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Comparison Of Effects On Gene Expression Activity Of Low-Molecular-Weight Lychee Fruit Polyphenol (Oligonol®), Adenosine, And Minoxidil In Human Dermal Papilla Cells

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Abstrak

Background: Oligonol® (OLG) is raw materials for functional foods or cosmetics which contains low-molecular-weight polyphenols derived from lychee fruit (*Litchi chinensis* Sonn.). It has been reported to many functions such as anti-inflammatory and anti-oxidants.

Aim: In this study, with the aim of exploring new functionalities of OLG on the scalp and hair growth, we investigate i ts effect on human dermal papilla cells (HDPC) by comparing it with adenosine and minoxidil at the genetic level.

Method: OLG, adenosine, and minoxidil were applied to HDPC culture lines for 24 h, after which VEGF, FGF-7, WNT5a, and WNT10a mRNA expression was measured by real-time PCR analysis. Additionally, using DNA microarrays, we investigated their effects on 205 inflammation-related genes.

Result: The FGF-7, and WNT10a mRNA expression were observed in HDPC added with OLG. The results of the DNA microarray analysis showed that the expression of 11 genes was suppressed by OLG. These functions of OLG on HDPC were not the same as adenosine and minoxidil.

Conclusions: OLG may affect the function of HDPC by regulating the expression of genes related to cell proliferation and inflammation.

Keywords: polyphenol, dermal papilla cell, FGF-7, WNT10a, DNA microarray

INTRODUCTION

Various treatments for hair growth are currently being developed. Finasteride, dutasteride, and minoxidil are representative hair loss treatment medicines used as pharmaceutical ingredients in Japan.

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Finasteride has been applied to treat male androgenic alopecia (AGA) as a substance inhibiting the conversion of the male hormone testosterone to dihydrotestosterone by 5α-reductase.Dutasteride was also developed as a substance having higher 5α-reductase inhibitory activity than finasteride.[1,2]

Minoxidil was originally developed as an antihypertensive drug. However, it was confirmed to have a side effect of promoting hair growth, so the developmental target was changed to a hair growth agent. Subsequent studies have reported that it

promotes the production of vascular endothelial growth factor (VEGF) at the cellular level.[3]

In the search for ingredients with an effect of promoting hair growth, attention has been drawn to natural substances. For example, Cotsarelis et al.[4] reported that cultured HDPC of the thin hair portion and non-thin hair portion from subject exhibiting male pattern epilation (AGA) and then analyzed gene expression of the HDPC by DNA microarray.The results showed that the gene expression level of fibroblast growth factor 7 (FGF-7) was greatly decreased in HDPC of the thin hair portion.[5]

Based on this research, a screening of components promoting the expression of the FGF-7 gene in HDPC was carried out, and adenosine became a candidate substance. Adenosine is currently approved as a quasi-drug component by the Japanese Ministry of Health, Labor and Welfare, and is marketed as an external preparation for hair growth.

Thus, a screening system for examining the gene expression of HDPC is useful, and various genes such as VEGF, FGF, hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and WNT are now attracting attention as target genes for hair growth function.[6,7]Regardingnatural substances other than adenosine that could be used, polyphenol procyanidin B2 from apple fruits has been reported to affect hair growth in a murine experiment and a human clinical trial.[8,9] However, the mechanism has not been fully clarified.

Meanwhile, we have developed raw materials for functional foods or cosmetics which industrially low-molecular-weight polyphenols (Oligonol; OLG) derived from lychee fruit (*Litchi chinensis* Sonn.).The polyphenols in OLG are oligomerized through a novel manufacturing process. It is made by technological oligomerization of polyphenol polymers in the extract and contains a relatively high proportion of catechin-type monomers and oligomers of proanthocyanidin. It has been reported that polyphenols such as epicate chin gallate (ECG), epigallo cate chin gallate (EGCG), and procyanidins A1, A2, B1, and B2 are contained in this OLG.[10,11] Previous studies showed that OLG is safe and that it has anti-oxidant capacity and relatively high bioavailability.Regarding other functional studies of OLG,anti-inflammatory, anti-diabetic, and anti-metabolic syndrome effects *in vitro*, *in vivo*, and in clinical trials has been reported.[12,13,14,15]

Against this background, we focused on the effect of promoting hair growth as a new functional search for OLG rich in polyphenols. As described above, several screening methods related to an effect of promoting hair growth has been developed. In this study, we first performed *in vitro* experiments using HDPC. Specifically, we carried out gene expression analysis on HDPC by real-time PCR, and then investigated other gene fluctuations by DNA microarray. And we discussed the possibility that OLG could be used as a substance promoting hair growth.

