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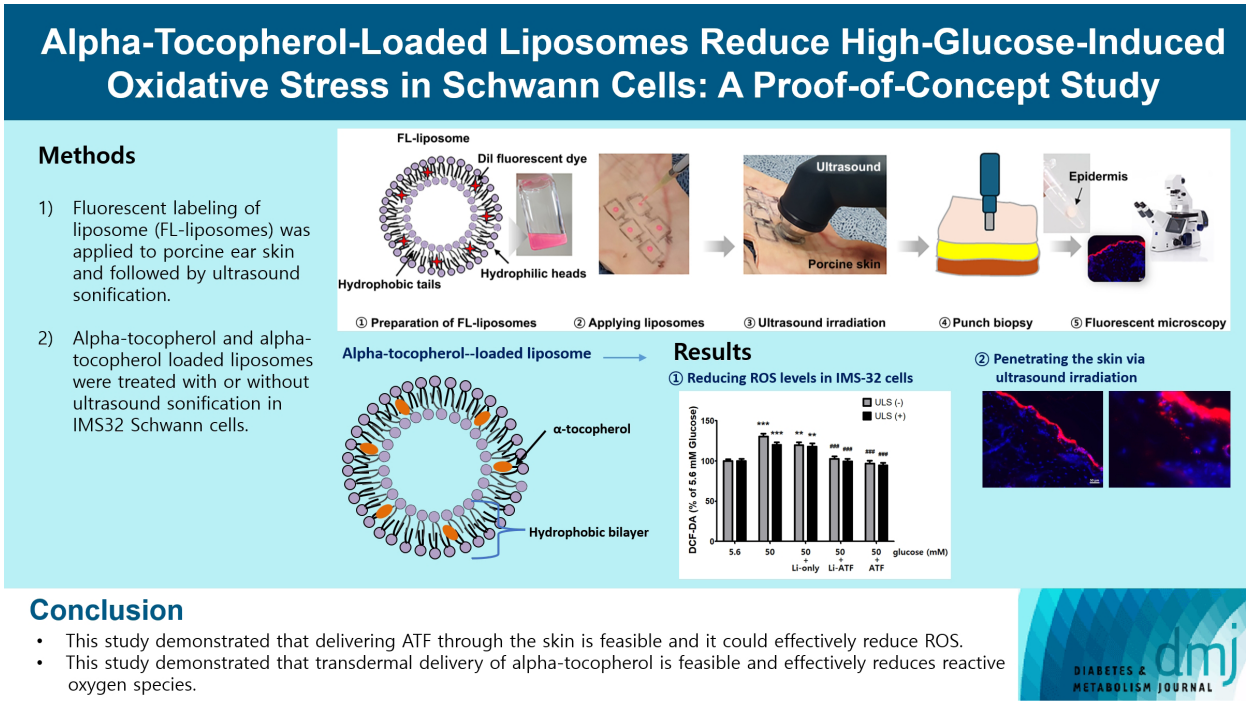

Highlights

- **Alpha-tocopherol (ATF) reduces reactive oxidative stress (ROS) in IMS-32 cells.**
- **ATF-liposomes effectively reduce ROS in IMS-32 cells.**
- **Ultrasound irradiation does not alter the antioxidant effect of ATF.**
- **Ultrasound enhances the transdermal delivery of molecules with liposomes.**

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Alpha-Tocopherol-Loaded Liposomes Reduce High Glucose Induced Oxidative Stress in Schwann Cells: A Proof of Concept Study

Jee-In Heo[1,*], Mi Jeong Kim[2,*], Daehyun Kim[2,3], Jimin Seo[1], Joon Ho Moon[1,4], Sung Hee Choi[1,4], Hak Jong Lee[2,5,6,7], Tae Jung Oh[1,4]

Departments of [1]Internal Medicine, [2]Radiology, Seoul National University Bundang Hospital, Seongnam,
[3]Department of Nano Science and Technology, Graduate School of Convergence Science and Technology, Seoul National University, Seoul,
Departments of [4]Internal Medicine, [5]Radiology, [6]Medical Device Development, Seoul National University College of Medicine, Seoul,
[7]Institute of Radiation Medicine, Seoul National University Medical Research Center, Seoul, Korea

