



## 2-Ketoglutaric acid, a component of rice fermented liquid, promotes skin hydration and barrier function

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### Introduction:

The tricarboxylic acid (TCA) cycle is a key metabolic pathway for driving the generation of mitochondrial energy in all oxidative organisms. 2-Ketoglutaric acid (2-KG), one of the TCA cycle intermediates, is a weak acid containing carboxyl groups and a ketone group (Fig.-1) and is involved in multiple metabolic processes [1]. A number of studies reported that 2-KG plays a pivotal role in the detoxification of reactive oxygen species (ROS) by improving antioxidative capacity and acts as an essential part of the oxidative defense machinery. In addition, 2-KG contributes to the oxidation of nutrients and the energy provision by producing plenty of ATP in the TCA cycle [2]. However, few studies have been reported on the efficacy of 2-KG in the maintenance of skin functions. Therefore, in this study, we aim to clarify whether 2-KG has beneficial effects on skin function and to find an effective way to enhance the production of 2-KG.

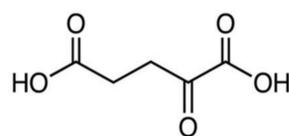


Fig.-1 Chemical structure of 2-KG

### Materials & Methods:

#### Test substance

2-Ketoglutaric acid (2-KG, Alfa Aesar, USA) and rice fermentation liquid fermented with *Saccharomyces veronae* were used for this study.

#### Cell culture

Normal human epidermal keratinocytes (NHEKs, KURABO, Japan) were cultured in keratinocyte basal medium (KBM) or keratinocyte growth medium (KGM) containing insulin, hydrocortisone, gentamycin/amphotericin B, and growth additives such as bovine pituitary extract and human epidermal growth factor (KURABO) at 37°C under an atmosphere of 5% CO<sub>2</sub> in air.

#### Evaluation of promoting effects on epidermal keratinocyte proliferation

NHEKs were seeded on a 96-well plate and cultured in KGM for overnight. The cells were then treated with or without 2-KG for 3 days. The promoting effects on cell proliferation was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay.

#### Evaluation of promoting effects on mRNA expression of genes related to skin hydration and barrier function

NHEKs were seeded on a 6-well plate and cultured in KGM for overnight. The medium was changed to KBM, and cells were cultured for 24 hours. The cells were then treated with or without 2-KG for 24 hours. Total RNAs were isolated in accordance with the standard operation method. Real-time RT-PCR reactions were performed by using a Thermal Cycler Dice® Real Time System III and TaKaRa SYBR® PrimeScript™ RT-PCR Kit (Perfect Real Time) (Takara Bio, Japan). The amount of mRNA expressions of filaggrin (FLG), serine palmitoyltransferase (SPT) and involucrin (IVL) was normalized by that of GAPDH used as an internal standard.

#### Analysis of 2-KG in rice fermented liquid using CE-TOFMS

To determine and quantify 2-KG in rice fermented liquid, capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) analysis was carried out using an Agilent G7100 CE system, an Agilent G6224AA LC/MSD TOF system, and an Agilent 1200 series isocratic HPLC pump. The system was controlled by Agilent ChemStation software for CE and MassHunter software for the Agilent TOFMS.

#### Statistical analysis

The data were statistically analyzed using parametric multiple comparison procedure of Williams by BellCurve for Excel (Social Survey Research Information, Japan). A p-value for rejection of less than 5% was considered statistically significant.

### Results & Discussion:

#### Promoting effects of 2-KG on epidermal keratinocyte proliferation

First, we examined whether 2-KG has an ability to promote keratinocyte proliferation. 2-KG significantly promoted epidermal keratinocyte proliferation (Fig.-2).

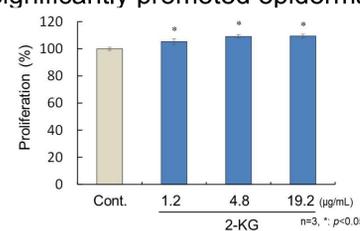


Fig.-2 Promoting effects of 2-KG on epidermal keratinocyte proliferation

#### Promoting effects of 2-KG on mRNA expressions of genes related to skin hydration and barrier function

To further examine whether 2-KG promotes the mRNA expressions of genes which relate to skin hydration and barrier function, promoting effects of 2-KG on mRNA expressions of FLG, SPT and IVL in keratinocytes were investigated. 2-KG significantly promoted mRNA expressions of FLG, SPT and IVL (Fig.-3).

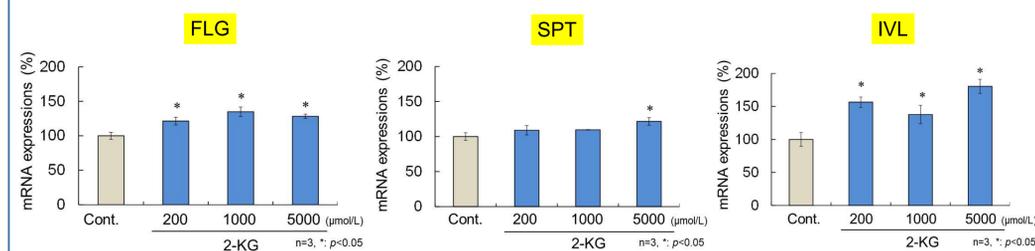


Fig.-3 Promoting effects of 2-KG on FLG, SPT and IVL mRNA expressions

#### Analysis of 2-KG in rice fermented liquid

As a candidate for cosmetic ingredients containing 2-KG, fermented materials were noticed. Five lots of rice fermentation liquid fermented with *Saccharomyces veronae* were analyzed. The analysis detected abundant 2-KG in the fermented liquid but not in the prefermented liquid (Table-1).

The amount of 2-KG (nmol/L)	
Rice prefermented liquid	N.D.
Rice fermented liquid	856.7 ± 12.8

N.D.: Not detected, Mean ± S.E., n=5

We focused on 2-KG which has been reported to possess many physiological functions, such as a natural scavenger of ROS [2]. However, few reports are available on the application of 2-KG for the maintenance of human skin functions. Thus, we investigated the efficacy of 2-KG on skin function in keratinocytes. As a result, 2-KG promoted keratinocyte proliferation and mRNA expressions of genes (FLG[3], SPT [4] [5] and IVL[6]) related to the maintenance of epidermal function. Next, we focused on fermentation with yeast as an efficient production method of 2-KG for developing a cosmetic ingredient containing 2-KG. Yeast cells have mitochondria in which the TCA cycle takes place and could boost the production of amino acids by fermentation [7] [8]. As a starting raw material, rice was selected and fermented by yeast. Analysis showed that the rice fermented liquid contained 2-KG abundantly. Taken together, it was considered that fermentation is one of the best ways to produce 2-KG effectively and to develop beneficial ingredients in cosmetics.

### Conclusions:

It is concluded that 2-KG promotes skin hydration and barrier function by activating cell proliferation and by up-regulating mRNA expressions of FLG, SPT and IVL in keratinocytes and that rice fermented liquid containing 2-KG could be a promising cosmetic ingredient for skin care products.

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