MATERIALSAND METHODS

Reagents and sample preparation

Oligonol® powder(OLG, lot OLG-F1502S) was supplied by Amino Up Chemical Co. Ltd. (Sapporo, Japan) and dissolved in dimethyl sulfoxide (DMSO; Nacalai Tesque Inc., Kyoto, Japan) to prepare a 500 mg/mL stock solution. Minoxidil was obtained from Sigma-Aldrich Japan (Tokyo, Japan) was dissolved in 50% ethanol to prepare a 15 mM (4.3mg/mL) stock solution. Adenosine was obtained from Wako Inc. (Tokyo, Japan) was dissolved in DMSO to prepare a 100 mM (26.7mg/mL) stock solution. These stock solutions were used for addition to cell culture. These minoxidil and adenosine were used as positive controls.

Dermal follicle papilla cell growth media (Toyobo Inc., Osaka, Japan), dermal follicle papilla cell subculturing unit (Toyobo Inc.), Ninety-six-well and Forty-eight-well Cell matrix Type I-C (Nitta-gelatin Inc., Osaka, Japan) culture plates, and live cell count reagent SF (Nacalai Tesque Inc., Kyoto, Japan) were purchased.

Fast Lane Cell® cDNA Kit (Qiagen Co., Ltd., Venlo, the Netherlands), 5 mL of SYBR® Premix Ex Taq(Takara Inc., Shiga, Japan), and oligonucleotides (primers) (FASMAC Inc., Kanagawa, Japan) were purchased for use in quantitative real-time PCR (qPCR) (Table 1). Message Amp II biotin-enhanced amplification kit and fragmentation reagents were pure chased from Applied Bio systems Japan (Tokyo, Japan).

All other laboratory chemicals were of the highest purity from commercial suppliers.

Table 1. Summary of primer sequences used for HDPC in qPCR analysis.

Gene	Primer name	Sequence (5'- 3')
FGF-7	FGF7-For1	tctgtcgaacacagtggtacctgag
	FGF7-Rev1	gccactgtcctgatttccatga
VEGF	VEGFA-For2	aaagcatttgtttgtacaagatccg
	VEGFA-Rev2	cttgtcacatctgcaagtacgttcg
WNT5a	WNT5A-For3	caagttggtacaggtcaacagccgc
	WNT5A-Rev3	gagccggtgctctcattgcgcacgc
WNT10a	WNT10A-For4	ggtcccggaacacccagccctgccc
	WNT10A-Rev4	ttagaaaagcgctccccgaagccc
GAPDH	GAPDH-RT-For1	catccctgcctctactggcgctgcc
	GAPDH-RT-Rev	ccaggatgcccttgaggggccctc

Cell viability test

HDPC were obtained from the skullcap of a 63-year-old Caucasian male (Code No. CA602t05a; Toyobo Inc.). HDPC (5,000 cells / well) were plated on cell culture plate (96 wells, 6 mm diameter) with the culture medium and incubated at 37 ℃ in 5 % CO2 / 95% air for 24h. The culture supernatant were substituted for the culture medium including OLG (0.032-100μg/mL, triplicate) and cultured at 37 ℃ in 5 % CO2 / 95% air for 24h. The cell viability of the cultures was determined by cell count reagent SF, modified method in MTT assay. Briefly, the culture supernatant were replaced by a conditioned medium

including SF (10 % WST-8,Tetrazolium Salts) reagent and incubated at 37 ℃ in 5 % CO2 / 95% air for 2h. The absorbance was measured at 450 nm by microplate reader. The cell viabilities were compared with vehicle (0.1% DMSO).

