Effects of squalene/squalane on dopamine levels, antioxidant enzyme activity, and fatty acid composition in the striatum of Parkinson’s disease mouse model

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Abstract: Active oxygen has been implicated in the pathogenesis of Parkinson’s disease (PD); therefore, antioxidants have attracted attention as a potential way to prevent this disease. Squalene, a natural triterpene and an intermediate in the biosynthesis of cholesterol, is known to have active oxygen scavenging activities. Squalane, synthesized by complete hydrogenation of squalene, does not have active oxygen scavenging activities. We examined the effects of oral administration of squalene or squalane on a PD mouse model, which was developed by intracerebroventricular injection of 6-hydroxydopamine (6-OHDA). Squalene administration 7 days before and 7 days after one 6-OHDA injection prevented a reduction in striatal dopamine (DA) levels, while the same administration of squalane enhanced the levels. Neither squalene nor squalane administration for 7 days changed the levels of catalase, glutathione peroxidase, or superoxide dismutase activities in the striatum. Squalane increased thiobarbituric acid reactive substances, a marker of lipid peroxidation, in the striatum. Both squalene and squalane increased the ratio of linoleic acid/linolenic acid in the striatum. These results suggest that the administration of squalene or squalane induces similar changes in the composition of fatty acids and has no effect on the activities of active oxygen scavenging enzymes in the striatum. However, squalane increases oxidative damage in the striatum and exacerbates the toxicity of 6-OHDA, while squalene prevents it. The effects of squalene or squalane treatment in this model suggest their possible uses and risks in the treatment of PD.

Key words: Parkinson’s disease, squalene, squalane, fatty acid composition, oxidative stress

1 INTRODUCTION

Parkinson’s disease (PD) is characterized by the progressive degeneration of dopaminergic neurons of the nigrostrial system and dopamine (DA) depletion in the striatum. While the pathogenesis of PD is not clear, damage to the dopaminergic neurons by oxygen-derived free radicals is considered to be an important contributing mechanism1. 6-Hydroxydopamine (6-OHDA) is used to produce an excellent animal model of PD2, and is considered to be an endogenous toxin because it is found in the urine of PD patients3. The toxicity of 6-OHDA is thought to be related to its ability to produce free radicals and to cause oxidative stress4–6. 6-OHDA is susceptible to auto-oxidation, resulting in the formation of 6-OHDA quinone and hydrogen peroxide, as well as superoxide and hydroxyl radicals4–5. These active oxygen species are neurotoxic because of their strong oxidizing potential. We have already reported that the phosphoric diester of alpha-tocopherol and ascorbic acid, which has active oxygen scavenging activity, can prevent 6-OHDA-induced neurotoxicity6. We also reported that some alkaloids, such as zingerone and eugenol, which are known antioxidants, provide protection against 6-OHDA-induced depletion7, 8.

DA is a neurotransmitter. Once released to synaptic cleft, it binds to and activates postsynaptic neuron, and is inactivated by reuptake into the presynaptic cell, then enzymatic breakdown by monoamine oxidase into 3,4-dihydroxyphenylacetic acid (DOPAC). Finally, catechol-O-methyl transferase reduces DOPAC to homovanillic acid (HVA).

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Accepted August 1, 2012 (received for review May 25, 2012)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/  http://mc.manuscriptcentral.com/jjocs
Squalene (SQLE) is an isoprenoid compound structurally similar to beta-carotene, coenzyme Q10, vitamin A, vitamin E, and vitamin K, which are physiologically active substances\(^\text{5}\). It has six double bonds and is a highly effective oxygen scavenging agent\(^\text{10}\), and protects human skin surfaces from lipid peroxidation as a quencher of singlet oxygen\(^\text{11}\). Furthermore, it is reported to have antioxidant properties and immune enhancing activities\(^\text{12,13}\). Squalane (SQLA) is found naturally in sebaceous secretions\(^\text{14}\). It is a saturated analogue of SQLE, from which it can also be produced by complete hydrogenation and possessing no active oxygen scavenging effects. Like SQLE, SQLA is widely employed as a skin lubricant, ingredient of suppositories, carrier of lipid-soluble drugs and adjuvants\(^\text{9,15}\).

In this study, we examined the effects of SQLE and SQLA on 6-OHDA-induced DA depletion in the mouse striatum, and on lipid peroxidation-inhibiting activity using an in vivo model. The purpose of this study is to investigate how SQLE/SQLA has the preventive effect against Parkinson’s disease pathogenesis and its mechanisms.