Although oxidative stress is the main pathophysiology of the development of diabetic neuropathy, oral administration of antioxidants has given disappointing results. Here, we hypothesized that local delivery of antioxidants would provide protective effects on Schwann cells due to the high concentration of local lesions. We prepared alpha-tocopherol (ATF)-loaded liposomes and tested their skin penetration after sonication. An *in vitro* study using IMS-32 cells was conducted to determine the level of reactive oxygen species (ROS) scavenging effects of ATF-liposomes. ATF reduced ROS in high-glucose-exposed IMS-32 cells in a dose-dependent manner. ATF-liposomes also reduced the ROS level *in vitro* and ultrasound irradiation enhanced delivery to the dermis in porcine ear skin. This study showed that it is feasible to deliver ATF through the skin and can effectively reduce ROS. This model is worthy of development for clinical use.

Keywords: Alpha-tocopherol; Antioxidants; Diabetic neuropathies; Liposomes; Reactive oxygen species

INTRODUCTION

Diabetic peripheral neuropathy (DPN) is a common microvascular complication with a prevalence that has been reported up to 50% in subjects with diabetes [1,2]. Both neurons and Schwann cells are vulnerable to hyperglycemia, and oxidative stress is the key pathophysiology of hyperglycemia-induced tissue damage [3]. Various antioxidant therapies have been introduced and studied extensively for the treatment of DPN [4]. Amongst these, alpha lipoic-acid treatment displayed beneficial effects by reducing pain associated with DPN [5]. However, symptom relief was observed generally after intravenous infusion [6], but not after oral treatment [7]. The oral administration of another antioxidant, mixed tocotrienols, naturally occurring subtypes of vitamin E, also failed to improve neuropathic symptoms in subjects with painful DPN [8]. These results suggest high-dose exposure to antioxidants in peripheral nervous tissue is important and there is a need for a novel drug delivery system aimed at enhancing the tissue concentration of antioxidants.

Alpha-tocopherol (ATF) is the most active and effective form of vitamin E, and we chose ATF from among various antioxidants because it can be easily formulated in liposome

Corresponding authors: Hak Jong Lee https://orcid.org/0000-0003-0858-7873
Department of Radiology, Seoul National University Bundang Hospital, 82 Gumi-ro 173beon-gil, Bundang-gu, Seongnam 13620, Korea
E-mail: hakjlee@snu.ac.kr

Tae Jung Oh https://orcid.org/0000-0002-5078-6123
Department of Internal Medicine, Seoul National University Bundang Hospital, Seoul National University College of Medicine, 82 Gumi-ro 173beon-gil, Bundang-gu, Seongnam 13620, Korea
E-mail: ohtjmd@gmail.com

*Jee-In Heo and Mi Jeong Kim contributed equally to this study as first authors.

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form. ATF is widely distributed in nature and used clinically as an antioxidant. The antioxidant properties of ATF lie in its ability to scavenge free radicals generated and ultimately disrupt free radical chain reactions [9]. However, because ATF has low solubility and is easily oxidized, its effectiveness in clinical application is low. To improve solubility and increase delivery efficiency to the disease lesion, we attempted to deliver ATF by encapsulating it in liposomes. Liposome-based drug delivery systems have potential for significant roles in a variety of therapeutic applications. Liposomes composed of lipid bilayers have the advantages of being biocompatible, biodegradable, nontoxic, and capable of encapsulating both hydrophilic and hydrophobic drugs. Drugs encapsulated in liposomes are reliably protected from physiological events [10]. In addition, ultrasound treatment has been proposed as a noninvasive method to increase the efficiency of drug delivery. Ultrasound therapy can improve the penetration of drugs into tissues through a temporary cavitation phenomenon and concentrate drug delivery on the specific area irradiated by ultrasound while minimizing off-target side effects.

In this study, we tested whether ATF, a major isoform of vitamin E, reduced reactive oxygen species (ROS) in a Schwann cell (IMS-32) line. Additionally, we developed a procedure to enhance the transdermal delivery of ATF using a liposome-incorporating formula under ultrasound irradiation.

