Novel glycerin compounds improve skin health

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Abstract

Background: Novel glycerin compounds (NGC) were developed which generated new reaction products, endoperoxide derivatives. NGC have been reported to exhibit various biological effects. In addition to their deodorizing, disinfecting and antiviral effects, NGC were found to increase type I collagen production and inhibit pro-inflammatory cytokine secretion.

Aims: This study is aimed to evaluate the effects of NGC on skin homeostasis by investigating antioxidant activity and barrier functions.

Methods: In this study, in order to clarify the effects of NGC on normal human skin, we quantified the mRNA gene expression of heme oxygenase-1 (HO-1) and DAD(P)H quinone dehydrogenase 1 (NQO-1) as antioxidant factors by PR-PCR and the protein content of intracellular glutathione (GSH) using a culture system of normal human epidermal keratinocyte. Moreover, gene expression of factors related to skin barrier functions (involucrin (INV), filaggrin (FLG), and serine palmitoyl transferase (SPT)) was examined to clarify the effects of NGC on epidermal cell differentiation. We investigated whether the superoxide anion radical can be scavenged.Hypoxantine-xantine oxidase system(HPX-XOD) was used to generate superoxide anion radicals.Superoxide anion radicals were detected by ESR (JES-FR80) as DMPO-O2-adduct using DMPO as a spin trapping agent.We investigated scavenge effect of superoxide anion radical by NGC.

Results and Discussion: NGC enhanced expression of HO-1, NQO-1 mRNA and GSH protein in a concentration-dependent manner from 20 ppm after 24 h of stimulation. These results imply that the oxidative property of NGCs activates Nrf2 (NF-E2-related factor 2) transcription factor, which is a biological defense sensor, and induces the expression of antioxidant factors. It was strongly suggested that their hormesis effect would be working protectively for the skin.

NGC almost scavenged superoxide anion radicals. NGC scavenges ROS include superoxide anion radical caused by dryness, it was suggested that NGC enhances the skin protect system from dryness.

Conclusion: Mild oxidative stimulation of NGCs could lead to healthy skin through their hormesis effect on antioxidant activity and barrier functions.

Keywords:barrier function,hormesis,mild oxidative stimulation,glycerin

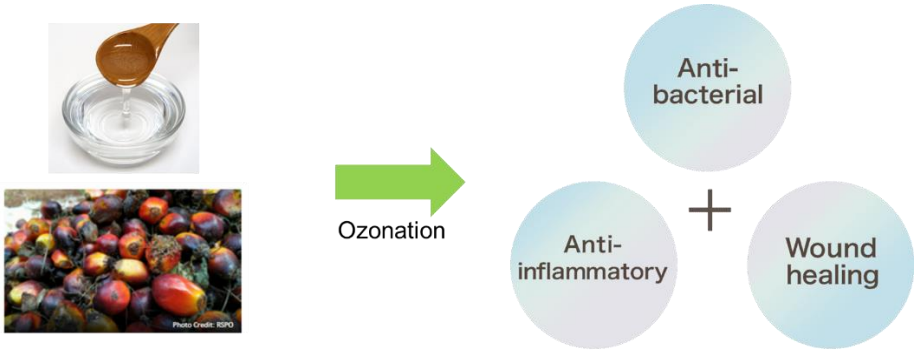
Introduction:

Novel glycerin compounds (NGC) were developed which generated new reaction products, endoperoxide derivatives. NGC was made by solubilizing ozone in high-purity glycerin (>99 %) using an ozone generator, and has been reported to have antiviral and antimicrobial effects due to its oxidizing action. For the skin, it has been reported to promote granulation and epithelial formation, synthesis of extracellular matrixes such as collagen fibers and hyaluronic acid.

And also NGC showed therapeutic efficacy for skin ulcers and dermatitis. Regarding safety for application of NGC to the skin, none of toxicities was found in human clinical study so far. Meanwhile, clinical trials were conducted in order to develop NGC as a cosmetic raw material and demonstrated that NGC directly decomposed synthetic melanin pigments and lightened facial age spots※1. However, the detailed mechanism of action of NGC on normal human skin is not well understood. In the present study, we investigated the effects of NGC using culture system of normal human epidermal keratinocytes (NHEK).

