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Transdermal Delivery of Anionic Nanoparticles of Resveratrol, Cannabidiol, Paclitaxel and Paclitaxel Glucoside to Epidermis and their Application for Treatment of Skin Cancer

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A B S T R A C T

Technol PG-resveratrol or paclitaxel glucoside nanoparticles were successfully prepared by dispersing Technol PG powder with cholic acid-based surfactants such as SC. Composite nanoparticles composed of an anionic phospholipid of 1,2-dipalmitoyl-sn-glycero-3-phosphorylglycerol (DPPG) and resveratrol or paclitaxel were successfully prepared by mixing them in water followed by a subsequent heating and cooling process. Upon addition of small-sized fluorescently labeled resveratrol nanoparticles with DPPG to rat skin tissue, fluorescently labeled resveratrol molecules permeated to the stratum corneum (*in vitro*). DPPG-paclitaxel nanoparticles exerted high anti-skin cancer activity (*in vitro*). Technol PG-paclitaxel glucoside nanoparticles also showed high anti-skin cancer activity (*in vitro*). During the anti-cancer test using mouse model of skin cancer, this study revealed that the numbers of papillomas of the mouse treated with paclitaxel glucoside nanoparticles stabilized with Technol PG were decreased, although those of the mouse treated with paclitaxel glucoside itself were increased. Thus, our study established that since paclitaxel glucoside nanoparticles stabilized with Technol PG could permeate stratum corneum and be incorporated into the epidermis layer of mouse, they could also treat skin cancer (*in vivo*). This study showed that since DPPG-resveratrol nanoparticles could permeate stratum corneum and be incorporated into the epidermis layer of mouse, they could also treat atopic dermatitis.

Introduction

Atopic dermatitis is one of the most common cutaneous inflammatory and pruritic diseases in dogs; it affects up to 27% of the canine population. Atopic dermatitis is associated with well-defined clinical signs and the overexpression of immunoglobulin IgE directed against environmental allergens, even if cases not due to IgE responses are known.

Several factors in both humans and dogs appear to contribute to skin inflammation and itching, such as increased exposure to pollutants, changes in dietary habits, stress, genetic factors, and cutaneous infections which predispose to the development of the disease (Chiocchetti *et al.,* 2022).

A recent study has demonstrated that cannabidiol, a non-psychotropic phyto-cannabinoid showing numerous health related benefits, including anti-inflammatory and anti-anxiety properties, may be useful in dogs with atopic dermatitis. Despite these promising clinical studies, there are still few studies dedicated to the histological localization of cannabinoid receptors in the canine inflammatory cells.

It is evident that knowing the cellular distribution of specific receptors is fundamental to understanding the action of a drug (Chiocchetti *et al.,* 2022). Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin found in grapes, peanuts and red wine. Resveratrol is a natural compound known for its anti-inflammatory properties and has several biological activities described, such as antioxidant, anti-inflammatory, anti-apoptotic, anti-atopic dermatitis, and antitumor capacity (Carlucci *et al.,* 2023).

Skin is frequently exposed to oxidative stress from ultraviolet radiation, which presents a risk for the development of cancers such as melanoma, squamous cell carcinoma, and basal cell carcinoma. Efficient transdermal delivery of cannabidiol and resveratrol would be useful to cure atopic dermatitis.

Skin tissue is composed of stratum corneum, epidermis, and dermis. However, the 10- to 40-μm-thick stratum corneum, consisting of densely packed cells, provides a barrier to protect the underlying tissue from infection, dehydration, chemicals, and mechanical stress. It is difficult to applicate cannabidiol and resveratrol for treatment of atopic dermatitis, because they cannot penetrate the stratum corneum. Paclitaxel is a tricyclic diterpenoid compound naturally produced in the bark and needles of *Taxus brevifolia*. It is one of the most successful and widely used natural anticancer drugs.

It is used in coronary heart disease, renal and hepatic fibrosis, inflammation, and axon regeneration (Gelmon, 1994; Luo *et al.,* 2017; Narayanan *et al.,* 2010; Perez, 2009; Zhu *et al.,* 2019). It is difficult to applicate paclitaxel for treatment of skin cancer, because it cannot penetrate the stratum corneum.