METHODS

Preparation of ATF-liposomes

Pig ears used in the study were obtained from deceased animals, and approval from the Institutional Animal Care and Use Committee was not required.

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), N-(carbonyl-methoxy polyethylene glycol-2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, and sodium salt (DSPE-mPEG 2000), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were purchased from Lipoid AG (Steinhausen, Switzerland). ATF-loaded liposomes were synthesized with modifications referred to previously [11]. We dissolved 2 mg of DSPC, 3.5 mg of DSPE-mPEG 2000, 1.7 mg of cholesterol, 12.8 mg of DOPE, and 2 mg of ATF in 4.4 mL of ethanol at 70°C, at a lipid concentration of 5 mg/mL. The lipid solution was mixed with 250 mM ammonium sulfate solution at 60°C, and the lipid concentration was 2 mg/mL. The mixture was stirred for 1 hour to hydrate the lipid bilayers. Liposomes were reduced in size and homogenized by serial extrusion cycles with polycarbonate filters using a high-pressure extruder (LIPEX, Evonik, BC, Canada). The extrusion process involved passing the mixture through polycarbonate filters in the high-pressure extruder, with the pore size gradually decreasing from 0.8 to 0.4 μm and finally to a pore size of 0.2 μm. This process was repeated until the size of the produced liposomes reached 100 to 200 nm.

Fluorescent labeling of liposomes

Fluorescent labeling (FL)-liposomes were prepared using 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Thermo Fisher Scientific, Franklin, MA, USA) and were used to detect the liposomes by fluorescence analysis. We dissolved 2 mg of DSPC, 3.5 mg of DSPE-mPEG 2000, 1.7 mg of cholesterol, 12.8 mg of DOPE, and 20 μg of DiI fluorescent dye together in chloroform instead of ethanol. Then, the steps of mixing with ammonium sulfate solution, hydrating the lipid bilayers, and homogenizing using a high-pressure extruder were performed in the same manner as described above. Subsequently, purification was conducted using prepacked disposable 10 mL (PD-10) gel filtration columns to remove the unloaded fluorescent dye.

Penetration assay

Ears of pigs were used as a human skin surrogate for penetration testing in this study. Pig ears were purchased from a local butcher. After washing the ears with running water, they were washed with 1×phosphate-buffered saline (PBS) and incubated for 1 hour at 37°C. Areas of 1.0 cm^2 were marked on the porcine ear skin and treated with 25 μL of fluorescent-tagged ATF-liposomes followed by ultrasound (1, 2, or 3 W/cm^2 of intensity, 1 MHz, 100 Hz of pulse repetition frequency for 5 minutes; Sonoplus 190, Enraf-Nonius B.V., Rotterdam, Netherlands). We designed the ultrasonic parameters based on the study of Levy et al. [12] and optimized the ultrasonic parameters by changing the ultrasonic intensity conditions. After incubating for 4 hours at 37°C and washing with 1×PBS, punch biopsies were embedded in optimal cutting temperature and immediately frozen with liquid N_2. After cryosection, fluorescence labeling was observed using a fluorescence microscope (Axio Observer 7, Zeiss, Oberkochen, Germany). Fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Other experimental methods are described in Supplementary Methods.