2 MATERIALS AND METHODS

2.1 Animals
Male ICR mice (Charles River Japan, Yokohama, Japan) weighing 30 to 35 g were used in all experiments. They were provided with water and a standard laboratory diet containing 24% protein (MF, Oriental Yeast, Tokyo, Japan) ad libitum, and housed at 25°C with a 12-h light, 12-h dark cycle (lights on 7 am to 7 pm). New mice were used for each assay. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kagawa Prefectural College of Health Sciences.

2.2 Effect of treatment with SQLE/SQLA on 6-OHDA toxicity
SQLE or SQLA was administered orally at a dose of 1.0 or 0.1 g/kg body weight for 14 days. After 7-day administration (once daily), 6-OHDA was injected intracerebroventricularly (i.c.v.) at a dose of 60 μg in 2 μL of 0.05% L-ascorbic acid/saline 30 min after SQLE or SQLA administration. SQLE/SQLA administration was continued for 7 days after the 6-OHDA injection. The control was given vehicle (0.1% Tween 20/water solution for SQLE/SQLA administration and 0.05% L-ascorbic acid/saline for 6-OHDA injection) in the same manner. Twenty-four hours after the final administration, mice were sacrificed under sodium pentobarbital anesthesia and the striatum was removed on an ice plate and stored at −80°C until analysis.

2.3 Effect of treatment with SQLE/SQLA on activity of striatal active oxygen scavenging enzymes, thio-barbituric acid reactive substances (TBARS), and fatty acids levels in mouse striatum
Mice were orally administered with SQLE or SQLA at a dose of 1 g/kg body weight. The control was given vehicle (0.1% Tween 20/water solution). Thirty minutes after the final administration of a 7-day course of SQLE, SQLA or vehicle (once daily), mice were sacrificed by deep sodium pentobarbital anesthesia. The brain was removed and the striatum was separated immediately on an ice plate, and stored at −80°C until analysis.

2.4 DA and its metabolites (DOPAC and HVA) levels
Striatal tissues were homogenized with 300 μL of 200 mM ice-cold perchloric acid containing 10 mM disodium EDTA. After centrifugation (12000 × g for 10 min at 4°C), the supernatant was filtered (0.45 μm pore size) and then injected directly into an HPLC column (Shimadzu, Kyoto, Japan) with an electrochemical detector (ECD; Eicom, Kyoto, Japan). The appendant potential of the ECD (carbon electrode vs. Ag/AgCl reference electrode) was set at 700 mV. The analytical column was TSKgel ODS-80TM (4.6 mm I.D. × 250 mm; Tosoh, Tokyo, Japan), and the mobile phase consisted of 0.1 M citrate-sodium acetate buffer (pH 3.9) containing methanol (18%, v/v), disodium EDTA (4 mg/L), and sodium octanesulfonate (0.8 mM).

2.5 SOD activity
Cytosolic SOD activity was assayed using a SOD assay kit (SOD Test Wako; Wako Pure Chemical, Osaka, Japan), based on the method described by Oberley and Spitz\(^\text{16}\). Briefly, tissues were homogenized in 20 volumes of ice-cold phosphate buffer (10 mM, pH 8.0) and centrifuged at 12000 × g for 10 min at 4°C. Xanthine (0.4 mM), nitroblue tetrazolium (0.24 mM), and xanthine oxidase (0.049 U/mL) dissolved in phosphate buffer (0.1 M, pH 8.0) were added to the supernatants, and the mixture was incubated for 20 min at 37°C. The reaction was stopped by adding sodium dodecyl sulfate (69 mM). Absorbance was measured at 560 nm. SOD from bovine erythrocytes (Sigma Life Science, St. Louis, MO, USA) was used as the standard.

2.6 Catalase activity
Catalase activity was assayed according to the method described by Aebi\(^\text{17}\). In brief, the striatum was homogenized in 20 volumes of ice-cold RIPA buffer (0.1 M phosphate buffer (pH 7.4) containing 5 mM EDTA, 0.01% digitonin, and 0.25% sodium cholate) and centrifuged at 12000 × g for 30 min at 4°C. Phosphate buffer (50 mM, pH 7.0) containing EDTA (5 mM) and H₂O₂ (10 mM) pre-incubated for 10 min at 37°C was added to the supernatant, and the decomposition of H₂O₂ was assayed directly by measuring the decrease in absorbance at 240 nm for 3 min. Catalase from bovine liver (Wako Pure Chemical, Osaka, Japan) was used as a standard.
2.7 GPx activity

GPx activity was assayed using a Bioxytech GPx-340 kit (OXIS International, Portland, OR, USA). The striatum was homogenized with 10 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 1 mM 2-mercaptoethanol, and centrifuged at 12000 × g for 10 min at 4°C. Supernatant was used for the analysis. GPx from bovine erythrocytes (Sigma Life Science, St. Louis, MO, USA) was used as a standard.

