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Resveratrol-loaded liposomes for topical treatment of the vaginal inflammation and infections

May Wenche Jøraholmen [a], Nataša Škalko-Basnet [a], Ganesh Acharya [b,c], Purusotam Basnet [b,c,*]

[a] Drug Transport and Delivery Research Group, Department of Pharmacy, Faculty of Health Sciences, Tromsø, Norway
[b] Department of Obstetrics and Gynecology, University Hospital of North Norway, Tromsø, Norway
[c] Women's Health and Perinatology Research Group, Department of Clinical Medicine, Tromsø, Norway

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Chemical compound studied in the article:
Resveratrol (3,5,4′-trihydroxy-*trans*-stilbene)
Vitamin C (ascorbic acid)
Vitamin E (α-tocopherol)
2,2′-Azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt (ABTS)
1,1-Diphenyl-2-picrylhydrazyl (DPPH)
Phosphatidylcholine
Chitosan
L-nitro-arginine methyl ester (NAME)

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ABSTRACT

Resveratrol (RES), chemically known as 3,5,4′-trihydroxy-*trans*-stilbene, is a promising multi-targeted anti-oxidative and anti-inflammatory natural polyphenol. Preclinical studies showed its biological activities against the pathogens of sexually transmitted diseases causing vaginal inflammation and infections. Due to its low solubility and poor bioavailability, the optimal therapeutic uses are limited. Therefore, a clinically acceptable topical vaginal formulation of RES exhibiting optimal therapeutic effects is highly desirable. For this purpose, we prepared and optimized chitosan-coated liposomes with RES. The coated vesicles (mean diameter 200 nm) entrapped up to 77% of RES, a sufficient load to assure required therapeutic outcome. *In vitro* drug release study showed the ability of liposomes to provide sustained release of RES. *In vitro* anti-oxidative activities of RES, namely DPPH and $ABTS^{\bullet+}$ radicals scavenging assays, confirmed RES to be as potent as standard anti-oxidants, vitamins C and E. The anti-oxidative activities of RES and its corresponding liposomal formulation were also compared by measuring enhanced superoxide dismutase (SOD) activities in lipopolysaccharide (LPS)-induced J774A.1 cells. *In vitro* anti-inflammatory activities were compared by measuring nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-1β production in LPS-induced J774A.1 cells. Liposomal RES was found to exhibit stronger anti-oxidative and anti-inflammatory activities than RES solution.

1. Introduction

Resveratrol (RES), a common natural compound produced by several plants in response to pathogenic infection (Houille et al., 2014), is identified chemically as 3,5,4′-trihydroxy-*trans*-stilbene. RES and its derivatives were reported to exhibit fungicidal and anti-microbial activities (Chan, 2002; Baur and Sinclair, 2006; Adrian and Jeandet, 2012; Houille et al., 2014). It also showed anti-viral effect (Docherty et al., 2005). In addition to this, diverse pharmacological activities such as anti-oxidative, anti-inflammatory, neuro-protective, anti-aging, anti-cancer and cardio-protective effects of RES have been reported which can carry potential therapeutic application to humans (Bhat et al., 2001; Smoliga et al., 2011; Vang et al., 2011; Lu et al., 2013). Due to the multi-targeted microbicide activities and significantly low toxic effects, RES might be the potential candidate in safe topical treatment of vaginal inflammation and infection especially in pregnant women. The vaginal environment and structure are highly vulnerable towards the pathogens such as various bacteria, fungi, viruses or protozoa (*Trichomonas*) which cause vaginal inflammation and infection and are often transmitted easily during sexual intercourse. Contamination and rapid growth of these pathogens lead not only to inflammation

Abbreviations: ABTS, 2,2′-azino-bis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt; CAT, catalase; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's modified eagle medium; GPX, glutathione peroxidase; HIV-1, human immunodeficiency virus-1; HPV, human papilloma virus; HSV-2, human simplex virus type 2; IL-1β, interleukin-1β; *i*NOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; L-NAME, L-nitro-arginine methyl ester; MDZ, metronidazole; NO, nitric oxide; PBS, phosphate buffer solution; PM, pig mucin; PC, phosphatidylcholine; PI, polydispersity index; ROS, reactive oxygen species; RES, resveratrol; STD, sexually transmitted diseases; SOD, superoxide dismutase; TH1, T-helper cell type 1; TNF-α, tumor necrosis factor-α.

