# Effects of Oxidative Stress Prevention Using DNA Aptamer (Aptamin C®) in Keratinocyte

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## Abstract

Oxidative stress is one of the leading causes of various diseases. Antioxidants are effective against reactive oxygen species (ROS), a major factor in oxidative stress. Antioxidants play important roles in our body by scavenging free radicals, thereby preventing them from damaging biological molecules. Vitamin C is essential for various physiological functions such as the synthesis of collagen and neurotransmitters, and it is a major antioxidant. Although vitamin C is widely used in cosmetic and therapeutic products, it easily undergoes oxidation by air, pH, temperature, and UV light, thereby decreasing its potency as an antioxidant and reducing the shelf-life of products containing vitamin C. To overcome this drawback, we developed Aptamin C®, an innovative single-strand DNA aptamer that maximizes the antioxidant efficacy of vitamin C by binding to its reduced form and delaying its oxidation. To investigate the effect of Aptamin C® and vitamin C complex on human skin, we performed both in vitro and clinical tests. We observed that the Aptamin C® and vitamin C complex had effective ROS-scavenging and anti-inflammatory effects.

**Keywords:** Aptamin C®; Vitamin C; Keratinocyte; DNA aptamer; Human skin

**Abbreviations:** ROS: Reactive Oxygen Species; AA: Ascorbic Acid; LPS: Lipopolysaccharide; DMSO: Dexamethasone and Dimethyl Sulfoxide; DMEM: Dulbecco's Modified Eagle Medium; FBS: Fetal Bovine Serum; NO: Nitric Oxide; CAA: Cellular Antioxidant Assay.

## Introduction

A variety of biochemical reactions such as photosynthesis and respiration in organelles generate reactive oxygen species (ROS) [1-4]. Oxidative stress conditions occur when ROS levels exceed cell defense mechanisms [5]. Oxidative stress directly impacts various diseases, especially skin diseases [6-16]. Besides, oxidative stress and inflammation are closely related. Several studies have shown that inflammation around the upper third of hair follicles suggests that inflammation has a pathogenic role in androgenic alopecia [17-21].

Biological antioxidants are present in low concentrations compared with an oxidizable substrate and substantially delay or prevent substrate oxidation [22]. The human body contains various antioxidants that prevent oxidative stress. Antioxidants such as vitamin C, vitamin E, coenzyme Q10, and polyphenolic compounds protect against oxidative stress-related diseases [23-25]. Vitamin C, also known as ascorbic acid (AA), is one of the many antioxidants that plays an important role in maintaining skin health. Normal skin cells contain higher concentrations of vitamin C than the blood. Vitamin C has roughly four roles in the skin. First, vitamin C is an extremely potent antioxidant that effectively counters skin damage-generated ROS. Second, vitamin C stimulates collagen synthesis by skin fibroblasts and stabilizes it. Third, vitamin C promotes keratinocyte differentiation, enhancing the barrier function. Fourth, vitamin C decreases melanin synthesis, which prevents hyperpigmentation of the skin

[26]. However, vitamin C quickly loses its function due to its antioxidant properties.

To slow down the oxidation of vitamin C, we developed Aptamin C®, a DNA aptamer that binds to vitamin C and inhibits its oxidation, through SELEX [27]. Here, we report that Aptamin C® increases cell proliferation and confirm its positive effect on inflammation in vitro.

## Materials and methods

### Chemicals

2′,7′-dichlorofluorescin diacetate (DCFH-DA), AA, lipopolysaccharide (LPS), dexamethasone, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Hydrogen peroxide ($H_2O_2$) was purchased from Wako. Aptamin C® was synthesized at IDT. Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, and distilled water were purchased from Gibco. 20X PBS was purchased from biosesang and diluted in gibco distilled water to obtain 1X PBS.

The nitric oxide (NO) detection kit was purchased from Intron. The Tumor Necrosis Factor alpha (TNF-α) ELISA kit was purchased from R&D system.

