA = 1)	90.7 ± 2.7	14.5 ± 6.0

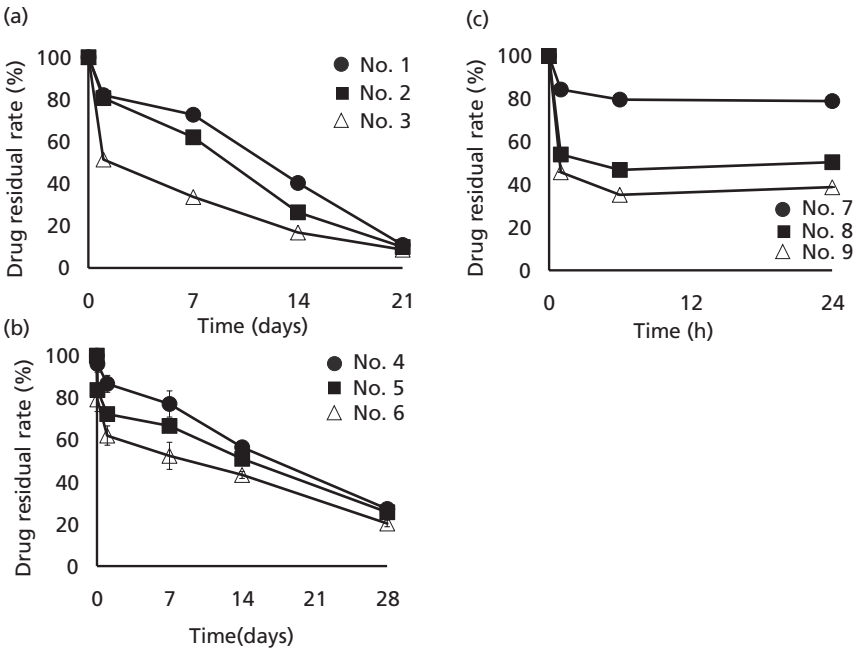

Figure 1 (a) In-vitro release profile of ONO-1301 acetate from microspheres made from three types of PLGA. No. 1, PLGA 5050; No. 2, PLGA 5020; No. 3, PLGA 5010. (b) In-vitro release profile of ONO-1301 acetate from microspheres with three different lactide/glycolide ratios in PLGA. No. 4, PLGA 0020; No. 5, PLGA 7520; No. 6, PLGA 5020. (c) In-vitro release profile of ONO-1301 acetate from microspheres made from three particle sizes of PLGA 5050. No. 7, 30.9 ± 12.7 μm; No. 8, 16.6 ± 8.1 μm; No. 9, 14.5 ± 6.0 μm. All values represent the mean ± SD.

Table 2 Fitting drug release profiles of ONO-1301 microspheres using PLGA differences in molecular weight to first- and zero-order release models

Microsphere formulation No.	First-order release model		Zero-order release model	
	Rate constant (min^{-1})	Correlation coefficient	Rate constant (% min^{-1})	Correlation coefficient
1	0.100	0.882	3.688	0.970
2	0.108	0.962	3.703	0.980
3	0.090	0.995	2.154	0.966

The drug was released from the PLGA matrix of the microspheres by hydrolysis *in vitro*. The drug release from microspheres using PLGA 5010 was restricted, mainly due to the small bulk of PLGA in the hydrolytic process. In contrast, PLGA 5050 and 5020, which have higher molecular weights than PLGA 5010, probably dissolved more gradually and had decreased drug release rates in the early stage of in-vitro drug release.

Effect of the L/G ratio in PLGA on in-vitro drug release

Drug release from prepared microspheres using different L/G ratios in PLGA was evaluated *in vitro* because the drug release profiles from PLGA microspheres changed with the type of PGLA encapsulated. A comparison of drug release from microsphere formulations using PLGA 0020, PLGA 7520 or PLGA 5020 in phosphate buffer is shown in Figure 1b. There were significant differences between batch No. 5 and 6 microspheres in Figure 1a ($P < 0.05$, 0.01 *versus* No.4, analysis of variance).