* Corresponding author at: IVF Unit, Department of Obstetrics and Gynecology, University Hospital of North Norway, Sykehusveien 38, 9019 Tromsø, Norway.
E-mail addresses: may.w.joraholmen@uit.no (M.W. Jøraholmen), natasa.skalko-basnet@uit.no (N. Škalko-Basnet), ganesh.acharya@uit.no (G. Acharya), purusotam.basnet@uit.no (P. Basnet).

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and infection but also facilitate human immunodeficiency virus (HIV-1), human simplex virus type 2 (HSV-2) and human papilloma virus (HPV) infections (Nikolic and Piguet, 2010). Therefore the understanding on the cross-links between various diseases and microbicides together with the link between HPV and cervical cancer was recognized by the award of the 2008 Physiology and Medicine Nobel Prize (Abbott and Brumfiel, 2008). Particularly, it is interesting that RES uses different mechanisms to induce cell death in cervical cancer cell lines (Garcia-Zepeda et al., 2013). In addition, pregnant women are vulnerable to vaginal infection because of the reduced T-helper cell type 1 (TH1) activities due to the development of protective mode towards the growing fetus. If they are not treated in time, the pregnancy might result in impaired fetal growth and development or even termination. Although anti-microbial agents are commonly used in the treatment of pathogenic vaginal infection, contemporary normal course of anti-microbial therapy cannot be applied during pregnancy. Moreover, the problem of anti-microbial resistance such as the one linked to metronidazole (MDZ) and other 5-nitroimidazoles (tinidazole, ornidazole, and secnidazole) used against trichomoniasis needs to be taken into consideration (van de Wijgert and Shattock, 2007).

Regarding the pathogen resistance and serious side effects linked to current anti-microbial options in vaginal therapy, especially in pregnant patients, a multi-targeted, less toxic and potential candidate, such as RES could be an ideal molecule. However, due to its low solubility and poor bioavailability, the possible clinical uses against vaginal inflammation and infection remain limited. Clinically applicable and safe formulation of RES assuring its optimal therapeutic value in the treatment of vaginal inflammation and infection in pregnancy is needed. By applying the chitosan-coated liposomal carrier for RES, we aimed to utilize the ability of chitosan not only as microbicide target but also to disrupt bacterial biofilms, which is of great importance in the treatment of vaginal bacterial inflammation and infections (Kandimalla et al., 2013). This paper describes the nanomedicine-based topical formulation of liposomal RES targeted to vaginal inflammation and infection. *In vitro* anti-oxidative and anti-inflammatory effects of free RES were compared with that of the corresponding liposomal formulation.

2. Materials and methods

2.1. Materials

Lipoid S 100 (PC, >94% phosphatidylcholine) was a gift from Lipoid GmbH, Ludwigshafen, Germany. Vitamin C (ascorbic acid), chitosan [low MW, Brookfield viscosity 20.000 cps, degree of deacetylation (DD) of 92], 1,1-diphenyl-2-picrylhydrazyl (DPPH), mucin from porcine stomach (type III, bound sialic acid 0.5%–1.5%, partially purified), phosphorus standard solution (0.65 mM), sodium chloride, resveratrol (RES: 3,5,4′-trihydroxy-*trans*-stilbene, purity ≥ 99%), vitamin E, 2,2′-azinobis(3-ethyl benzothiazoline)-6-sulfonic acid diammonium salt (ABTS) and potassium peroxodisulphate were the products of Sigma-Aldrich, Chemie GmbH, Steinheim, Germany. Acetic acid (glacial), anhydrous potassium phosphate, and sodium hydrogen phosphate were purchased from Merck KGaA, Darmstadt, Germany. Ammonium acetate was obtained from BHD Prolab, Leuven, Belgium. Cibacron brilliant red 3B-A was purchased from Santa Cruz Biotech, Dallas, TX, USA. Glycine hydrochloride Plusone® was obtained from Pharmacia Biotec, Uppsala, Sweden. Dulbecco's modified eagle medium (DMEM), trypsin–ethylenediaminetetraacetic acid, lipopolysaccharide (LPS; *Escherichia coli*, 055:B5), L-nitro-arginine methyl ester (L-NAME), sulfanilamide, naphthylethylenediamine dihydrochloride, and phosphoric acid were purchased from Sigma Life Science (Sigma–Aldrich Norway AS, Oslo). Assay kit for SOD activity measurement was from Abnova GmbH EMBLEM, Heidelberg, Germany, and TNF-α, and IL-1β measured spectrophotometrically with the assay kits were from Cell Biolabs, Inc., San Diego, CA, USA. All chemicals and solvents used were of analytical grade.