### HaCaT and RAW264.7 Cell Culture

HaCaT and RAW264.7 cells were grown in a growth medium (DMEM supplemented with 5% FBS and 1% penicillin–streptomycin) and maintained at 37°C and 5% $CO_2$. Cells between passages 1 and 10 were used in this study.

### Cellular Antioxidant Assay (CAA) for ROS Production Detection

Human epithelial cells, HaCaT, were seeded at a density of $2.5 \times 10^4$ /well in a 96-well microplate with 100 μL of growth medium/well. We used only the inner wells of the plates because the results from the outer wells showed greater variation. 24 hours after seeding, the growth medium was removed and the wells were washed with PBS. All wells were treated with 100 μL of 10 μM DCFH-DA dissolved in PBS, wrapped in foil, and incubated for 1 h at 37°C under $CO_2$ atmosphere. 10 mM DCFH-DA stock was dissolved in DMSO. Then, all wells were washed with PBS and treated three times with 50 μL of 100 μM $H_2O_2$ plus 50 μL of AA with or without Aptamin C® for 1 h at 37°C under $CO_2$ atmosphere. After incubation, the 96-well microplate was read with a Synergy H1 Hybrid Multi-Mode Reader (BioTek Instruments, Inc.). Wavelengths: excitation 485 nm, emission 535 nm. The plate included triplicate control and blank wells: blank wells contained cells treated with DCFH-DA dye and 10% FBS without $H_2O_2$; control wells contained cells treated with DCFH-DA dye and 10% FBS with $H_2O_2$.

Oxidative conditions: 10 μM AA was mixed with Aptamin C® (100:1, mass ratio) at room temperature for 10 minutes, then 30 μM $H_2O_2$ was added and the reaction mixture was placed at 37°C for 1 h.

Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements (2007)

### High-Performance Liquid Chromatography (HPLC) Analysis

To quantify AA by HPLC, we followed the general test method of the Korean Pharmacopoeia. The AA solution was filtered with a 0.2 mm PVDF filter and 10 μL was injected in a reverse phase C-18 HPLC column (Agilent, USA) (4. 6 × 250 mm, 5 μm diameter). The mobile phase consisted of sodium-1-hexanesulfonate, glacial acetic acid, and methanol with a flow rate of 1 ml/min. The sample was detected at 254 nm on an Agilent 1260 Infinity II multiple wavelength detector. The retention time for standard AA was recorded as 3.41 min.

Oxidative conditions: 10 μM AA was mixed with Aptamin C® (100:1, mass ratio) at room temperature for 10 minutes, then 30 μM H2O2 was added and the reaction mixture was placed at 37°C for 1 h.

Antioxidant phytochemicals in cabbage (Brassica oleracea L. var. capitata) (2006).

### Statistical analyses

All results are presented as mean ± SD. Pairs of means were compared using unpaired Student's t-test. Multiple means were compared by ANOVA followed by Fisher's least significant difference test. Differences were considered significant when $p < 0.05$ and $p < 0.001$.

### Measurement of NO Production and TNF-α Production

The RAW264.7 cells were seeded into 24-well plates ($1.6 \times 10^5$ cells/well) and incubated for 16 h. The cells were then treated with various concentrations of Aptamin C® and vitamin C in the presence or absence of 1 μg/mL LPS. After 24 h, 100 μL of the supernatants and 100 μL of the NO detection reagent were added to a 96-well plate and reacted for 10 min in the absence of light. The absorbance at 540 nm was measured with a microplate reader. The value was corrected by total protein value. Dexamethasone (7.84 μg/mL) was used as a positive control.

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## Results

### *In vitro* ROS inhibition ability using the CAA assay

The CAA assay enables quantifying ROS via the production of DCF. DCFDA penetrates the cell membrane and cellular esterases convert it to non-fluorescent DCF. ROS then oxidizes non-fluorescent DCF, which becomes fluorescent DCF.