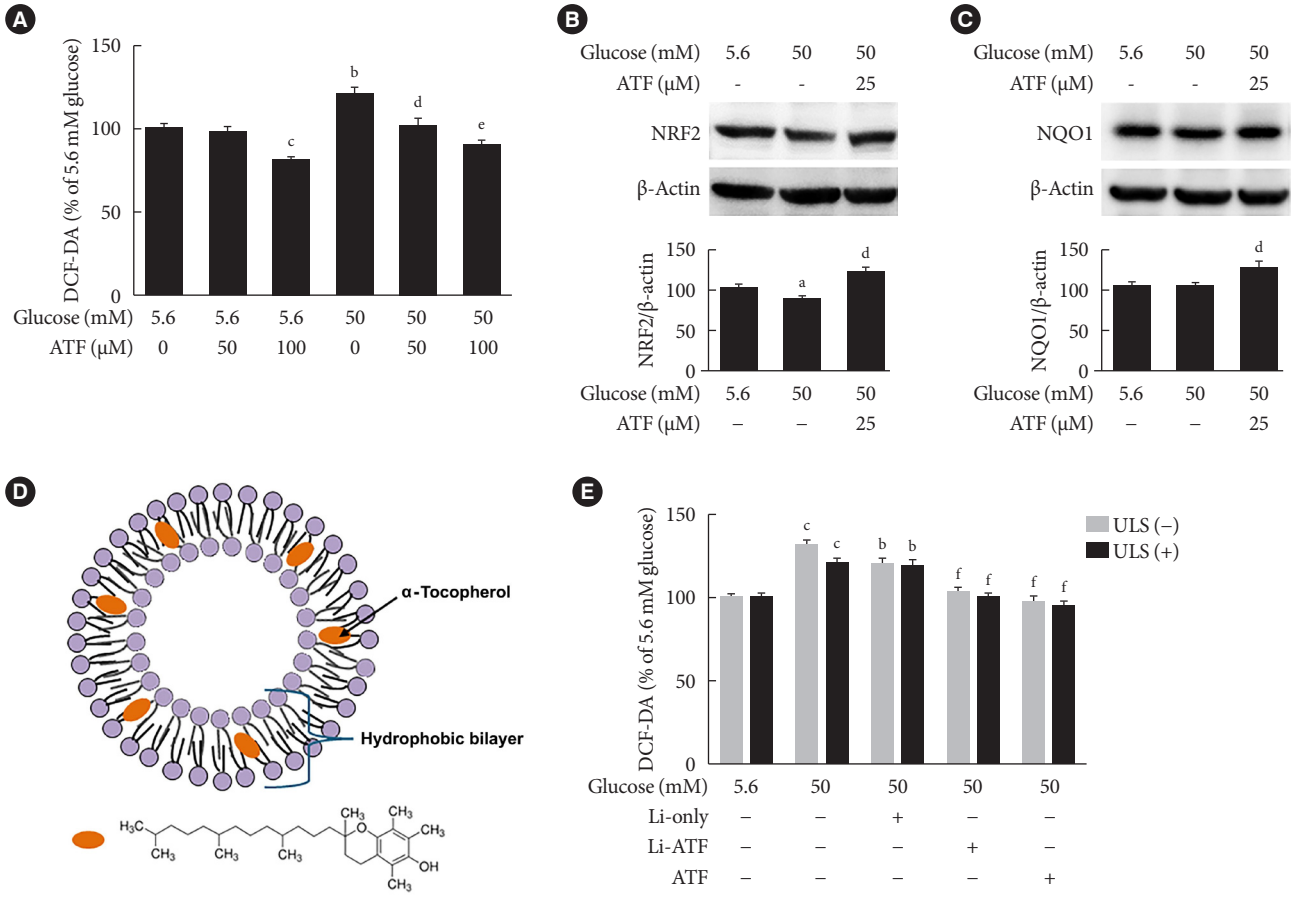

Fig. 1. Effect of alpha-tocopherol (ATF) and ATF-liposomes in high glucose induced reactive oxidative stress (ROS) production. (A) Intracellular ROS levels measured by 2'-7'-dichlorofluorescein diacetate (DCF-DA) in IMS32 cells. (B) Nuclear factor erythroid 2-related factor 2 (NRF2), and (C) NAD(P)H quinone oxidoreductase 1 (NQO1) protein expression in IMS32 cells. (D) Illustration of ATF-loaded liposome (Li). (E) Intracellular ROS levels measured by DCF-DA with or without ultrasound (ULS) irradiation in IMS32 cells. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$ vs. ATF 0 μM and glucose 5.6 mM group; [d]$P<0.05$, [e]$P<0.01$, [f]$P<0.001$ vs. ATF 0 μM and glucose 50 mM group.