Cell culture and cDNA preparation

For the gene expression tests regarding qPCR and DNA microarray, a control (medium, duplicate), vehicle (0.1% DMSO, duplicate), adenosine (final concentration of 100μM, duplicate), minoxidil (final concentration of 30μM, duplicate), and OLG (final concentrations of 4, 20, and 100μg/mL, duplicate)were added to cell culture plates and incubated for 24h.

Fast Lane Cell® cDNA Kit was used to extract total RNA, and 2 μL of gDNA Wipeout Buffer, 1μl of Fast Lane Lysate, and 1μL of RNase-free water were added to a PCR tube, and incubated at 42°C for 5 min. Then, 6μL of reverse transcription master mix solution (1 μL of Quantiscript Reverse Transcriptase, 4 μL of Quantiscript RT Buffer, 1 μL of RT Primer Mix) was added and incubated at 42°C for 30 min. Finally, the mixture was incubated at 95°C for 3 min to inactivate the reverse transcriptase, and the product was used as synthesized cDNA for the quantitative real-time polymerase chain reaction (qPCR) and DNA microarray analysis.

Quantitative real-time polymerase chain reaction

qPCR was performed using SYBR® Premix Ex Taq. Previously reported primers were used. PCR conditions were set at 95°C for 10 s and 60°C for 30 s. Relative quantification was performed by normalizing target expression to the housekeeping gene GADPH. Data are expressed as change in FGF-7, VEGF, WNT5a, and WNT10a mRNA expression compared with that in HDPC incubated without samples (control).

DNA microarray Human-allergy Chip®

cDNA was prepared from the RNA, and biotin-labeled RNA was transcribed and amplified using a Message Amp II biotin-enhanced amplification kit, in accordance with the manufacturer's instructions. Biotinylated amplified RNA (aRNA) was fragmented using fragmentation reagents and then incubated at 94°C for 7.5 min. The fragmentation reaction was terminated by the addition of stop solution. Hybridization was carried out with a DNA microarray (Genopal®; Mitsubishi Rayon Co., Yokohama, Japan). The special chip used for the DNA microarray was the Human-allergy Chip® (equipped with 205 types of genes on the surface of chip).Hybridization signal acquisition was performed using a DNA microarray reader adopting multi beam excitation technology (Yokogawa Electric Co., Tokyo, Japan). These experimental flows are shown in Figure1.

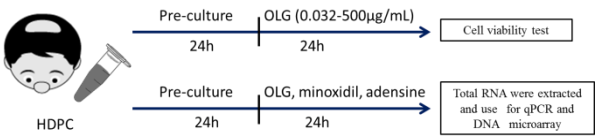

Figure 1. Experimental flow for cell viability test and genes expression analysis on HDPC cultured with samples.

Statistical analysis

Results are expressed as means ± standard error of the mean (S.E). Statistical significance was performed using EXCEL 2015 ver.1.15software. The Dunnett's multiple comparison test was used to compare relative gene expression levels and cell viability. A *p*-value of < 0.05was considered statistically significant. In the DNA microarray analysis, a 2.0-fold change in expression or suppression, as measured by the signal intensity ratio (SIR, log2 [Sample Signal intensity/Control Signal intensity]), was interpreted as differential expression. Level of SIR (+1 or more, -1 or less; 2.0-fold change) is defined as differential expression genes (DEGs).

RESULTS

Cell viability test: The results of OLG on the viability of HDPC revealed safety of cytotoxicity, even when a maximum concentration of 100μg/mL was used (Figure2). Therefore, 4, 20, and 100 μg/mL OLG were used in the qPCR and DNA microarray analysis.

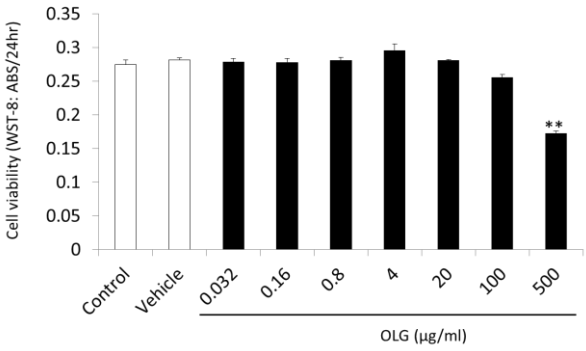

Figure 2. Measurement of viability of HDPC cultured with OLG. The HDPC were cultured with OLG (0.032-500 μg/mL, triplicate) for 24 h. Significant differences (**P < 0.01) were obtained between the group of OLG and control. Vehicle = 0.1% DMSO.