Phospholipids are promising molecules because they are synthesized in the body, and therefore, highly biocompatible. However, frequently utilized neutral phospholipids tend to form large-sized vesicles, which sometimes result in insufficient skin penetration.

Attention is drawn on nanotechnology and the usefulness of nanoparticles containing cannabidiol, resveratrol, or paclitaxel. Thus, preparation of phospholipid-based small-sized nanoparticles containing cannabidiol, resveratrol, or paclitaxel is still a challenging problem (Uchida *et al.,* 2020; Uchida *et al.,* 2022a; Uchida *et al.,* 2022b).

Here, the report nanoparticles of cannabidiol, resveratrol, or paclitaxel stabilized with anionic phospholipids of Technol PG and 1,2-dipalmitoyl-sn-glycero-3-phospho-1′-rac-glycerol (DPPG). Also, their applications for treatment of atopic dermatitis and skin cancer are reported.

Materials and Methods

General

Ultrasonication was performed by using a QSonica model ultrasonic homogenizer. Sonication was performed using a Branson model sonicator. Particle sizes were measured using either a Malvern model Zetasizer Nano ZSP zeta potential analyzer (DLS) or a Horiba model LA-960 laser diffraction particle size analyzer (SALD). 1,2-Dipalmitoyl-sn-glycero-3-phospho-(1-rac-glycerol), sodium salt (DPPG) was purchased from Avanti Polar Lipids.

Resveratrol was coupled with *N*-Boc-bromoethylamine by Williamson ether synthesis in DMF solvent containing potassium carbonate. After an incubation of the mixture at 50°C for 2 days, the solvent and potassium

carbonate were removed by a separation operation. The reaction proceeded properly as confirmed by TLC. The resulting compounds were purified by silica gel column chromatography although the mixture contained 2 regioisomers, where *N*-Boc-bromoethylamine reacted with different hydroxyl groups of resveratrol.

As the second step, the Boc protecting group was deprotected by trifluoroacetic acid (TFA) in EtOAc. The reaction proceeded quickly and the present study could obtain Resveratrol-NH_2•HCl after a separation. As a final step, the Resveratrol-NH_2•HCl was allowed to react with NBD-F in an aqueous solution to give NBD-labeled resveratrol (Fig. 1) (Yanagi *et al.,* 2019).

Preparation of DPPG-resveratrol, DPPG-fluorescent resveratrol, DPPG-paclitaxel, Technol PG-resveratrol, Technol PG-cannabidiol, and Technol PG-paclitaxel glucoside

Resveratrol or paclitaxel (or paclitaxel glucoside) was mixed with DPPG powder in water and sonicated for 2 minutes to disperse homogeneously, and then heated at 60°C for 15 minutes, when the solution turned clear. The resulting mixture was kept at room temperature for 1 hour before use. DPPG-fluorescently labeled resveratrol was prepared in the same way. To tune the size of DPPG-resveratrol and DPPG-fluorescently labeled resveratrol nanoparticles, the samples were ultrasonicated at 50 W for 3 hours at 4°C.

For the preparation of Technol PG nanoparticle, Technol PG (5 wt%) was mixed with cholic acid-based surfactants of SC (0.5-5 wt%), CA (5 wt%), or CHAPSO (5 wt%) in the presence of resveratrol, cannabidiol, or paclitaxel glucoside in water and sonicated for 2 min (Uchida *et al.,* 2022a; Uchida *et al.,* 2022b).

Invitro transdermal delivery

In vitro skin permeation tests were performed using a vertical Franz diffusion cell with an effective diffusion area of 0.95 cm^2 (Uchida *et al.,* 2020). Skin tissues were obtained from the abdominal hair of rats. The subcutaneous fat and other extraneous tissues of rat skin were trimmed and removed.

A piece of excised skin (area 3.14 cm^2, diameter 20 mm) was mounted on the Franz diffusion cell with the stratum corneum facing the donor compartment, in which DPPG-fluorescent resveratrol nanoparticles (DPPG-NBD-labelled resveratrol nanoparticles) located. One circular SS Nikasol or SS HGA patch (area 0.785 cm^2, diameter 10 mm) was applied to the stratum corneum side of the skin. The receptor compartment was filled with 3 mL of water and maintained at 32°C using a circulating water bath stirred with magnetic bars. For microscopic observations, skin tissue was embedded into OCT compound, frozen, and cryosectioned.