RESULTS

Both ATF and ATF-liposomes reduce high glucose induced ROS in IMS32 cells

ROS generation monitored by 2'-7'-dichlorofluorescein diacetate (DCF-DA) was increased significantly after high glucose exposure. This was attenuated in a dose-dependent manner by ATF treatment (Fig. 1A). Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates genes related to antioxidants [13]. NRF2 and nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase 1 (NQO1) protein, a target of NRF2, were decreased by high glucose, but increased in cells treated with ATF (Fig. 1B and C), indicating that ATF treatment inhibits the cellular oxidative stress response. Fig. 1D presents the structure of the ATF-liposome, showing that ATF is incorporated into the hydrophobic bilayer. This configuration imparts hydrophobic properties to its outer surface. When we examined ROS production in response to varying glucose concentrations and the presence of ATF or ATF-liposomes, with or without ultrasound irradiation, we consistently observed a reduction in ROS levels irrespective of the ATF formulation or ultrasound irradiation (Fig. 1E).

Ultrasound irradiation facilitates the infiltration of FL-liposomes into the dermis

We tested the efficacy of the ultrasound-mediated drug delivery system using porcine skin experiments (Fig. 2A). The readout was the quantity of fluorescent material present in the der-

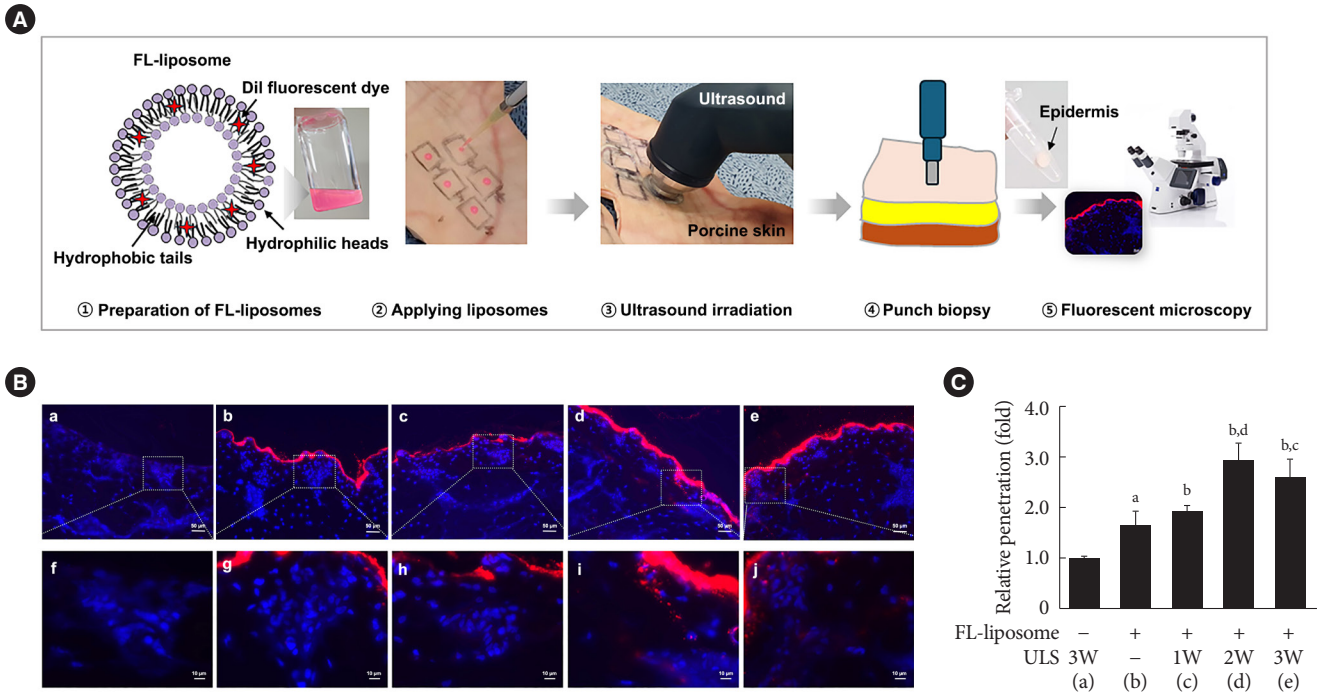

Fig. 2. Ultrasound-induced liposome penetration. (A) Experimental design using porcine skin. (B) Visualization of dermal penetration of liposome. Scale represents: 50 μm (a–e) and 10 μm (f–j). (C) Quantification of dermal penetration of liposome with or without ultrasound irradiation. FL, fluorescent labeling; ULS, ultrasound. [a]$P<0.01$, [b]$P<0.001$ vs. FL-liposome– and ULS 3W group; [c]$P<0.05$, [d]$P<0.01$ vs. FL-liposome+ and ULS– group.

mis (Fig. 2B and C). We successfully facilitated the penetration of FL-liposomes into the dermis and confirmed their superior efficacy at 2 W or higher.