Quantitative real-time polymerase chain reaction:

Figure 3 shows the results from the analysis of FGF-7, VEGF, WINT5a, and WINT10a mRNA expression. For FGF-7 mRNA, significant expression (P < 0.01) was observed after adenosine addition; similarly, significant expression (*P < 0.05) was confirmed upon OLG (100 μg/mL) treatment (A).For

VEGF, no significant mRNA expression was confirmed upon minoxidil or OLG treatment (B).For WNT5a mRNA, no significant expression was observed after the addition of each of samples (C); on the other hand, significant WNT10a mRNA expression was confirmed with adenosine (*P < 0.05) and OLG(100 µg/mL) (**P < 0.01)treatment (D).

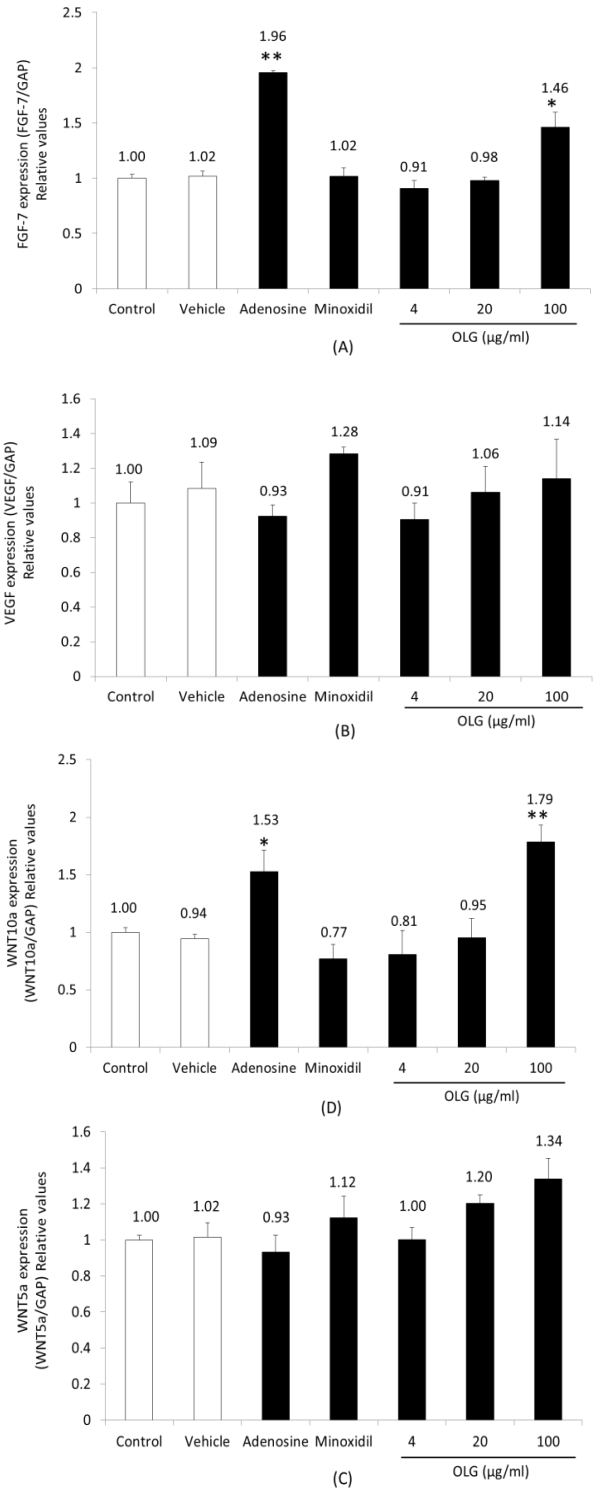

Figure 3. The mRNA expression of FGF-7, VEGF, WNT5a, and WNT10a in HDPC treated with each of samples. The mRNA expression levels in HDPC cultured with adenosine (100 µM), minoxidil (30 µM), and OLG (4, 20, and 100 µg/mL). The FGF-7 (A), VEGF (B), WNT5a (C), and WNT10a (D) mRNA levels measured qPCR. Significant differences (*P < 0.05, **P < 0.01) were obtained between the group of samples and control .vehicle = 0.1% DMSO.