What's NGC

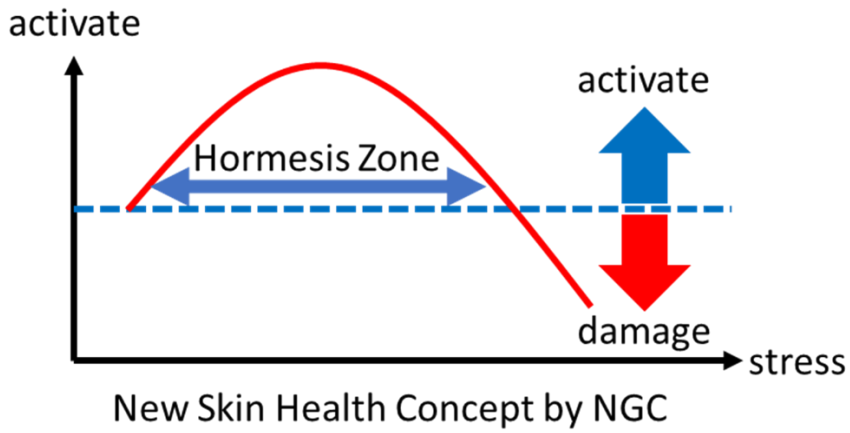
NGC has been variously reported to be antibacterial, anti-inflammatory, and promote wound healing for medical use.On the other hand, there have been few reports on NGC for normal skin and cosmetics.



Glycerin from RSPO

HYPOTHESIS

For many years, oxidative stress has been the focus of toxicity research.Antioxidants alone have limited ability to activate biological functions.On the other hand, as shown in Nrf2 and redox studies, mild oxidative stress contributes to body homeostasis.The hormesis effect of mild oxidative stress is very important for skin health.We thought that mild oxidative stress may contribute to the strengthening of the skin itself.



New Skin Health Concept by NGC

Material and Methods:

GSH and GSSG contents were measured in a recycling assay using 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB). HNEK were plated onto 96-well culture plates at 2 x104 cells/well and maintained in a 100 µL culture medium (HuMedia-KG2, Kurabo, Japan) for 24 h. The cells were treated with vehicle or OG and cultured in the absence for further 24 and 48 h. Cells were extracted using 0.5% Triton X-100/PBS (100 µL per well) . The extracted solution (25 µL) was mixed with reaction solution (2 mM NADPH, 0.12 units/mL glutathione reductase, 0.1 mM PBS containing 0.5mM EDTA, 175 µL). After incubation at 37 C for 10 min, 10 mM DNTB (25 µL) was added and then measured for absorbance at 405 nm.Measurement of mRNA expression of antioxidant and differentiation markers in HNEK.NHEK cells were seeded in 96-well plates (IWAKI, Japan) at a density of 2.0x104 cells/100 µL/well using HuMedia-KG2 and incubated with 0, 1 % , 2 % and 4 % of NGC for 24 hours at 37°C and 5% CO2. After incubation, the cells were washed with PBS(-), total RNA was extracted using the Ambion Cells-to-CT kit (Thermo Fisher Scientific, USA), and cDNA was synthesized by reverse transcription reaction (37°C, 60 min→95°C, 5 min) using the StepOne Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific, USA). The mRNAs of heme oxygenase-1 (HO-1), DAD(P)H quinone dehydrogenase 1 (NQO1), involucrin (INV), filaggrin (FLG) and serine palmytoiltransferase (SPTLC2), differentiation markers of NHEK, were amplified using the primer (Takara, Japan) below and relative quantification was performed using the ΔΔCt method. The relative expression levels of each gene were normalized to the house keeping gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Name	NCBI Gene ID	forward primer	reverse primer	product size
HO-1	GeneID (3162)	TTGCCAGTGCCACCAAGTTC	TCAGCAGCTCCTGCAACTCC	150
NQO1		GTGGCAGTGGCTCCATGTACTC	GAGTGTGCCCAATGCTATATGTCAG	
IVL	GeneID (3713)	GCTGGAGCAGCCTGTGTTTG	CTGGACACTGCGGGTGGTTA	159
FLG		CATGGCAGCTATGGTAGTGCAGA	ACCAAACGCACTTGCTTTACAGA	
SPTLC2	GeneID (9517)	GCCTGTCAGCAGCTCATACCAA	GGCCTGTCCAGTAGAGGTACCAA	121
GAPDH	GeneID (2597)	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	138