DISCUSSION

In this *in vitro* study, exposure to high glucose concentrations induced elevated levels of ROS in Schwann cells. ATF decreased ROS levels dose-dependently in hyperglycemic conditions. We then tested specially designed ATF-loaded liposomes and the antioxidative effect of ATF remained uncompromised. Furthermore, the application of ultrasound did not alter the effect of ATF. Rather, ultrasound irradiation enhanced liposome transmission to the dermis.

To date, there has been no disease-modifying treatment of DPN other than risk factor management [5]. Aldose reductase converts glucose into sorbitol, leading to elevated levels of oxidative stress accompanied by a decrease in cytosolic NADPH [14]. Against this background, aldose reductase inhibitors have been developed as a therapy for DPN. Despite the effectiveness of this class of drug in animal models, these inhibitors exhibited disappointing results in humans [15,16]. Aldose reductase inhibitors improved nerve conduction velocity (NCV), but DPN symptoms were not improved compared with placebo. Some possible explanations for these unexpected results include the following. First, the phenotype of rodent models is not well matched with human DPN [17]. Second, the improvement of NCV does not ensure recovery of small fiber neuropathy or nonneuronal cells, which can be primarily associated with pain sensation [18]. Thus, it is essential to develop a new treatment for DPN considering these limitations. Therefore, we tested the effect of ATF-liposomes using a Schwann cell *in vitro* model rather than a rodent animal model. Because Schwann cells can contribute to DPN pathology [19], we can test the molecular function efficiently through this *in vitro* study. Additionally, we used porcine skin to demonstrate the effects of ultrasound-irradiation-induced penetration because of its structural similarity to human skin [20]. We reasoned that this step-by-step approach could contribute to the successful development of a novel treatment modality for patients with DPN.

In this study, we showed that both ATF and ATF-liposomes

effectively decreased ROS production, and that ultrasound irradiation enhanced the tissue filtration of FL-liposomes. The next step involves applying this treatment in human DPN subjects, bypassing the need for other rodent models. This is a huge advantage as it eliminates the need for unnecessary animal studies. However, we address our study limitations as follows. First, we could not measure tissue concentrations of ATF in porcine dermis. Therefore, we were unable to confirm the appropriate concentration of ATF for the recovery of nerve damage. We conceptually speculated that the tissue concentration might be higher to some degree than the distributed amount after oral ingestion. Second, we did not perform any functional study to test nerve function, and did not evaluate damaged cells, which may create a gap between the observed decrease in ROS production and its direct effect on cell protection. In the upcoming clinical trial, data on the parameters for small fiber nerves such as density and length should be collected [21]. Furthermore, targeting ROS alone might not be sufficient to overcome the underlying pathogenic mechanism of neuropathy, given the influence of other factors such as chronic inflammation, osmotic pressure, and altered intracellular signaling [22]. Lastly, we used porcine skin instead of human skin because porcine skin is widely used in *ex vivo* studies due to its similarity to human skin in terms of histology and physiology. Future studies applying this method to human skin are necessary.

We demonstrated that ATF alone and when incorporated into liposomes can reduce ROS production in a hyperglycemia-induced neuropathy model. Ultrasound enhanced the efficacy of liposomal drug delivery without compromising original antioxidative property of ATF. This proof of concept study suggests that liposome-incorporated antioxidants can be developed as a disease-modifying treatment for DPN. Based on the model developed in this study, we anticipate that other antioxidants, such as coenzyme Q10, N-acetylcysteine, and alpha-lipoic acid, can also be tested.