Invitro anti-cancer activity

The sensitivity of KB cells to paclitaxel (or paclitaxel glucoside) incorporated in DPPG (or Technol PG) was determined as follows. Cells were diluted with culture medium to the seeding density, suspended in 96-well tissue culture plates, preincubated at 37°C for 4 h, and then treated for 24 h with paclitaxel (or paclitaxel glucoside) incorporated in DPPG at various concentrations. After incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole) solution was added to each well and the plates were further incubated for 4 h. Absorbance at 570 nm was measured with a microplate reader model 450 (BIO-RAD).

Invivo transdermal delivery of paclitaxel glucoside to skin cancer

All animals were housed individually in cages under specific pathogen-free conditions during the experiments. Age- and sex-matched mice were used for the experiments (Ishida *et al.,* 2020). 8-Week-old male C57BL mice were used. Skin tumors were induced by two-step application of DMBA and 12-*O*-tetradecanoylphorbol-13-acetate. First, 25 µg of DMBA in 100 µL of acetone was applied onto the shaved dorsal skin of the mice on day 7 (1 week).

On day 0, topical application of 30 µg of 12-*O*-tetradecanoylphorbol-13-acetate in 100 µL of acetone was initiated and was continued for 20 weeks with a frequency of twice a week. Tumor development was monitored on a weekly basis and lesions greater than 2 mm in length were counted as positive. Technol PG-paclitaxel glucoside (paclitaxel glucoside incorporated in anionic nanoparticles (0.2 g/kg)) was applied to the rostral part of the back of mice five times a week. In the control experiment, paclitaxel glucoside was administrated.

Results and Discussion

Preparation of anionic nanoparticles

DPPG-resveratrol nanoparticles can be easily prepared by ultrasonic fragmentation. When nanoparticles of fluorescent NBD-labeled resveratrol with DPPG are added to rat skin tissue, a part of the fluorescent NBD-labeled resveratrol molecules permeate to the stratum corneum. Materials with unique functions making full use of electrostatic repulsion between colloidal particles have recently emerged. DPPG is an anionic phospholipid synthesized in the body and molecularly similar to DPPC. DPPG is known to control lung pressure and functions of mitochondria by using a repulsive force because of the anionic charge. In addition, DPPG molecules are supposed to form kinetically stable nanoparticles maintaining the assemblies in physiological conditions because the bilayer melting temperature) of DPPG is higher than body temperature.

DPPG-resveratrol nanoparticles could be prepared by incubating a water dispersed powder sample of DPPG and resveratrol. In this study dispersed DPPG powder and resveratrol, and heated for 15 minutes at 60°C, and then cooled to room temperature. The present study observed that the resveratrol molecules, forming precipitates without DPPG, were well dispersed to show a transparent solution after stabilization with DPPG. In this study treated the sample by ultrasonication for 3 hours to prepare small-sized nanoparticles. As a result, the DPPG-resveratrol nanoparticles were fractionated to 60 nm sized nanoparticles, as confirmed by dynamic light scattering (DLS) analysis. DPPG-paclitaxel nanoparticles were prepared in the same method as DPPG-resveratrol nanoparticles.

