



# Melanin-Decolorizing Activity of Antioxidant Enzymes, Glutathione Peroxidase, Thiol Peroxidase, and Catalase

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## Abstract

Melanin is the most important factor to determine skin color. Many research efforts are being undertaken to decompose the already-produced melanin compounds in skin for beauty. This research investigated the effects on reducing melanin color of the three antioxidant enzymes, Glutathione peroxidase (GPX), Thiol peroxidase (TPX), and Catalase, in lysosomal fraction. Melanin solution was treated with the enzymes and hydrogen peroxide, then reacted for 48 h. GPX and TPX decolorized melanin, and between them, GPX was more efficient, but Catalase was not effective. GPX also inhibited the production of melanin in B16F10 melanoma cells. GPX, which is present in almost all microorganisms, plays an important role in the cellular defense mechanism by reactive oxygen species. In addition, it was not cytotoxic, but was significantly effective in decolorizing melanin color. Therefore, in the biological and microbiological field, its possibility of utilization in skin whitening cosmetic is high.

**Keywords** Melanin · Glutathione peroxidase (GPX) · Thiol peroxidase (TPX) · Catalase · B16F10 · Lysosome

## Introduction

A clean face is one of the essential elements of beauty for modern people, especially Asians are enthusiastic about white skin. Melanin compound is the most important factor in determining skin color. It is distinguished from eumelanin having black or brown color, and pheomelanin, which results in a pink to red hue [1]. Melanin compound is synthesized by tyrosine oxidation in melanocyte cells, which are found in the basal layer of the epidermis; and the main role of this pigment in the skin is to protect the dermis by blocking harmful ultraviolet radiation [2, 3]. However, if

too many melanin compounds are present in skin, it makes skin dark and unclean, and can cause hyperpigmentation, like melasma [4].

There have been many studies related to skin care cosmetics that solve these problems, but most of the currently developed skin whitening cosmetics have a function for inhibiting melanin synthesis through several mechanisms, like blocking the oxidation of tyrosine, or UV ray that can promote melanin synthesis [5]. These products take a long time to be effective, and are not effective for specific symptoms, such as a hyperpigmentation [6]. At present, a typical method of removing melanin that has already been

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made is laser treatment [7]. If materials that can solve these problems without such procedures are developed, they will have great utility in the biological and cosmetic field.

In previous research, we tried to find a way to decolorize the melanin compounds already produced. There have been research reports regarding lysosome and melanin correlation. It is very likely that differentiated keratinocytes particularly degrade the extrinsic melanosomes according to the sort of basic autophagy functions that control the amount of cellular organelles, or remove the defective organelles, such as peroxisome [8]. The autophagic pathway, from cytoplasm to lysosomes, plays a substantial role in the degradation of melanosomes in human epidermal keratinocytes [9]. These data provide evidence that lysosomal specific enzymes contribute to melanin degradation. Previously, we found melanin color reduction activity of enzyme in lysosomal fraction, a complex containing hydrolysates to break down waste material and cellular debris in peroxisomes and lysosomes [10, 11]. This paper studied the effects of the three selected enzymes, Glutathione peroxidase, Thiol peroxidase, Catalase, in lysosomal fraction to reduce melanin color. These peroxidases act to defend against cell damage caused by reactive oxygen species (ROS), and are present in all eukaryotes [12].

Reactive oxygen species (ROS) are formed as a natural byproduct of the normal metabolism of oxygen in cells, and can be increased by environmental stress. Highly generated ROS can damage cells. The harmful effects of ROS on the cell are as follows [12, 13]: damage of DNA or RNA, oxidations of polyunsaturated fatty acids in lipids, oxidations of amino acids in proteins, and oxidative deactivation of specific enzymes by oxidation of co-factors. The cells neutralize the ROS by an antioxidation system to prevent such tissue damage. In this system, the superoxide anion produced by the metabolism is highly devastating; although it is converted to hydrogen peroxide by Superoxide dismutase (SOD), the hydrogen peroxide is still reactive. The hydrogen peroxide is transformed to H<sub>2</sub>O by Catalase, and consumed in the production of Glutathione disulfide (GSSG) from Glutathione by the catalysis of GPX. TPX works similarly to GPX [13].