A mixture of 50 μL of 2 mM HPX solution and 35 μL of 5.5 mM DETAPAC solution in 0.1 M PBS buffer was mixed with 15 μL of undiluted DMPO and 50 μL of PBS buffer, and 50 μL of xantine-oxidase solution prepared in PBS was added and injected into the ESR.

Result:

Compared to glycerin, NGC enhanced HO-1 expression in a concentration-dependent manner. In particular, HO-1 expression was enhanced at ≥2% NGC, and for NQO-1, especially at 4% NGC.

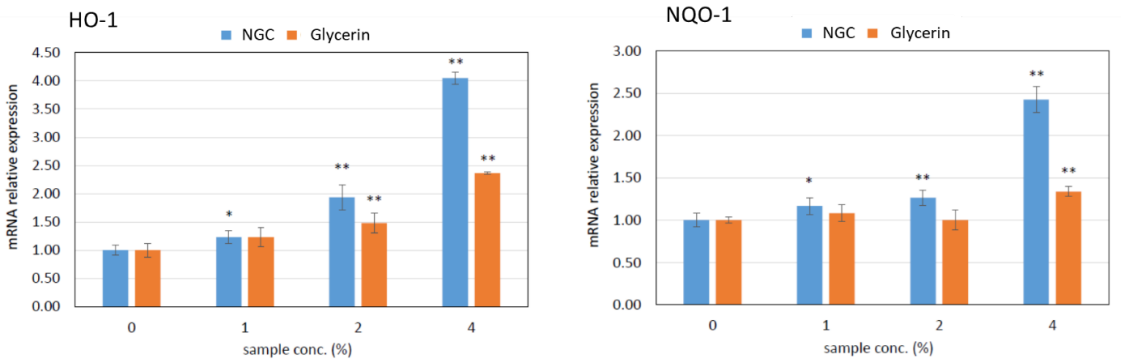


Figure 1.Effects of NGC on differentiation markers of human normal epidermal keratinocytes (NHEKs). NHEKs were treated with 0, 1, 2 and 4 % of vehicle (glycerin) or Novel Glycerin compound (NGC) for 24 hours. Relative HO-1,NQO-1 mRNA levels were analyzed by real-time PCR. Data are presented as mean ± standard error (n=3 or 4) in one out of two repeated experiments.P value was statistically determined (*P<0.05, **P<0.01).

Glutathione expression was not increased by glycerin at 24 h, but was increased by NGC in a concentration-dependent manner. increased, especially at 4%.