2.8 Thiobarbituric acid reactive substance (TBARS) levels

The tissue concentration of TBARS was measured according to the method described by Ohishi. Two hundred μL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% sodium acetate buffer (pH 3.5), 1.5 mL of 0.8% sodium thiobisbiturate, and 600 μL of distilled water were added to 200 μL of the homogenate and the mixture was heated at 100°C for 60 min. TBARS were extracted with a mixture of n-butanol:pyridine (15:1, V/V), and the fluorescence was measured (Ex. 515 nm, Em. 532 nm). Malonaldehyde (Tokyo Kasei Kogyo, Tokyo, Japan) was used as a standard.

2.9 Measurement of fatty acids concentrations in the striatum

The tissue was homogenized with 10 volumes of chloroform (67%) / methanol (33%) solution containing 1 μg heptadecanoic acid as an internal standard. After centrifugation, the supernatant was added to 2 mL of distilled water, mixed, and centrifuged again. The chloroform layer was isolated, evaporated, and dissolved in 0.2 mL of n-hexane. The extracted samples were methylated using a fatty acid methylation kit (Nacalai Tesque, Kyoto, Japan). Samples were injected into a gas chromatography-mass spectrometer (GC/MS, Shimazu GCMS-QP5050A, Shimazu, Kyoto, Japan), using a DB-5 column (J and W Scientific, Folsom, CA, USA) and helium as the carrier gas.

2.10 Protein levels

The protein assay was performed by the method of Lowry et al. using bovine serum albumin as the standard.

2.11 Chemicals

All chemicals and reagents were of the highest quality available and obtained either from Wako Pure Chemical (Osaka, Japan) or Sigma Life Science (St. Louis, MO, USA).

2.12 Statistical analysis

All data are expressed as means ± SEM. Differences between each groups were examined for statistical significance using two-way analysis of variance (DA, DOPAC, HVA), and one-way analysis of variance (TBARS, SOD, catalase, GPx, fatty acids) followed by Scheffe’s F-test. The ratio of linoleic acid/linolenic acid was examined for statistical significance using Mann-Whitney U test. A p-value of less than 0.05 was considered to indicate statistical significance.

3 RESULTS

3.1 Effect of SQLE/SQLA administration for 7 days before and 7 days following one 6-OHDA i.c.v. injection on DA, DOPAC, and HVA levels in mouse striatum

The mean tissue concentrations of DA and its metabolites (DOPAC and HVA) in the mouse striatum are shown in Figs. 1 and 2. DA and DOPAC levels were significantly decreased by 6-OHDA injection compared with those in the striatum of the control (Fig. 1). SQLE treatment blunted the 6-OHDA-induced DA and DOPAC reduction. DA recovered but not to a significant level compared with the control when 1.0 mg/kg of SQLE was administered (Fig. 1). SQLA treatment enhanced the 6-OHDA-induced DA and DOPAC reduction (Fig. 2). SQLE or SQLA alone did not significantly change the levels of striatal DA or its metabolites at this time point (Figs. 1 and 2).

3.2 Effect of SQLE/SQLA administration for 7 days on the levels of TBARS in the mouse striatum

The mean tissue concentrations of TBARS in the mouse striatum are shown in Fig. 3. SQLE treatment did not change the level of TBARS. However, SQLA treatment increased the level of TBARS significantly compared with that in the control striatum.

3.3 Effect of SQLE/SQLA administration for 7 days on the activities of SOD, catalase and GPx in mouse striatum

The activities of SOD, catalase, and GPx in the mouse striatum after the administration of SQLE or SQLA for 7 days are shown in Table 1. No significant changes in SOD, catalase, or GPx activity were found.

3.4 Effect of SQLE/SQLA administration for 7 days on the levels of fatty acids in the mouse brain

The mean tissue concentrations of DHA, EPA, arachidonic acid, linolenic acid, linoleic acid, and the ratio of linoleic acid/linolenic acid in the mouse brain are shown in Table 2. SQLE or SQLA treatment did not change the levels of these fatty acids; however, there was a significant decrease in the ratio of linoleic acid/linolenic acid.

4 DISCUSSION

Previous studies reported the presence of increased oxidative stress, low levels of endogenous antioxidants, and
low activity of free radical-scavenging enzymes in the brains of patients with PD\(^2\). These findings suggest that free radicals and oxidative stress are important contributors to the pathogenesis of PD\(^3\).