This study researched the melanin-decolorizing effect of three antioxidant enzymes, GPX, TPX, and Catalase. As a result, GPX and TPX were effective in reducing melanin color. Among them, GPX had no cytotoxicity at the optimum concentration of melanin-decolorizing activity, and effectively inhibited melanin synthesis. Therefore, it provides evidence that GPX plays an important role in the mechanism of melanin decolorization of lysosomal fraction, not only antioxidant activity, and offers the potential of a new whitening cosmetic agent as a single enzyme.

## Materials and Methods

### Peroxidases and Peroxidase Activity Assay

The concentration of the three peroxidases (Glutathione Peroxidase, Thiol Peroxidase, and Catalase) was determined by assay kit (Peroxidase assay kit, D2PD-100, QuantiChrom™) purchased from Bioassay Systems. Glutathione peroxidase 2 (GPX2, NBP1-78,824, Novus) and Thiol peroxidase (TPX, NBP1-78,805, Novus) were purchased from Novus, and Catalase (9001-05-2, Sigma-Aldrich) was purchased from Sigma-Aldrich.

This assay kit uses H<sub>2</sub>O<sub>2</sub> and an electron donor dye that forms resorufin during the peroxidase. The assay was performed at room temperature (RT), and the intensity of the color was measured at 570 nm. The peroxidases were reacted with the electron donor dye with 0.6% hydrogen peroxide for 10 min at RT. After reaction, the stop reagent was added, and the absorbance of sample was measured by microplate spectrophotometry. The activity was calculated as follows: Peroxidase activity =  $\frac{R_{\text{SAMPLE}} - R_{\text{BLANK}}}{R_{\text{RESORUFIN}} - R_{\text{H}_2\text{O}}} \times \frac{[\text{Resorufin}](\mu\text{M})}{t(\text{min})} \times \frac{\text{Reaction Vol}(\mu\text{L})}{\text{Sample Vol}(\mu\text{L})}$  (U/L). One unit (U) of enzyme will catalyze the formation of 1 μmole resorufin per min under the assay conditions.

Here  $R_{\text{SAMPLE}}$ ,  $R_{\text{BLANK}}$ ,  $R_{\text{RESORUFIN}}$ , and  $R_{\text{H}_2\text{O}}$  are the absorbance of the Sample, Sample Blank, Resorufin, and Water, respectively; and  $n$  is the sample dilution factor. The [Resorufin] for this assay is 50 μM. The Reaction Vol is 100 μL, and the Sample Vol in this study is 10 μL.

### Melanin Decolorization by Glutathione Peroxidase (GPX), Thiol Peroxidase (TPX), and Catalase

The melanin solution contains 0.1 mM PBS (Phosphate-buffered saline, pH: 7.0) and melanin powder (Synthetic, Sigma) that was prepared by oxidation of tyrosine with hydrogen peroxide. The melanin contents were measured by the absorbance at 450 nm. To determine the melanin concentration, a standard curve was prepared from an authentic standard of synthetic melanin ( $R^2 = 0.999$ , data not shown).

To verify the melanin decolorization, 100 ppm melanin solution containing (50 to 1000) μU/L Peroxidases (GPX, TPX, Catalase) with 1 mM hydrogen peroxide was reacted at RT, and measured everyday. Melanin decolorization value was determined by subtracting the melanin concentration after the reaction, from the initial value.

### Cell Culture

The B16F10 mice melanoma cells (KCLB 80008) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were cultured in Dulbecco's Modified

Eagle's Medium (DMEM) containing 4500 mg/L glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, to which was added 10% fetal bovine serum (FBS), 20 mM HEPES, 1% penicillin, and the cells were incubated in a humidified condition containing 5% CO<sub>2</sub> at 37 °C. The medium was exchanged every 2 days (d), and the cells were harvested by using trypsin/EDTA solution.

### Cell Viability Assay (MTT Assay)

The toxic effect of glutathione peroxidase on B16F10 melanoma cell was confirmed by MTT assay, a general method used to determine the effect of a sample on the viability of adherent cell. This assay uses 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) that can be reduced to formazan by mitochondrial enzyme in viable cell. The 3 × 10<sup>3</sup> cells were seeded per well in a 96-well plate, and incubated for 24 h. Then a sample was added to each well, and incubated for 72 h. After that, the medium was exchanged to one containing 0.5 mg/mL of MTT, and incubated for 2 h. Next, the MTT dye reagent was removed, and the generated formazan was dissolved by DMSO for 30 min. Finally, the concentrations of formazan were measured at 570 nm using microplate spectrophotometry. The cell viability was calculated as follows: cell viability (%) = (A<sub>sample</sub> - A<sub>blank</sub>) / (A<sub>control</sub> - A<sub>blank</sub>) \* 100.