The drug encapsulation efficiencies of prepared microspheres using the various types of PLGA, together with their respective particle diameters, are shown in Table 1. The drug residual ratios of the microspheres in the 24-h burst release and after 1, 2 and 3 weeks are also given. The drug release became slower as the L/G ratio in PLGA increased. These data correspond with those of Berkland *et al*.[19] and Ravivarapu *et al*.[20] Drug release from microspheres using PLGA 5020 was mainly restricted by the rate of hydrolysis of PL. In contrast, PLGA 0020 and PLGA 7520, which had a high L/G ratio in PLGA, probably dissolved more gradually and drug release rates were lower during the early stages of in-vitro drug release.

Effect of average particle diameter of microspheres on in-vitro drug release

The comparisons of drug release from microsphere formulations using PLGA 5050, in which the average particle size was 30.9 ± 12.7, 16.6 ± 8.1 or 14.5 ± 6.0 μm, are shown in Figure 1c. There were significant differences between batch No. 8 and 9 microspheres in Figure 1a ($P < 0.01$ *versus* No.7, analysis of variance). In-vitro release of ONO-1301 from PLGA microspheres was dependent on the particle size. The drug encapsulation efficiency of No. 7 microspheres was 89.1%, No. 8 microspheres was 81.4% and No. 9 microspheres was 99.9% (Table 1). The drug residual ratios of No. 7 microspheres after 1, 6 and 24 h of in-vitro drug release were 84.33% (±0.1), 79.72% (±0.1) and 79.02% (±0.2), respectively; those of No. 8 microspheres were 54.03% (±0.1), 46.86% (±0.1) and 50.49% (±0.1), respectively, and those of No. 9 microspheres were 45.82% (±0.1), 35.31% (±0.1) and 38.83% (±0.1), respectively. Thus, the release

rate increased as the average diameter of the microspheres decreased. It was considered probable that the surface area of microspheres facing the buffer would be greater when the size of microspheres is smaller. The results showed that release of ONO-1301 from small PLGA microspheres was increased because of an increase in the area exposed to the solvent.

Improvement of nerve conduction velocity *in vivo*

In a previous study, the plasma ONO-1301 level after injection of ONO-1301 microspheres lasted for over 2 weeks,[9] and this result almost coincided with the in-vitro release result in the present study. Optimized ONO-1301 PLGA microspheres (containing PLGA 5050, with an average diameter of about 30 μm) were used in the in-vivo study. The drug encapsulation efficiency of the microspheres was 74.3% (±0.2) and the drug residual ratio after 1, 4, 7, 14, 21 and 28 days was 84.6% (±1.6), 75.7% (±0.4), 71.6% (±0.6), 40.4% (±4.1), 10.3% (±2.7) and 6.9% (±0.7), respectively. During the treatment period, the healthy control rats had the expected slight increase in NCV caused by physiological growth. The administration of 40 mg/kg STZ induced a significant reduction in lower limb NCV in the vehicle group compared with controls, confirming the onset of diabetic peripheral neuropathy.

A significant increase in NCV in the lower left (drug-treated side) limb was observed in animals treated with oral ONO-1301 after 12 weeks compared with the vehicle group ($P < 0.05$). A significant increase was observed in animals treated intramuscularly with ONO-1301 PLGA microspheres after 8 and 12 weeks compared with vehicle ($P < 0.01$). In animals treated subcutaneously with ONO-1301 PLGA microspheres, a significant increase was observed after 8 ($P < 0.05$) and 12 weeks ($P < 0.01$), both compared with vehicle (Figure 2).

No significant difference was observed in the untreated limb between the ONO-1301 PLGA microsphere-treated (intramuscular) and vehicle-treated groups during the experimental period, but a significant increase was observed in animals treated subcutaneously with ONO-1301 PLGA microspheres after 8 and 12 weeks compared with the vehicle-treated group ($P < 0.05$; Figure 3). These results showed that treatment with ONO-1301 PLGA microspheres (both intramuscularly and subcutaneously) can improve the delayed NCV induced by diabetes. The intramuscular injection route was the more effective.