2.2. Cell culture

Murine macrophage, J774A.1 (ATCC® TIB67™) cells were purchased from ATCC and used in the *in vitro* anti-oxidative and anti-inflammatory studies. Cells (1×10^5 cells/ml) were cultured in 24-well plates with DMEM medium containing glutamine and 10% calf serum by incubating at 37 °C in 5% CO_2 for 24 h to stabilize and adhere on the plate. After 24 h the cell medium was replaced with the LPS (1 μg/ml) and/or test samples (RES/corresponding liposomal formulations) containing medium. The effects of the test samples on the LPS-induced pro-inflammatory cytokines (TNF-α, IL-1β) and NO expressed in the medium and SOD activity were measured after 24 h according to the instruction provided in the commercial kits.

2.3. Preparation of stock solutions and application to cells

Stock solutions (10 mg/ml) of RES and vitamin E were prepared by dissolving with dimethyl sulfoxide (DMSO). They were diluted with DMEM medium to the desired concentration before the treatment with the cells. In case of DMSO solution (for vitamin E and RES), maximum concentrations of DMSO were not more than 0.2% (*v*/*v*). All other samples were prepared and with the medium and applied directly into the cells.

2.4. Anti-oxidative assays

2.4.1. Measurement of DPPH radical scavenging activity

Effect of RES on DPPH free radical was determined by the similar method as reported previously (Basnet et al., 2012). In brief, DPPH solution (60 μM, 0.3 ml) in ethanol was mixed with an equal volume of each sample solution (1, 5, 10 or 20 μg/ml as the final concentrations). The reaction mixture was thoroughly mixed and kept in the dark for 30 min at room temperature. The anti-oxidative activity of RES was expressed by measuring the decreased absorbance intensity at 519 nm with UV spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) using following formula: Radical scavenging activity (%) $= 100 \times (A - B) / A$, where A is the control (absorbance of DPPH radicals without sample) and B is the absorbance of radicals after reacting with the sample. The anti-oxidative activity of RES was also compared to that of vitamin C and vitamin E under the same experimental conditions. The results are expressed as mean ± S.D. of three experiments.

2.4.2. Measurement of $ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ radicals were generated by mixing equal volumes (3 ml) from each of the stock solutions of ABTS (7.4 μM) and potassium peroxodisulphate (2.6 μM) in distilled water. The reaction mixture was allowed to stabilize for 3 h at room temperature and then diluted with ethanol to 100 ml as the $ABTS^{\bullet+}$ radicals working solution. The green colour $ABTS^{\bullet+}$ radicals working solution (0.3 ml) was mixed with an equal volume of sample solutions at the 1, 5, 10 and 20 μg/ml concentration. After mixing, it was kept in the dark at room temperature. After 30 min, optical density was measured with UV spectrophotometer at 731 nm. As the number of $ABTS^{\bullet+}$ radicals decreases, the intensity of green colour reduces. Results were expressed as described for DPPH radical assay. The anti-oxidative activity of RES was compared to that of the vitamin C and vitamin E under the same experimental conditions.

2.5. Preparation of liposomes

Liposomes were prepared by the film hydration method as described earlier (Jøraholmen et al., 2014). Briefly, RES (10 or 20 mg) was dissolved in methanol and mixed with phosphatidylcoline (PC, 200 or 400 mg) in methanol and solvents were evaporated on Büchi rotavapor R-124 (with vacuum controller B-721, Büchi Vac® V-500, Büchi Labortechnik, Flawil, Switzerland) for at least 3 h at 50 mm Hg