SQLE is easily absorbed when it is orally administrated\(^4\). It is transported in serum, generally in association with very low density lipoproteins, until it is distributed ubiquitously in tissues\(^5\). SQLE has been reported to possess antioxidant properties due to double bonds in the structure, and in vitro experimental evidence indicates that it is a highly effective oxygen-scavenging agent\(^6,7\). Furthermore, SQLE is a unique antioxidant molecule with high scavenging activity toward singlet oxygen\(^8\). The high singlet oxygen quenching ability is due to its chemical structure consisting of a six 2-methyl-2-pentene unit\(^9\).

In the present study, we first examined the effects of
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SQLE/SQLA on 6-OHDA neurotoxicity by measuring changes in DA, DOPAC, and HVA as markers of dopaminergic neuronal injury. Treatment with SQLE (for 7 days before and 7 days after 6-OHDA injection) inhibited 6-OHDA-induced neurotoxicity, lessening the reductions in striatal tissue DA and DOPAC. However, treatment with SQLA enhanced 6-OHDA-induced neurotoxicity, inducing a further reduction in striatal tissue DA and DOPAC.

The lipid peroxidation levels, and the antioxidative condition of the striatum at the time 6-OHDA was injected were important because 6-OHDA is susceptible to autooxidation, resulting in the formation of 6-OHDA quinine and hydrogen peroxide, as well as superoxide and hydroxyl radicals. Administration of 1.0 g/kg/day of SQLE/SQLA was effective to the 6-OHDA induced DA depression in the striatum. Therefore, we measured the effects of SQLE/SQLA (1.0 mg/kg/day administration for 7 days) on lipid peroxidation levels and the activities of active oxygen scavenging enzymes, such as SOD, catalase, and GPx in the striatum. Treatment with SQLA for 7 days increased the striatal TBARS level but SQLE treatment had no effect.

6-OHDA is taken in the DA neuron specifically and formed 6-OHDA quinine and hydrogen peroxide. Therefore, oxidative damage by 6-OHDA is induced in the DA neuron specifically. However, SQLA may be ingested into all cells in the brain. SQLA may change lipid composition and make the cells sensitive to oxidative stress, and increased TBARS levels. This increase in oxidative damage sensitivity induced by SQLA administration, combined with the toxicity of 6-OHDA, may exacerbate the level of DA depression in the striatum. Santhilkumar et al. reported that the oral administration of SQLE did not change the active oxygen scavenging activities of SOD, catalase, and GPx in heart tissue and erythrocyte hemolysate in rats. Likewise, we did not observe significant differences in the activities of these enzymes in the mouse striatum after oral administration of SQLE for 7 days.

We measured the effect of SQLE/SQLA on the composition of fatty acids in the striatum. SQLE or SQLA administration, carried out daily for one week, did not change the levels of these fatty acids in the striatum in a statistical sense. We compared the ratio of linolenic acid and linoleic acid. That of the SQLE and SQLA groups was significantly different.

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**Fig. 3** Effects of oral administration of SQLE and SQLA for 7 days on TBARS levels in the mouse striatum. Data are expressed as means ± SEM of 6-8 mice in each group. p <0.05, compared with each other using one-way ANOVA.

**Table 1** Activities of SOD, catalase, and GPx in mouse striatum after one week administration of squalene or squalane.

<table>
<thead>
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<th>control</th>
<th>SQLE</th>
<th>SQLA</th>
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<tbody>
<tr>
<td>SOD</td>
<td>473.6 ± 38.9</td>
<td>465.5 ± 22.6</td>
<td>464.4 ± 12.7</td>
</tr>
<tr>
<td>catalase</td>
<td>549.9 ± 22.9</td>
<td>549.4 ± 16.1</td>
<td>605.0 ± 29.7</td>
</tr>
<tr>
<td>GPx</td>
<td>2.09 ± 0.20</td>
<td>2.20 ± 0.08</td>
<td>2.18 ± 0.16</td>
</tr>
</tbody>
</table>

Data (U/mg protein) are expressed as the mean ± SEM. n=5-8. Significance was determined at p<0.05 level using one-way ANOVA followed by Sheffe’s F test. No significant difference was observed.

SQLE/SQLA on 6-OHDA neurotoxicity by measuring changes in DA, DOPAC, and HVA as markers of dopaminergic neuronal injury. Treatment with SQLE (for 7 days before and 7 days after 6-OHDA injection) inhibited 6-OHDA-induced neurotoxicity, lessening the reductions in striatal tissue DA and DOPAC. However, treatment with SQLA enhanced 6-OHDA-induced neurotoxicity, inducing a further reduction in striatal tissue DA and DOPAC.