Previous studies have reported that biodegradable microspheres for controlled delivery of the somatostatin analogue vapreotide *in vitro* have sustained plasma levels over 2–4 weeks after a single injection in rats.[21] Some studies that attempted to demonstrate a relationship between in-vitro and in-vivo release, restricted their analysis to a single microsphere formulation exhibiting a specific in-vitro release pattern under controlled release conditions.[22] The results of the present study are in accordance with these reports.

The plasma ONO-1301 level was measured after treatment with several treatment methods (oral, intramuscular, subcutaneous) to compare the ONO-1301 plasma concentration level and investigate the level of residual ONO-1301 in topical tissue in STZ-induced diabetic rats. Plasma ONO-1301 levels were measured 12 h after a single oral administration of 3 mg/kg ONO-1301 to STZ-induced diabetic rats. The mean ± SD was 45.14 ± 26.94 ng/ml. ONO-1301 was detected in plasma on Day 14 after a single intramuscular injection of 10 mg/kg ONO-1301 PLGA microspheres (mean 5.64 ± 1.55 ng/ml), while on Day 14 after a single subcutaneous injection, the mean value was 4.62 ± 1.81 ng/ml. The ONO-1301 plasma concentration level after oral treatment was higher than that after intramuscular and subcutaneous treatment. However, the improvement effect on the neurological disorder of the intramuscular and subcutaneous treatment groups was greater than that of the oral treatment group at 12 weeks. This suggests that intramuscular and subcutaneous treatment of ONO-1301MS has long-lasting activity and ONO-1301 might show sustained released from the

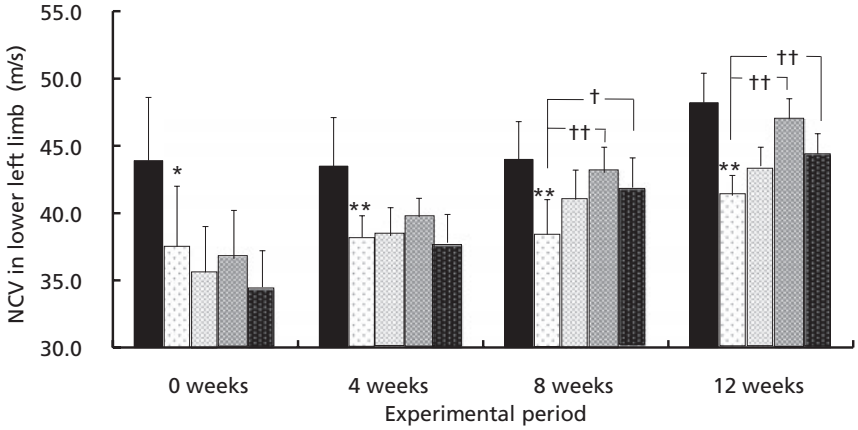

Figure 2 Nerve conduction velocity in the lower left (drug-treated side) limb of streptozotocin-induced diabetic rats. ■, Vehicle was injected intramuscularly in normal rats once every 3 weeks; □, vehicle was injected intramuscularly in streptozotocin-induced diabetic rats once every 3 weeks; ▨, ONO-1301 was administered orally to streptozotocin-induced diabetic rats (3 mg/kg) twice daily; ▩, ONO-1301 PLGA microspheres (No. 1) (10 mg/kg) were injected intramuscularly in streptozotocin-induced diabetic rats once every 3 weeks; ■, ONO-1301 PLGA microspheres (No. 1) (10 mg/kg) were injected subcutaneously in streptozotocin-induced diabetic rats once every 3 weeks. Values represent the mean ± SD ($n = 10$). *$P < 0.05$, **$P < 0.01$ compared with normal rats; †$P < 0.05$, ††$P < 0.01$ compared with vehicle (Bonferroni/Dunn test).