SUPPLEMENTARY MATERIALS

Supplementary materials related to this article can be found online at https://doi.org/10.4093/dmj.2024.0489.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

AUTHOR CONTRIBUTIONS

Conception or design: J.I.H., H.J.L., T.J.O.
Acquisition, analysis, or interpretation of data: J.I.H., M.J.K., D.K., J.S.
Drafting the work or revising: J.I.H., H.J.L., T.J.O.
Final approval of the manuscript: all authors.

ORCID

Jee-In Heo *https://orcid.org/0000-0003-4315-5603*
Mi Jeong Kim *https://orcid.org/0000-0003-0078-0409*
Hak Jong Lee *https://orcid.org/0000-0003-0858-7873*
Tae Jung Oh *https://orcid.org/0000-0002-5078-6123*

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REFERENCES

1. Lee CG, Ciarleglio A, Edelstein SL, Crandall JP, Dabelea D, Goldberg RB, et al. Prevalence of distal symmetrical polyneuropathy by diabetes prevention program treatment group, diabetes status, duration of diabetes, and cumulative glycemic exposure. Diabetes Care 2024;47:810-7.
2. Elafros MA, Andersen H, Bennett DL, Savelieff MG, Viswanathan V, Callaghan BC, et al. Towards prevention of diabetic peripheral neuropathy: clinical presentation, pathogenesis, and new treatments. Lancet Neurol 2022;21:922-36.
3. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes 2005;54:1615-25.
4. Lin Q, Li K, Chen Y, Xie J, Wu C, Cui C, et al. Oxidative stress in diabetic peripheral neuropathy: pathway and mechanism-based treatment. Mol Neurobiol 2023;60:4574-94.

5. Jang HN, Oh TJ. Pharmacological and nonpharmacological treatments for painful diabetic peripheral neuropathy. Diabetes Metab J 2023;47:743-56.
6. Ziegler D, Nowak H, Kempler P, Vargha P, Low PA. Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: a meta-analysis. Diabet Med 2004;21:114-21.
7. Ziegler D, Hanefeld M, Ruhnau KJ, Hasche H, Lobisch M, Schutte K, et al. Treatment of symptomatic diabetic polyneuropathy with the antioxidant alpha-lipoic acid: a 7-month multicenter randomized controlled trial (ALADIN III Study). ALADIN III Study Group. Alpha-Lipoic Acid in Diabetic Neuropathy. Diabetes Care 1999;22:1296-301.
8. Vitamin E in Neuroprotection Study (VENUS) Investigators; Hor CP, Fung WY, Ang HA, Lim SC, Kam LY, et al. Efficacy of oral mixed tocotrienols in diabetic peripheral neuropathy: a randomized clinical trial. JAMA Neurol 2018;75:444-52.
9. Jain AK, Mehra NK, Swarnakar NK. Role of antioxidants for the treatment of cardiovascular diseases: challenges and opportunities. Curr Pharm Des 2015;21:4441-55.
10. Guimaraes D, Cavaco-Paulo A, Nogueira E. Design of liposomes as drug delivery system for therapeutic applications. Int J Pharm 2021;601:120571.
11. Kim YS, Ko MJ, Moon H, Sim W, Cho AS, Gil G, et al. Ultrasound-responsive liposomes for targeted drug delivery combined with focused ultrasound. Pharmaceutics 2022;14:1314.
12. Levy D, Kost J, Meshulam Y, Langer R. Effect of ultrasound on transdermal drug delivery to rats and guinea pigs. J Clin Invest 1989;83:2074-8.
13. Tonelli C, Chio II, Tuveson DA. Transcriptional regulation by Nrf2. Antioxid Redox Signal 2018;29:1727-45.
14. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414:813-20.
15. Dutta D, Mohindra R, Kumar M, Kumar A, Sharma M. Ranirestat improves electrophysiologic but not clinical measures of diabetic polyneuropathy: a meta-analysis. Indian J Endocrinol Metab 2022;26:399-406.
16. Sekiguchi K, Kohara N, Baba M, Komori T, Naito Y, Imai T, et al. Aldose reductase inhibitor ranirestat significantly improves nerve conduction velocity in diabetic polyneuropathy: a randomized double-blind placebo-controlled study in Japan. J Diabetes Investig 2019;10:466-74.
17. Hossain MJ, Kendig MD, Letton ME, Morris MJ, Arnold R. Peripheral neuropathy phenotyping in rat models of type 2 diabetes mellitus: evaluating uptake of the neurodiab guidelines and identifying future directions. Diabetes Metab J 2022;46:198-221.
18. Ji RR, Chamessian A, Zhang YQ. Pain regulation by non-neuronal cells and inflammation. Science 2016;354:572-7.
19. Feldman EL, Nave KA, Jensen TS, Bennett DL. New horizons in diabetic neuropathy: mechanisms, bioenergetics, and pain. Neuron 2017;93:1296-313.
20. Uhm C, Jeong H, Lee SH, Hwang JS, Lim KM, Nam KT. Comparison of structural characteristics and molecular markers of rabbit skin, pig skin, and reconstructed human epidermis for an ex vivo human skin model. Toxicol Res 2023;39:477-84.
21. Tesfaye S, Boulton AJ, Dyck PJ, Freeman R, Horowitz M, Kempler P, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care 2010;33:2285-93.
22. Feldman EL, Callaghan BC, Pop-Busui R, Zochodne DW, Wright DE, Bennett DL, et al. Diabetic neuropathy. Nat Rev Dis Primers 2019;5:42.