### Melanin Contents Assay

The 3 × 10<sup>5</sup> B16F10 cells were seeded per well in a 6-well plate, and incubated for 24 h to adhere. The medium of each well was exchanged with one containing various concentration of enzyme and 10 nM α-MSH, then incubated for 72 h. After that, the wells were all washed with DPBS buffer, and harvested using 0.5% trypsin/EDTA solution. The pellets of cells obtained through centrifugation were lysed with 200 μL of 1 N NaOH containing 10% DMSO for 1 h at 80 °C. Finally, the lysed melanin content was measured by the absorbance at 450 nm. The melanin production inhibition efficiency of enzyme was determined by comparison with positive control using 100 mg/mL of arbutin.

## Results

### Peroxidase Activity of Glutathione Peroxidase (GPX), Thiol Peroxidase (TPX), and Catalase

This study is extension research of the application of lysosomal fraction treatment for decolorizing melanin compounds in vitro and in vivo. In previous study, we found that lysosomal fraction isolated from Hela cells, *S. cerevisiae*, egg of hen has the effect of reducing melanin color, and the

efficiency of melanin decolorization was associated with the peroxidase activity of the lysosomal fraction non-membrane bound microsomal fraction, including lysosomes and peroxisomes. To confirm the melanin color reduction effect of peroxidases, three peroxidases were selected, Glutathione peroxidase (GPX), Thiol peroxidase (TPX), and Catalase. These enzymes play an important role in reducing oxidative stress caused by reactive oxygen species in cells [14].

The activity of these enzymes was determined by assay kit (Peroxidase assay kit, D2PD-100, QuantiChrom™). This method was based on the oxidation of electron donor dye that forms a pink color during the peroxidase reaction [15]. The peroxidase activity was expressed using the following units: 1 Unit = 1 U = the formation of 1 μmole resorufin per min under the assay conditions. Table 1 summarizes the activity and concentration of the three peroxidases, GPX, TPX, and Catalase.

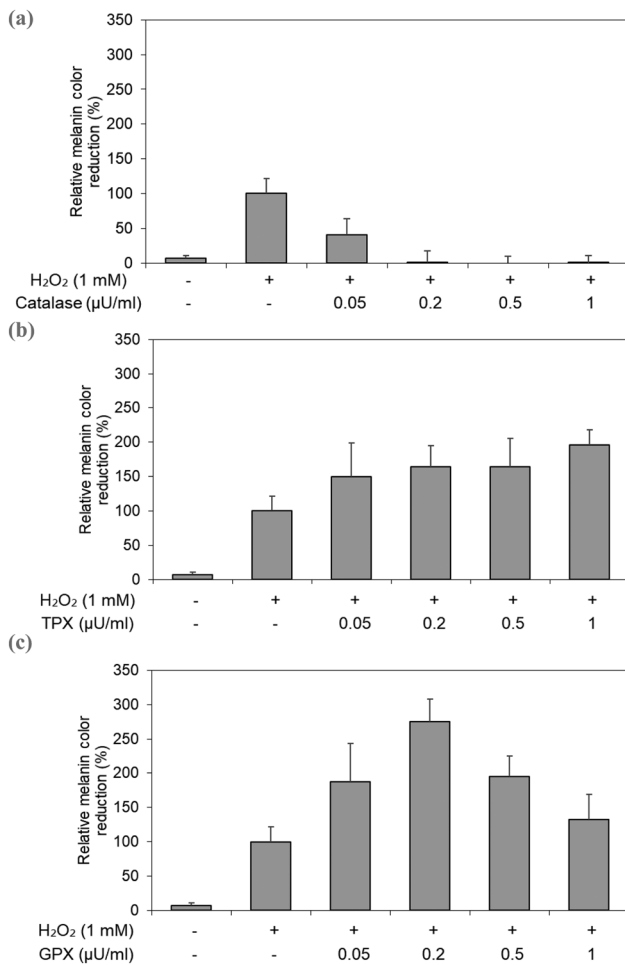
### Melanin Decolorization Activity of Glutathione Peroxidase (GPX), Thiol Peroxidase (TPX), and Catalase