Technol PG consists of a mixture of phosphatidylglycerol having fatty acids which contain C16 and C18 with 0 (C18-0), 1 (C18-1), 2 (C18-2), and 3 (C18-3) of double bonds. The percentage of each component (C16, C18-0, C18-1, C18-2, C18-3) is 14.4%, 4.7%, 13.5%, 61.0%, and 6.0%, respectively. Since Technol PG is less expensive than DPPG, nano-formulation with Technol PG could be more practical. However, the utility of Technol PG has been not well explored. In this study, prepared Technol PG-based nanoparticles using easily accessible cholic acid-derived surfactants. Dispersion of Technol PG with sodium cholate (SC) enabled the encapsulation of resveratrol or paclitaxel glucoside. As a typical method, Technol PG powder (5 wt%) was dispersed in water, mixed with SC (0.5-5 wt%), and sonicated for 2 min. When 0.5 wt% of SC was mixed with Technol PG, a cloudy dispersion was observed, similar to Technol PG before the addition of SC. However, when SC was added at 2 wt% and 5 wt%, the Technol PG dispersion became transparent. In this study performed a dynamic light scattering (DLS) analysis to investigate how the SC surfactant affected the size of Technol PG particles. The hydrodynamic diameter of Technol PG, around 0.1 to 10 μm, decreased slightly after the addition of 0.5 wt% of SC. The addition of 2 wt% and 5 wt% of SC resulted in Technol PG particles with 1 to 5 nm size as observed by DLS. To clarify the effects of molecular structure of the surfactants. Technol PG with noncharged cholic acid (CA) and 3-[(3-cholamidopropyl) (CHAPSO) having a zwitterionic group. As a result, a cloudy dispersion was observed when CA was added, while a clear dispersion was observed when CHAPSO was mixed, suggesting that the ionic groups in the surfactants were important for the dispersion of Technol PG. In good agreement with the DLS result, microscopic observation of Technol PG (5 wt%) with 2 wt% of SC revealed that Technol PG was well dispersed in the solution, while it showed large aggregates before the addition of SC. When resveratrol was added to the SC-dispersed Technol PG sample and sonicated for 1 min, a clear aqueous solution was observed, suggesting an encapsulation of resveratrol into the Technol PG nanoparticles (Uchida *et al.,* 2022a; 2022b). DLS analysis of the Technol PG-resveratrol nanoparticles showed a hydrodynamic diameter of 1 to 5 nm, and no peak derived from an aggregate of resveratrol was observed (Fig. 2). Technol PG-cannabidiol or paclitaxel glucoside nanoparticles were prepared in the same method as Technol PG-resveratrol nanoparticles. DLS analysis of the Technol PG-cannabidiol nanoparticles showed a hydrodynamic diameter of 3 nm (Uchida *et al.,* 2022a; Uchida *et al.,* 2022b).

Invitro transdermal delivery and in vitro anti-cancer activity

The present study investigated skin permeability of DPPG-resveratrol nanoparticles (Uchida *et al.,* 2020). For this, first checked that smaller-sized DPPG-resveratrol nanoparticles could be prepared even in phosphate buffered saline (PBS) buffer. Then, prepared DPPG-fluorescent labeled resveratrol nanoparticles using the same method as that used for DPPG-resveratrol and incubated them with rat skin tissue placed on Franz diffusion cells.

Figure.1 Chemical structures of resveratrol, NBD-labelled resveratrol, cannabidiol, paclitaxel, and paclitaxel glucoside.