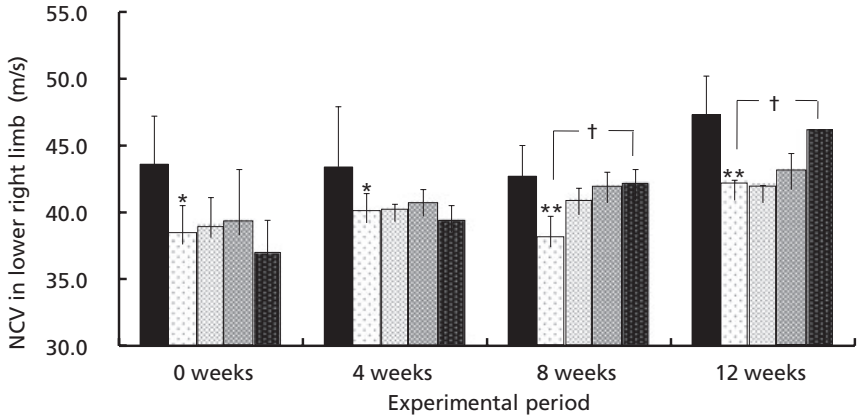

Figure 3 Nerve conduction velocity in lower right (non-drug-treated side) limb of streptozotocin-induced diabetic rats. ■, Vehicle was injected intramuscularly in normal rats once every 3 weeks; ▫, vehicle was injected intramuscularly in streptozotocin-induced diabetic rats once every 3 weeks; ▫, ONO-1301 was administered orally (3 mg/kg) to streptozotocin-induced diabetic rats twice a day; ▫, ONO-1301 PLGA microspheres (No. 1) were injected intramuscularly (10 mg/kg) in streptozotocin-induced diabetic rats once every 3 weeks; ■, ONO-1301 PLGA microspheres (No. 1) were injected subcutaneously (10 mg/kg) in streptozotocin-induced diabetic rats once every 3 weeks. Values represent the mean ± SD ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with normal rats; $^{\dagger}P < 0.05$ compared with vehicle (Bonferroni/Dunn test).

microspheres *in vivo*. The results suggest that controlled drug release, providing ONO-1301 plasma levels of 4–5 ng/ml, could provide an effective therapeutic strategy for peripheral neuropathy. The plasma ONO-1301 level was determined as described in a previous study,[9] in which subcutaneous treatment of ONO-1301 microspheres was applied in an in-vivo study. Furthermore, no significant difference was observed between plasma ONO-1301 levels at 2 weeks after treatment in the intramuscular and subcutaneous treatment groups. However, microspheres may be distributed in the topical site such as the lower limb, since the improvement effect on the neurological disorder by intramuscular treatment was greater than that obtained after subcutaneous treatment. Thus ONO-1301 loaded microspheres might affected in lower leg ischaemia without systemic action such as the level of vascular endothelial growth factor.[23]

In the present study, it seems likely that drug release from PLGA microspheres implanted subcutaneously or intraperitoneally may be directly due to enzymatic degradation, while release from intramuscular implants appears to be mostly dependent on hydrolytic cleavage. Intramuscular implants behaved in a similar way to their behaviour *in vitro*.[24] Intramuscular injection of ONO-1301 PLGA microspheres in the affected area was more effective than subcutaneous injection, providing an easily controlled drug release from microspheres as evidenced by the in-vitro study.

Conclusion

The purpose of present study was to investigate the preparative conditions of ONO-1301 microspheres as 3-week long-lasting formulations *in vitro*, and to assess their clinical effects on diabetic neuropathy *in vivo*. The selection of appropriate polymer types (molecular weight and the L/G ratio in PLGA) and particle sizes allowed the set criteria regarding encapsulation efficiency and in-vitro release over 1 month to be met. In particular, microspheres prepared from PLGA 5050, with an average particle diameter of 30 µm, fully satisfied the targets set in the in-vivo study. The results of the in-vivo study showed that intramuscular treatment with ONO-1301 PLGA microspheres could significantly improve delayed NCV induced by diabetes. Our results suggest that the prepared ONO-1301 microspheres may be a new therapeutic strategy for the treatment of diabetic neuropathy as a 3-weekly injectable delivery system for ONO-1301.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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