To confirm the melanin decolorization effect of the peroxidases, 100 ppm melanin solution was treated with 1 mM hydrogen peroxide and (50 to 1000) μU/L concentration of each peroxidase. The samples were reacted for 2 days at RT, then compared with the initial concentration. Figure 1 shows the melanin decrease after 48 h. Figure 1 shows the evidence that TPX and GPX have melanin decolorization activity. The catalase had no effect on reducing melanin color (Fig. 1a). The TPX was effective at decolorizing melanin compounds, and the efficiency was best when treated at a concentration of 1 μU/mL (Fig. 1b). The GPX also had a melanin color reduction activity, which was best at concentration of 0.2 μU/mL, and gradually decreased at higher concentrations (Fig. 1c). This value represents 175% improved melanin color reduction, compared to the control treated with hydrogen peroxide only. The GPX was more efficient in terms of concentration and peroxidase activity, compared to TPX. The treatment of 0.2 μU/mL GPX and 1 mM hydrogen peroxide to

**Table 1** Specific data of the enzymes, Glutathione Peroxidase 2 (GPX2, NBPI-78824, Novus), Thiol Peroxidase (TPX, NBPI-78805, Novus), and Catalase (9001-05-2, Sigma-Aldrich)

	Concentration (mg/mL)	Peroxidase activity	
GPX 2	0.25	2.44 U/L	9.76 U/g
TPX	1.0	2.56 U/L	2.56 U/g
Catalase	10	0.775 U/L	0.0755 U/g

The peroxidase activity of enzymes was measured by assay kit (Peroxidase assay kit, D2PD-100, QuantiChrom™), according to the manufacturer's instructions

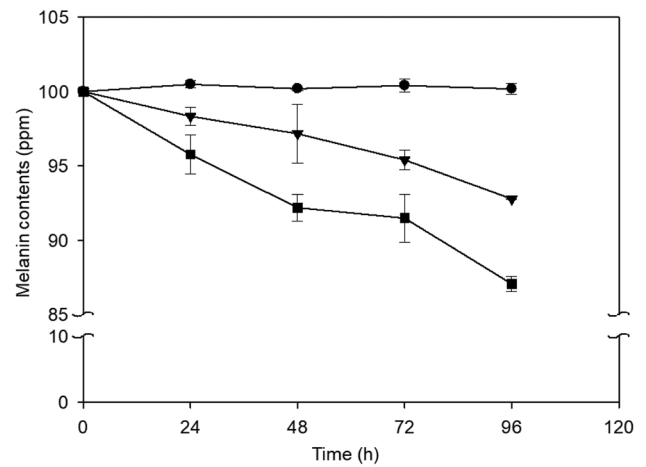


**Fig. 1** The relative melanin color reduction compared to treating only hydrogen peroxide. The 100 ppm melanin solution was treated with enzymes and H<sub>2</sub>O<sub>2</sub> for 48 h. **a** Relative melanin reduction of Catalase, **b** TPX, and **c** GPX

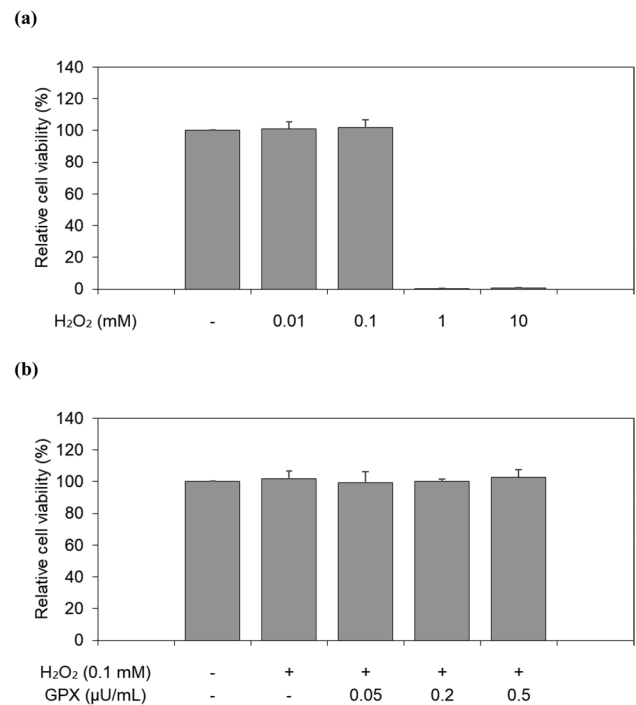
melanin solution decolorized 13% of 100 ppm melanin for 4 d (Fig. 2). However, neither GPX nor TPX showed any color reduction effect under the absence of hydrogen peroxide (data not shown).