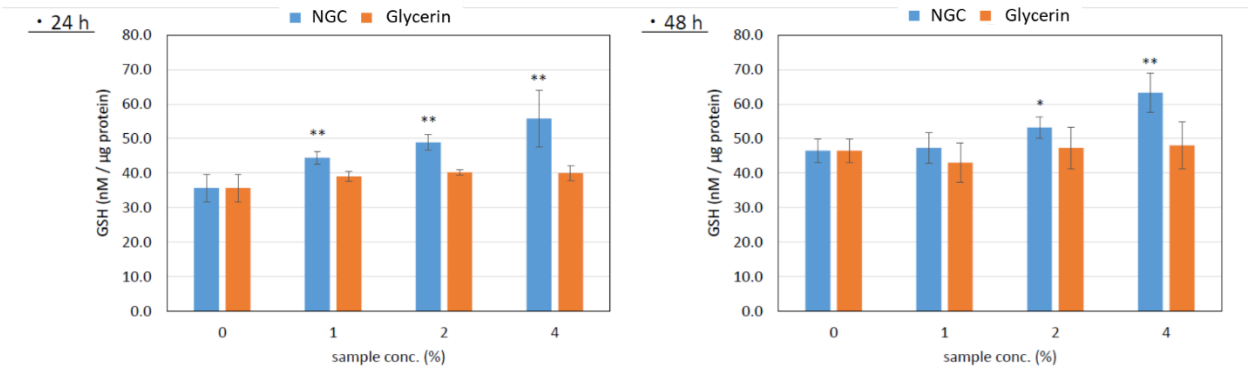


Figure 2.Effects of NGC on differentiation markers of human normal epidermal keratinocytes (NHEKs). NHEKs were treated with 0, 1, 2 and 4 % of vehicle (glycerin) or Novel Glycerin compounds (NGC) for 24hours,48hours. Relative GSH mRNA levels were analyzed by real-time PCR. Data are presented as mean ± standard error (n=3 or 4) in one out of two repeated experiments. P value was statistically determined (*P<0.05, **P<0.01).

FLG expression was significantly increased by NGC, especially by about 4 times increased at 2% than control; SPT was increased by NGC, especially at 2%; INV was increased in each

concentration range of NGC, especially at 4%; and glutathione expression was increased in each concentration range of NGC, especially at 4%.

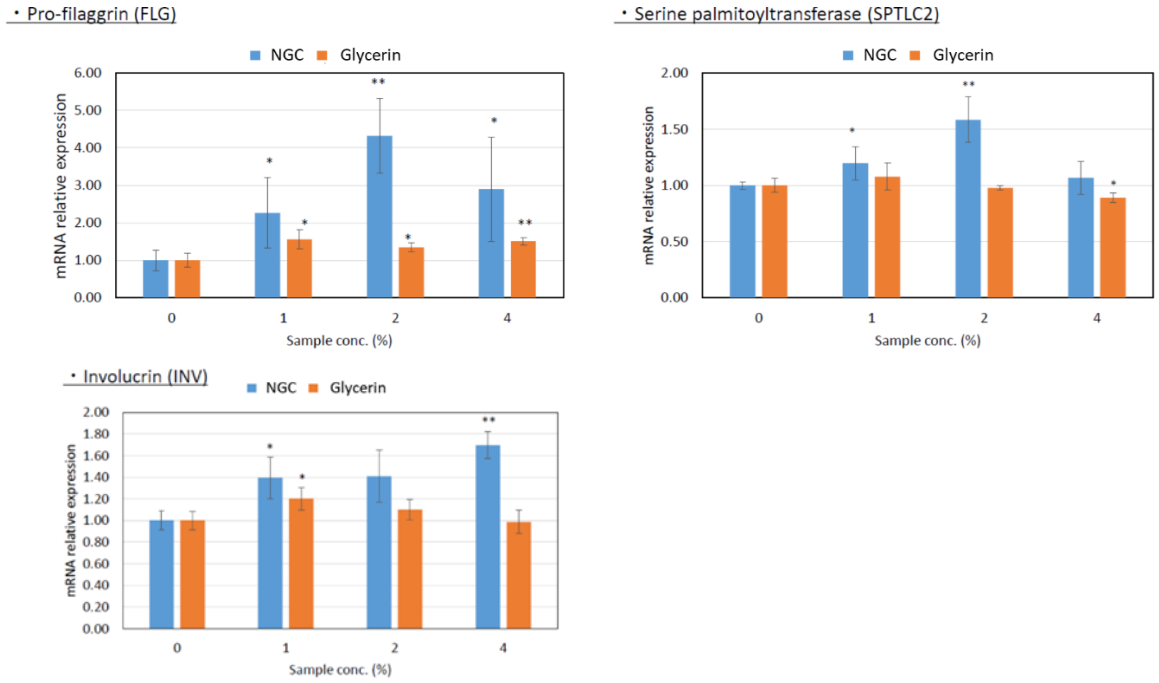


Figure 5.Effects of NGC on differentiation markers of human normal epidermal keratinocytes (NHEKs). NHEKs were treated with 0, 1, 2 and 4 % of vehicle (glycerine) or Novel Glycerine compound (NGC) for 24 hours. Relative Involucrin,FLG and SPTLC2 mRNA levels were analyzed by real-time PCR. Data are presented as mean ± standard error (n=3 or 4) in one out of two repeated experiments.P value was statistically determined (*P<0.05, **P<0.01).