DNA microarray Human-allergy Chip®.

The results of the Human-allergy Chip® analysis showed that 74 of 205 genes were suppressed and 4 of 205 genes were expressed in the adenosine treatment. In the minoxidil treatment, 74 of 205 genes were suppressed, while in the OLG treatment 11 of 205 genes were suppressed (Figure4).Among these DEGs, 53 of 74 genes were found to be commonly inhibited by adenosine and minoxidil. One gene (1 of 11) for which the expression was suppressed by both OLG and minoxidil was interleukin 8 (IL-8) (Figure5).

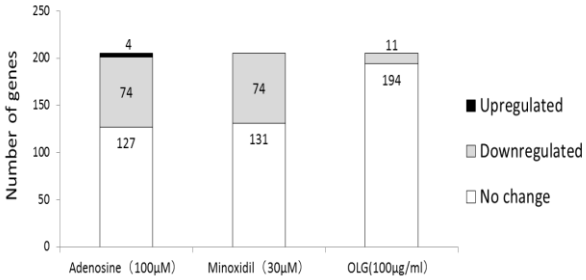

Figure 4. Number of DEGs (up regulated and down regulated genes) in HDPC treatment with adenosine (n=1), mioxidil (n=1), and OLG(n=1), as analyzed by DNA microarrays

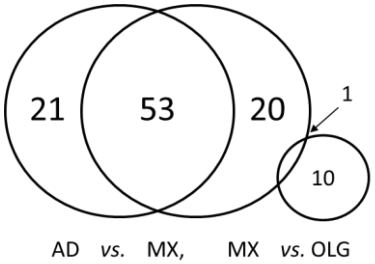

Figure 5. Venn diagram of DEGs from DNA microarray on each samples treatments HDPC. The number in each area shows the number of DEGs. This venn diagram summarizes the overlap of DEGs from the left circle (AD vs. MX) and the right circle (MX vs. OLG). Fifty-three DEGs were shared between AD and MX, while only one was shared between DEG and MX. AD; adenosine, MX; minoxidil

Additionally, 4 of 205 genes expressed as DEGs in the adenosine treatment were confirmed,as follows: nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1, transcript variant 2 (NFATC1), tumor necrosis factor receptor super family, member 4 (TNFRSF4/OX40), CD82 molecule, transcript variant 1 (CD82/Kai1), and signal transducer and activator of transcription 4 (STAT4).

Moreover, 11 of 205 genes suppressed in the OLG (100 µg/mL) treatment were confirmed: interleukin 1 beta (IL-1β), interleukin 6(IL-6), interleukin 8 (IL-8),

interleukin 11 (IL-11),interleukin 18 receptor 1 (IL18r1), Jun B proto-oncogene (JUNB), FOS-like 1 (FOSL1), intercellular adhesion molecule 1 (ICAM1), leukocyte-associated Ig-like receptor 1 (LAIr1),prostaglandin-endoperoxide synthase 2 (PTGS2), and TNF receptor-associated factor 1 (TRAF1) mRNA (Table 2).

Table 2. List of DEGs due to OLG treatment using DNA microarrays (Human-allergy Chip)in HDPC.

Gene symbol	Gene name	SIR
IL-1β	Homo sapiens interleukin 1, beta (IL1B), mRNA	-1.48
IL-6	Homo sapiens interleukin 6 (interferon, beta 2) (IL6), mRNA	-2.07
IL-8	Homo sapiens interleukin 8 (IL8), mRNA	-3.15
IL11	Homo sapiens interleukin 11 (IL11), mRNA	-2.07
IL18r1	Homo sapiens interleukin 18 receptor 1 (IL18R1), mRNA	-2.12
JUNB	Homo sapiens jun B proto-oncogene (JUNB), mRNA	-2.02
FOSL1	Homo sapiens FOS-like antigen 1 (FOSL1), mRNA	-1.08
ICAM1	Homo sapiens intercellular adhesion molecule 1 (CD54), human rhinovirus receptor (ICAM1), mRNA	-1.27
LAIr1	Homo sapiens leukocyte-associated immunoglobulin-like receptor 1 (LAIR1), transcript variant a, mRNA	-1.19
PTGS2	Homo sapiens prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA	-1.04
TRAF1	Homo sapiens TNF receptor-associated factor 1 (TRAF1), mRNA	-1.01