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We measured the effect of SQLE/SQLA on the composition of fatty acids in the striatum. SQLE or SQLA administration, carried out daily for one week, did not change the levels of these fatty acids in the striatum in a statistical sense. We compared the ratio of linolenic acid and linoleic acid. That of the SQLE and SQLA groups was significantly different.

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**Table 2** Concentrations of fatty acids in mouse striatum after one week administration of squalene or squalane.

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>SQLE</th>
<th>SQLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stearic Acid (C18)</td>
<td>703.7 ± 78.2</td>
<td>820.9 ± 127.2</td>
<td>656.7 ± 87.1</td>
</tr>
<tr>
<td>Linolenic Acid (C18:2n-3)</td>
<td>64.3 ± 7.6</td>
<td>78.0 ± 9.7</td>
<td>61.9 ± 4.6</td>
</tr>
<tr>
<td>EPA (C20:5n-3)</td>
<td>12.6 ± 1.5</td>
<td>19.5 ± 2.7</td>
<td>15.8 ± 2.2</td>
</tr>
<tr>
<td>DHA (C22:6n-3)</td>
<td>1517.1 ± 184.8</td>
<td>1933.4 ± 305.8</td>
<td>1517.1 ± 183.0</td>
</tr>
<tr>
<td>Linoleic Acid (C18:3n-6)</td>
<td>55.3 ± 5.5</td>
<td>50.7 ± 6.6</td>
<td>42.7 ± 2.3</td>
</tr>
<tr>
<td>Arachidonic Acid (C20:4n-6)</td>
<td>1256.3 ± 139.2</td>
<td>1542.1 ± 227.9</td>
<td>1106.6 ± 140.2</td>
</tr>
<tr>
<td>Linoleic Acid/Linolenic Acid</td>
<td>0.944 ± 0.069</td>
<td>0.651 ± 0.026 a</td>
<td>0.697 ± 0.027 a</td>
</tr>
</tbody>
</table>

Data (ng/mg wet weight and the ratio) are expressed as the mean ± SEM. n=5-8. Differences were examined using ANOVA followed by Sheffe’s F test (fatty acids) and Mann-Mann-Whitney’s U test (Linoleic Acid/Linolenic Acid). a:p<0.05
lower than that of the control group. Although it was not statistically significant, the mean value of stearic acid, linolenic acid, EPA, and DHA in the SQLE-administered group seemed to increase, and that of linoleic acid in both the SQLE- and SQUALA-administered groups seemed to decrease. Hauss et al. reported that SQLE occurs in the midplane of the lipid bilayer and stabilizes the layers in cellular and subcellular membranes through the formation of complexes with the fatty acids in the phospholipid bilayer membranes. SQLE/SQUALA administration may induce changes in the fatty acids in the phospholipid bilayer membranes. The change in the ratio of linolenic acid and linoleic acid may be induced by increases in the levels of SQLE and SQUALA entering the midplane of the lipid bilayer. SQLE has six double bonds, and both the steric structure and the electron configuration are very different from those of SQUALA. The minor distinctions in fatty acid composition between the SQLE and SQUALA groups may be caused by these differences. SQLE in the midplane of the lipid bilayer may quench free radicals and contribute to the stabilization of cellular and subcellular regions during oxidative stress. The distribution of SQLE in the phospholipid bilayer and the changes in the composition of fatty acids may have rendered stability to the membranes of the striatum against induced oxidative injury.

Oral administration of SQLE/SQUALA may cause stress in the mouse. Stress induces monoamine metabolism disorders in the striatum and increases oxidative stress. SQLE has active oxygen scavenging activities; therefore, lipid peroxidation in the striatum was compensated when SQLE was administered in our study. SQUALA does not have active oxygen scavenging activities and induced lipid peroxidation. Differences in the steric structure and the electron configuration between SQLE and SQUALA might have induced changes in fatty acid composition in the phospholipid bilayer membranes. The membrane stabilizing and active oxygen scavenging properties of SQLE may have diminished the toxicity of 6-OHDA. When SQLE was administered, the oxidative stress induced by monoamine metabolism and increased by emotional stress, enhanced 6-OHDA-induced DA depression.

Our study demonstrated that the administration of SQLE has preventive effects against 6-OHDA-induced DA depression in the mouse striatum; however, the administration of SQUALA enhanced neurotoxicity.

ACKNOWLEDGMENTS

This study was supported by a grant from the Kagawa Prefectural College of Health Science and a Grant-in-Aid (No. 24500989) for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan.

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