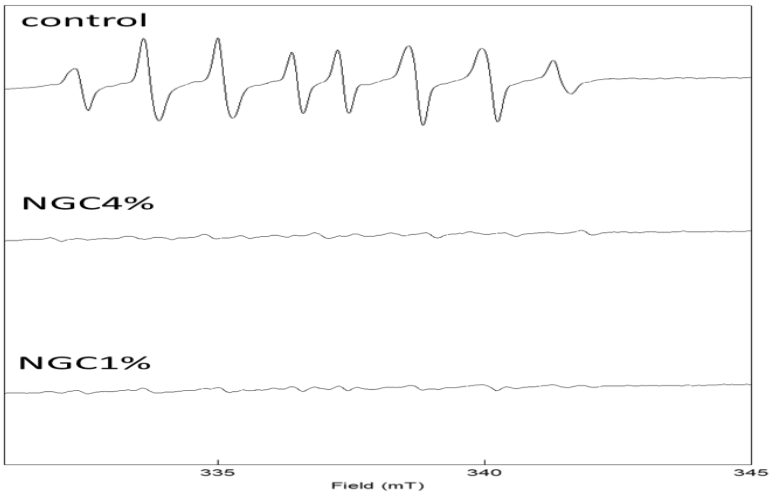


Figure 6. Scavenge effect of superoxide anion radical by NGC. Superoxide anion radical generated by HPX-XOD reaction system.Detection of radical with DMPO(spin trap agent) by ESR.

NGC almost scavenged superoxide anion radicals at 1% and 4%, and the scavenging ability was not significantly different between 1% and 4%.

Discussion:

These results suggest that the oxidative property of NGC activates Nrf2 transcription factor, which is a biological defense sensor, and induces the expression of antioxidant factors. Since their expression was caused by oxidative stimulation at low concentrations of NGC, it was strongly suggested that their hormesis effect would be working protectively for the skin. Consistent with this, the expression of INV, FLG, and SPT genes also increased at low concentrations of NGCs: FLG showed a threefold increase compared to the control group. These results suggest that NGC promote epidermal cell differentiation and have positive effects on skin barrier functions. We are currently conducting detailed analysis of the effects of NGC using three-dimensional cultures of human epidermis. Mild oxidative stimulation of NGC can lead to healthy skin through hormesis effect.