SUPPLEMENTARY METHODS

Cell culture

IMS32 mouse Schwann cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Thermo Fisher Scientific, Franklin, MA, USA) containing 10% fetal calf serum (FCS; Gibco), 1% penicillin/streptomycin and 5.6 mM glucose at 37°C in a humidified atmosphere containing 5% CO_2. The day before each experiment, IMS32 cells were seeded into 96-well plates at a density of 2×10^4 cells/well and 6-well plates at a density of 3.5×10^5 cells/well. Cells were incubated with 1% FCS-containing medium for 24 hours, followed by incubation with 5.6 mM glucose (normal) or 50 mM glucose (high) with or without alpha-tocopherol (ATF; Sigma-Aldrich, Merck, MA, USA). To apply ultrasound to the cells, the cells were treated with ATF-liposomes followed by ultrasound (1.5 W/cm^2 of intensity, 1 MHz, 100 Hz of pulse repetition frequency for 1 minute; Sonoplus 190, Enraf-Nonius B.V., Rotterdam, Netherlands).

Measurement of cellular reactive oxidative stress level

Reagent 2',7'-dichlorofluorescein diacetate (DCFDA) is a method that can measure intracellular reactive oxygen species (ROS). It measures ROS by measuring the fluorescent signal that is transformed when ROS is generated within the cell. Cells were washed with 1×phosphate-buffered saline (PBS) and incubated with DCF-DA (Sigma-Aldrich) at 37°C for 30 minutes. Cells were washed again with 1×PBS. Fluorescence intensity was measured using a fluorescence spectrometer (SpectraMax iD3, Molecular Devices, San Jose, CA, USA) (λ_{ex}, 485 nm; λ_{em}, 530 nm).

Western blot

Cells were lysed in lysis buffer (10 mmol/L Tris-hydrochloride [HCl], pH 7.4, 100 mmol/L sodium chloride [NaCl], 5 mmol/L ethylenediaminetetraacetic acid [EDTA], 10% glycerol, and 1% NP-40 containing a mixture of protease inhibitors). Forty micrograms of protein were separated on a sodium dodecyl sulfate polyacrylamide gel and then transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies and then incubated with the secondary antibodies conjugated with anti-mouse or anti-rabbit immunoglobulin G-horseradish peroxidases. Protein detection was performed using the enhanced chemiluminescence (ECL) system (Thermo Fisher Scientific). The nuclear factor erythroid 2-related factor 2 (NRF2) and NAD(P)H quinone oxidoreductase 1 (NQO1) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, TX, USA). The anti-actin antibody was purchased from Sigma-Aldrich. Horseradish peroxidase-coupled anti-rabbit and anti-mouse antibodies were obtained from Santa Cruz Biotechnology.

Statistical analysis

Parameters were analyzed using GraphPad Prism version. 5.02 (GraphPad Software, San Diego, CA, USA). Comparison of the two groups was evaluated by Mann–Whitney *U* test. *P* values <0.05 were considered significant.