### Effect of Glutathione Peroxidase (GPX) and H<sub>2</sub>O<sub>2</sub> on B16F10 Cell Viability

The effect of GPX on the viability of B16F10 melanoma cells was confirmed by MTT assay, before the melanin synthesis inhibition test. To find the proper concentration of hydrogen peroxide to treat, Cytotoxicity was assessed by treatment with hydrogen peroxide for 72 h at different concentrations. Under the concentration of 0.1 mM, H<sub>2</sub>O<sub>2</sub> had no cell toxicity to the cells for 72 h, while at concentration above 1 mM, great toxicity was observed. (Fig. 3a). The effect of GPX on cell survival was then assessed in the presence of 0.1 mM hydrogen peroxide, which was not cytotoxic.



**Fig. 2** The variation of concentration of residual melanin upon treatment of 100 ppm melanin without any drug (circles), treated with 1 mM of H<sub>2</sub>O<sub>2</sub> alone (reversed triangle), and both 0.2 μU/mL of Glutathione peroxidase and 1 mM of H<sub>2</sub>O<sub>2</sub> (squares)



**Fig. 3** The effect of H<sub>2</sub>O<sub>2</sub> and GPX on the 16F10 melanoma cell viability analyzed by MTT assay. The cells were treated for 72 h with each sample. **a** Hydrogen peroxide at (0.01 and 0.1) mM had viability of 100% compared to the control (no treatment), but at (1 and 10) mM, the viability was 0%. **b** In the case of treatment with both GPX and 0.1 mM H<sub>2</sub>O<sub>2</sub>, (0.05, 0.2, and 0.5) μU/mL of GPX had no cytotoxicity

The GPX had no cytotoxicity to B16F10 cells below the concentration of 0.5  $\mu\text{U}/\text{mL}$ , which was effective in melanin decolorization studies (Fig. 3b).

### Effect of Glutathione Peroxidase (GPX) and $\text{H}_2\text{O}_2$ on Melanin Synthesis in B16F10 Melanoma Cells

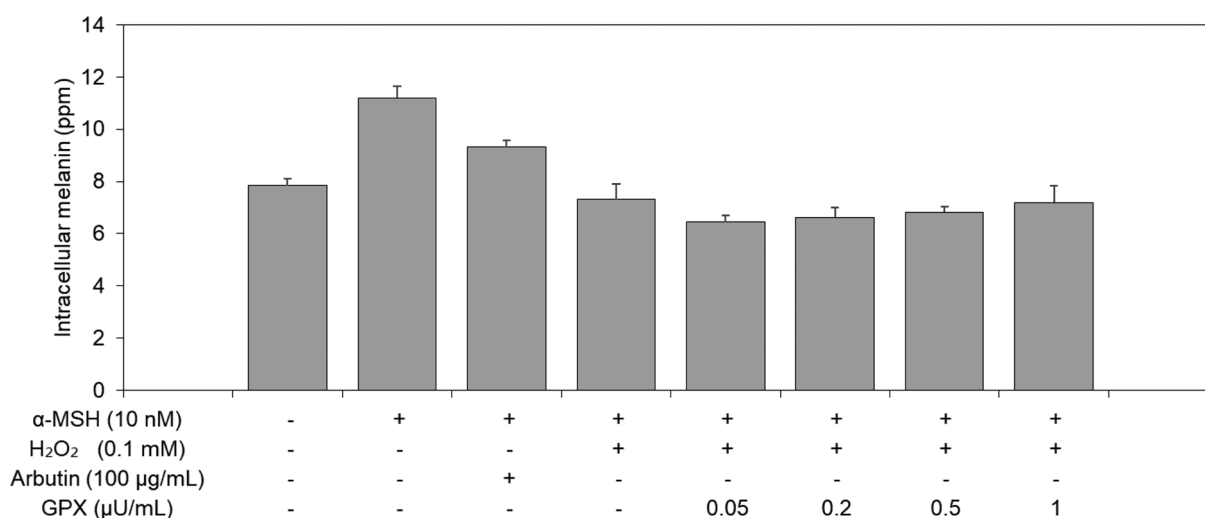
We researched the effect of GPX, which was most effective on melanin decolorization, on melanin synthesis in melanocytes. Figure 4 shows the melanin contents in B16F10 melanoma cells treated with each sample for 72 h. The treatment with 0.05  $\mu\text{U}/\text{mL}$  of GPX and 0.1 mM  $\text{H}_2\text{O}_2$  induced the most inhibition of melanogenesis by 43% compared to control (only treatment with  $\alpha\text{-MSH}$ ). This was 153% higher than the positive control (treatment with Arbutin and  $\alpha\text{-MSH}$ ), and 22% higher than that of hydrogen peroxide without enzyme, while treatment of GPX did not show dramatic melanin reduction in the absence of hydrogen peroxide also supports this view (data not shown). As a result, GPX inhibited the production of melanin compounds, but melanin synthesis in cells was more affected by hydrogen peroxide than by GPX.