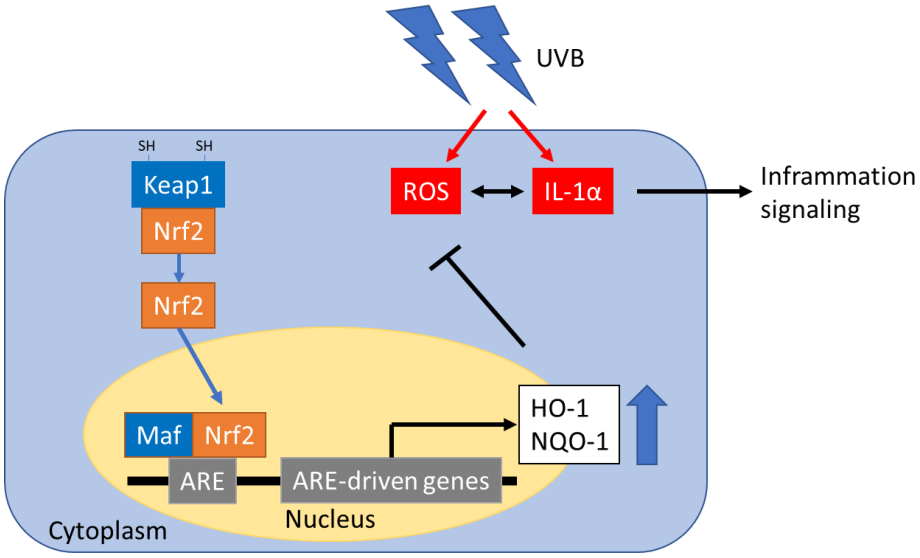


Figure 7. Expression of HO-1,NQO-1 and GSH by Nrf2-Keap1 signal

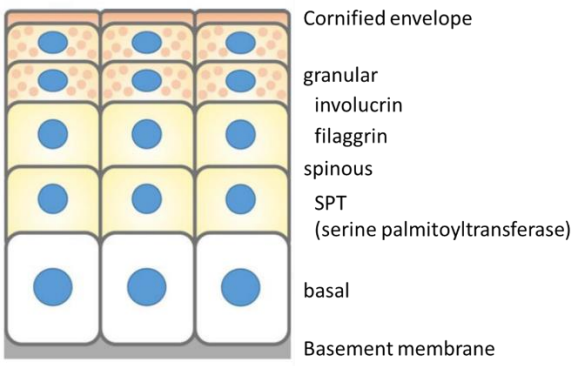


Figure 8. INV,FLG and SPTLC2 of epidermal cells

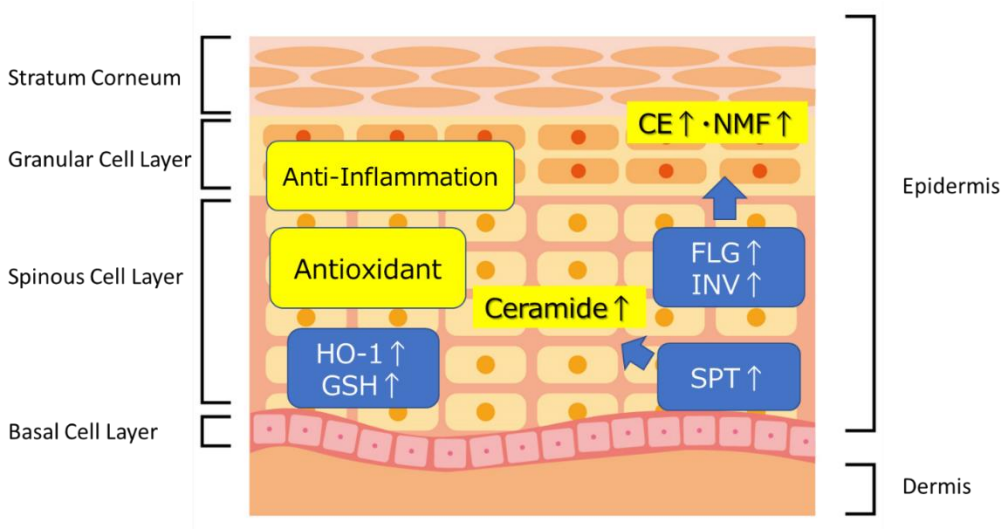


Figure9. Multi effect of NGC to improve skin health.

Conclusion:

Mild oxidative stimulation of NGCs could lead to healthy skin through their hormesis effect on antioxidant activity and barrier functions.

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Conflict of Interest Statement:

None to declare.

References:

1)Hanada K,Okuda D,Ogi R,Kojima S,Tsuruoka R,Shiota G,Ozonized glycerin (OG)-based cosmetic products lighten age spots on human facial skin: Journal of Dermatology :18 April 2022

2) Takeda Y, Jamsransuren D, Makita Y, Kaneko A, Matsuda S, Ogawa H, Wang P：Inactivation of SARS-CoV-2 by Ozonated Glycerol：Food and Environmental Virology 2021 Jun 26:1-6

3) Wang P, Tachi Y, Masuno K, Okusa K, Imamura Y：The Effect Ozone Gel Bone Matrix Production by Human Osteosarcoma Cell Line Saos-2：J Hard Tissue Biology. 27(3)195-198, 2018

4) Makita Y, Imamura Y, Masuno K, Fujiwara S, Shiota G, Shiba A and Wang PL. The Effect of Ozone on Collagen Type-1 and Inflammatory Cytokine Production in Human Gingival Fibroblasts. Dentistry 5:339.

5) Fukui T, Masuno K, Makita Y, Fujiwara S and Shiota G. Antimicrobial effects of ozone gel against periodontal bacteria. J Hard Tissue Biol 23: 445-448, 2014

6) Philos Trans R Soc Lond B Biol Sci. 2013 Sep 23;368(1629):20130016.

7)Izutu Y,Masaki H, FragranceJournal;10-15,49,12,2021