DISCUSSION

In the present study, we investigated the influence of OLG on HDPC collected from the scalps of healthy donors with hair loss, as primary screening for the effect of OLG on hair growth. First, we used qPCR to investigate the expression of VEGF, FGF-7, WNT5a, and WNT10amRNA in HDPC.

Adenosine and minoxidil are reported to affect hair growth, as well as the expression of FGF-7 and VEGF mRNA, which has been a recent focus in this field. Therefore, we also used adenosine and minoxidil as positive controls in this gene expression experiment. It has been reported that the WNT gene induces downstream activation (of the β-catenin gene), which promotes hair follicle formation.[16]Therefore, the VEGF, FGF-7, WNT 5a, and WNT 10a genes have attracted attention as target genes of hair growth activity and were focused on in an *in vitro* experiment.

VEGF promotes vascularization of the hair-bulb region and improves circulation to the hair. This is believed to increase the flow of necessary nutrients to the hair and thus promotes hairgrowth.[17,18] However, the results showed that the difference was not found to be significant. The lack of a significant difference for minoxidil is likely to be associated with the culture conditions or individual differences of the donors of the HDPC.

In contrast, the expression of FGF-7 mRNA was found to be significantly promoted by adenosine and OLG treatments. FGF-7 was reported to promote hair growth by accelerating cell proliferation, such as both hair matrix and hair root sheath cells from dermal papilla cells. Adenosine has also been reported to facilitate FGF-7 production via the adenosine A2b receptor in dermal papilla cells. Immuno histo chemical staining confirmed that the adenosine A2b receptor protein was expressed in the surrounding HDPC and outer root sheath in the hair follicles of healthy humans.[5]

Additionally, OLG significantly promoted the expression of WNT10a mRNA. This WNT signal is related to cellular proliferation and differentiation and is well known in the context of protein networks related to embryogenesis and cancer, particularly with respect to the β-catenin pathway, which suppresses gene expression through β-catenin. Consequently, recent research has raised expectations that the expression of the WNT gene in HDPCwould promote hair growth by participating in cellular proliferation.[19] These results suggested that OLG induces the mRNA of FGF-7 and WNT 10a of HDPC to exhibit hair growth activity.

Normal DNA microarrays allow comprehensive analysis of the expression of more than 20,000 genes; however, by using the DNA microarray (Genopal®) used in the present study, which is refined to gene probes for around 200 genes in each experiment, it is possible to conduct genetic analyses more economically and easily. The chip used in the present study was the Human-allergy Chip® (equipped with 205 types of genes associated with inflammation).[20]The results showed that 11of 205 DEGs were suppressed by the OLG (100 μg/mL) treatment, namely, IL-1β, IL-6, IL-8, IL-11, IL18r1, JUNB, FOSL1, ICAM1, LAIr1, PTGS2,and TRAF1.

From these data, it is difficult to consider the meaning of the suppression of these genes, but it is interesting that the genes suppressed by minoxidil and adenosine are different from those suppressed by OLG. In addition, since the number of genes suppressed by minoxidil and adenosine is greater than the number of genes suppressed by OLG, minoxidil and adenosine may have stronger anti-inflammatory effects than OLG. Regarding the association between hair growth and anti-inflammatory effects, it has been reported that the suppression of inflammation improves the condition of the scalp, and suppresses apoptosis in HDPC.[21,22.]

CONCLUSIONS

These results finds that OLG promotes the expression of the mRNA of FGF-7 and WNT10a and inhibits several genes related to inflammation, so further research on hair growth-promoting substances

exhibiting different mechanisms from minoxidil and adenosine is expected.

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