### Discussion

In this study, our researchers found the optimal concentration and the effect of GPX, TPX, and Catalase, antioxidant enzymes that reduce oxidative stress in cell, on the decolorization of melanin compound. As a result of extracellular

experiments using synthesized melanin compounds, GPX and TPX have melanin-decolorizing effect, but Catalase does not. There are reports that lignin-degrading enzymes in some fungi, like a laccase, lignin peroxidase, can break down melanin in the presence of hydrogen peroxide [16–18]. Through this study, it was confirmed that the antioxidant enzymes, which are in all eukaryotes and not lignin-degrading enzymes, have melanin-decolorizing activity. Although GPX and TPX could also reduce melanin color in the presence of hydrogen peroxide, these enzymes have the possibility of acting as a major component of the melanin decolorization mechanism of lysosomal fraction.

GPX, which was most effective to reduce melanin color in vitro, was treated with B16F10 melanoma cells to determine its effect on melanin synthesis. In B16F10 melanoma cells, GPX was not cytotoxic at concentrations that were effective in the decolorization of melanin compounds, and the concentration of hydrogen peroxide treated together was 0.1 mM, with no toxicity. Treatment of hydrogen peroxide greatly inhibited the melanin synthesis, and when working with GPX, the efficiency was better, while there was no concentration dependence of the drug. Since hydrogen peroxide is quickly consumed by enzymes, it can be interpreted that the higher the concentration of enzyme to be treated, the lower the inhibition of melanin synthesis by hydrogen peroxide. The fact that treatment of GPX did not show dramatic melanin reduction in the absence of hydrogen peroxide also supports this view (data not shown). As a result, GPX inhibited the production of melanin compounds, but melanin synthesis in cells was more affected by hydrogen peroxide than by GPX.



**Fig. 4** The melanin content in B16F10 melanoma cells treated with drug for 72 h. The cells reacted in 6-well plate were dissolved in 200  $\mu\text{L}$  of 1 N NaOH containing 10% DMSO, and the concentration was measured by a standard curve prepared with synthetic melanin



These results suggest that antioxidant enzymes induce melanin degradation in autophagy mechanisms that digest melanosomes.

## Conclusion

Our study shows that Glutathione peroxidase (GPX) and Thiol peroxidase (TPX) contribute melanin decolorization, and GPX effectively inhibited melanin synthesis. The results support the hypothesis that antioxidant enzymes induce melanin degradation in autophagy mechanisms that digest melanosomes. To reach a definite conclusion, study on the mechanism of melanosome digestion in skin cells, keratinocyte and melanocyte, may be required.

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**Author Contributions** All authors contributed to writing of the manuscript.

## Compliance with Ethical Standards

**Conflict of interest** Gyeongchan Jeon, Chinhan Kim, Uk Min Cho, Ee Take Hwang, Hyung Seo Hwang, and Jiho Min declare that they have no conflict of interest.

**Research Involving Human and Animals Rights** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Informed consent was obtained from all individual participants included in the study.

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