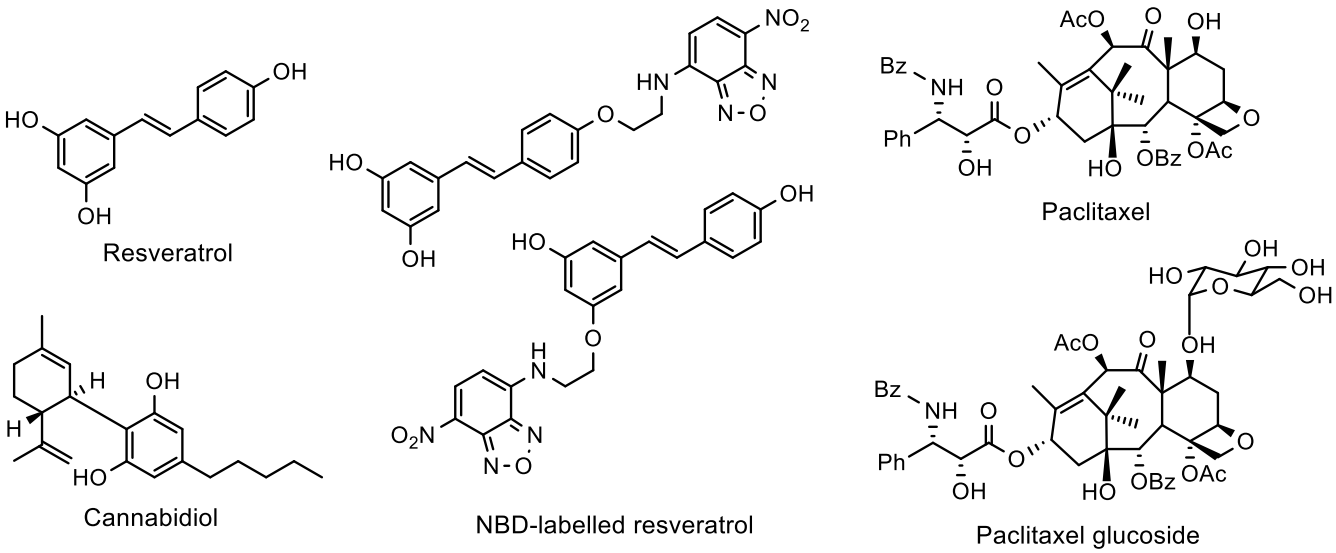


Figure.2 Particle size analysis of Technol PG-resveratrol nanoparticles.

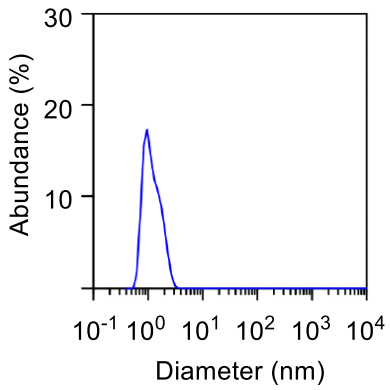


Figure.3 Fluorescent nanoscopic observation of skin sample (A) without and (B) with DPPG-fluorescent resveratrol nanoparticles.

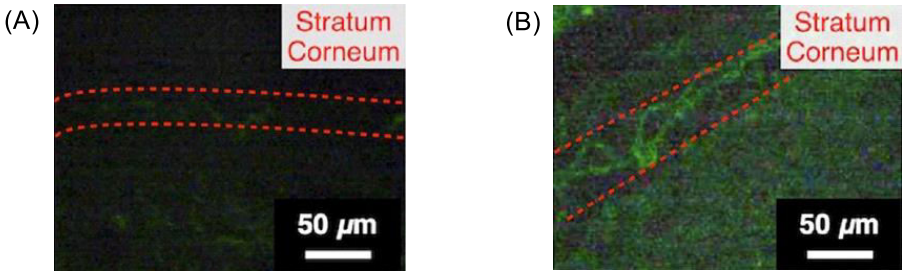
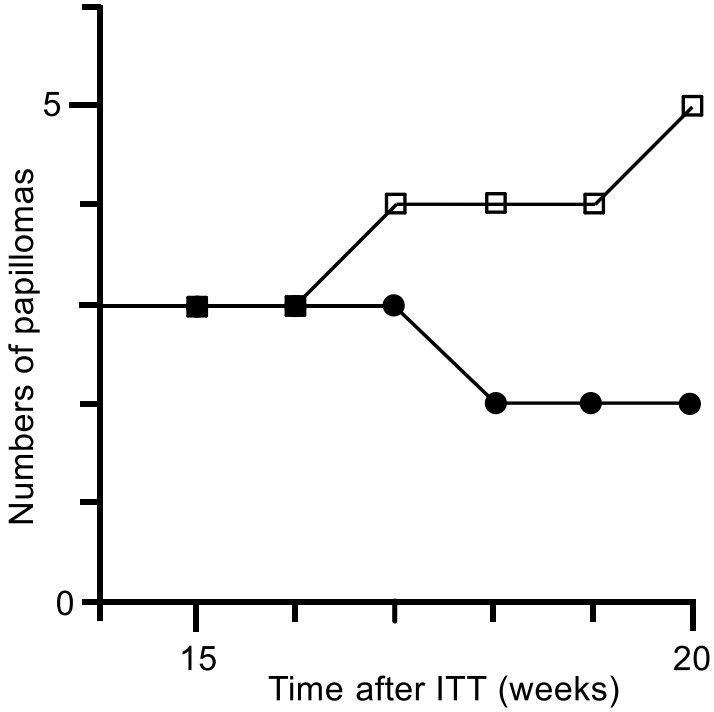


Figure.4 Numbers of papillomas of Technol PG-paclitaxel glucoside nanoparticles-treated mouse (●) and paclitaxel glucoside-treated mouse (control mouse, □). ITT: initial 12-*O*-tetradecanoylphorbol-13-acetate treatment



In the present study prepared a histological section of the skin sample after 24 hours incubation and performed a fluorescent microscopic observation. Strong fluorescence derived from penetration of fluorescent labeled resveratrol molecules into the stratum corneum was observed (Fig. 3B) as compared with the sample without DPPG-fluorescent labeled resveratrol (Fig. 3A). It was noteworthy that could observe fluorescence not only in the stratum corneum but also in the epidermis layer suggesting that fluorescent labeled resveratrol molecules penetrate the stratum corneum. Although the molecular structure of fluorescent labeled resveratrol is not exactly the same as that of resveratrol, the present study envisaged that DPPG-resveratrol nanoparticles would have rather high skin permeation capability because the molecular structure of resveratrol is much smaller than that of fluorescent labeled resveratrol (Uchida *et al.,* 2022a; Uchida *et al.,* 2022b).

The cytotoxic activity of paclitaxel (or paclitaxel glucoside) incorporated in DPPG (or Technol PG) nanoparticles toward human KB cells was examined. Human KB cells were diluted, suspended in 96-well tissue culture plates, preincubated, and then treated with paclitaxel incorporated in DPPG nanoparticles.

After incubation, MTT solution was added to each well and the plates were further incubated. Absorbance at 570 nm was measured. The cytotoxic activity of paclitaxel incorporated in DPPG nanoparticles (IC_{50}=19 μM) was higher than paclitaxel itself (IC_{50}=25 μM). The cytotoxic activity of paclitaxel glucoside incorporated in Technol PG nanoparticles (IC_{50}=15 μM) was higher than paclitaxel glucoside itself (IC_{50}=22 μM).

Invivo transdermal delivery of paclitaxel glucoside to skin cancer

At 14 weeks after initial 12-*O*-tetradecanoylphorbol-13-acetate treatment, mice developed three papillomas and were used for the in vivo transdermal delivery experiment. The numbers of papillomas in Technol PG-paclitaxel glucoside nanoparticles-treated mouse were decreased, although those in paclitaxel glucoside-treated mouse (control mouse) were increased (Fig. 4). These explain that Technol PG-paclitaxel glucoside

nanoparticles may contribute as chemo-preventive and anti-skin cancer agents.

In the present work reported resveratrol nanoparticles stabilized by anionic phospholipids of DPPG. The DPPG-resveratrol nanoparticles can be easily prepared by heating followed by cooling of aqueous mixtures of DPPG and resveratrol, and the nanoparticles can be fractionated by an ultrasonication treatment to prepare small-sized nanoparticles. Upon addition to rat skin tissue, the small-sized DPPG-resveratrol nanoparticles penetrated into the stratum corneum.

Although resveratrol is a well-known anti-atopic dermatitis agent, applications of resveratrol to skin diseases have been challenging compared with diseases in other tissues because of difficulty in transdermal delivery of resveratrol. The DPPG-resveratrol nanoparticles having skin permeability demonstrated in this study would be a new candidate as atopic dermatitis therapeutic agent. In this study successfully prepared Technol PG nanoparticles by dispersing Technol PG powder with cholic acid-based surfactants such as SC. Technol PG-based nanoparticles in this study could be applicable to anti-atopic dermatitis materials by evaluating its skin permeability in the future. The Technol PG-paclitaxel glucoside nanoparticles having skin permeability demonstrated in this study would be a new candidate as effective anti-skin cancer materials, which can decrease numbers of papillomas.

Further studies on the anti-atopic dermatitis property of anionic DPPG-resveratrol nanoparticles are now in progress in our laboratory.

Author Contribution

Hiroki Hamada: Investigation, formal analysis, writing—original draft. Daisuke Uesugi: Validation, methodology, writing—reviewing. Kohji Ishihara:—Formal analysis, writing—review and editing. Ryusuke Hosoda: Investigation, writing—reviewing. Kei Shimoda: Resources, investigation writing—reviewing. Atsuhito Kuboki: Validation, formal analysis, writing—reviewing. Yuya Kiriake: Conceptualization, methodology, data curation, supervision, writing—reviewing the final version of the manuscript.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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