To determine whether AA inhibits ROS production, we performed the CAA assay on HaCaT cells. Under oxidative conditions, the control cells generated ROS (Figure 1A). AA inhibited ROS production in a dose-dependent manner. Moreover, we confirmed that low concentrations of the oxidized form of AA, dehydroascorbic acid, did not inhibit ROS production (Figure 1B).
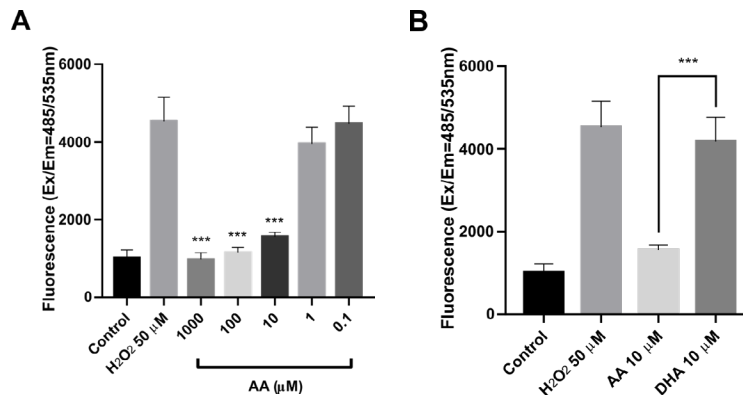


**Figure 1:** ROS production in HaCaT cells. (A) ROS production in the presence of various concentrations of ascorbic acid. (B) ROS production in the presence of ascorbic acid or dehydroascorbic acid. Values are presented as mean ± SD (n = 3). ***, P-value < 0.001 (compared to other groups and as indicated).

### Antioxidant Effect of AA with/without Aptamin C® under Oxidative Conditions

To evaluate the stabilization effect of AA by Aptamin C®, we treated AA with an oxidant ($H_2O_2$) with or without Aptamin C® and quantified it by HPLC. Aptamin C® did not change the retention time of AA (Figure 2A). With Aptamin C®, the stabilization effect of AA was about 36% higher than without Aptamin C® (Figure 2B).

We also conducted CAA assay in HaCaT cell line to evaluate the stabilization effect of AA in cells with or without Aptamin C®. Under non-oxidative conditions, Aptamin C® did not affect the ROS inhibitory ability of AA. Whereas, under oxidative conditions, Aptamin C® enhanced the inhibition of ROS production (Figure 2C).
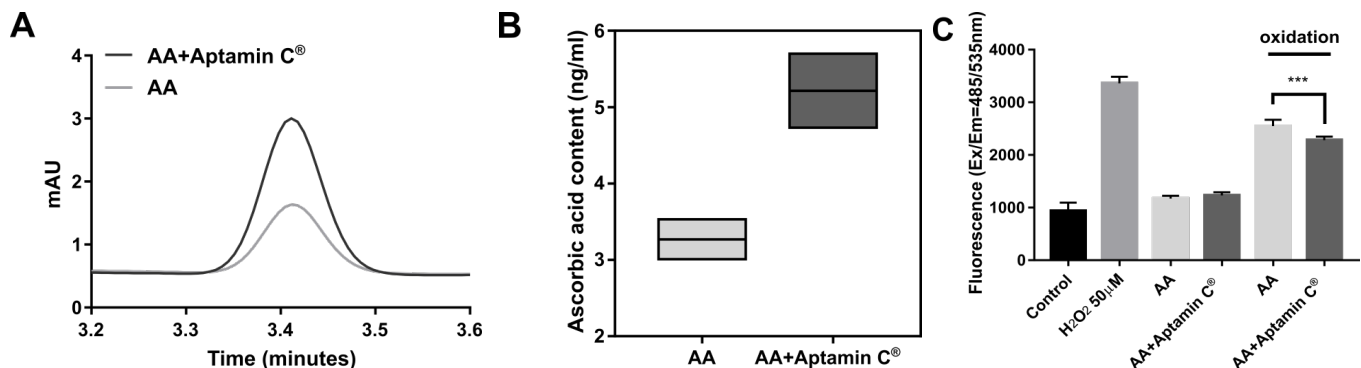


**Figure 2:** Stability of ascorbic acid with and without Aptamin C® under oxidative conditions. (A) HPLC chromatograms of ascorbic acid with or without Aptamin C®. (B) Ascorbic acid amount with or without Aptamin C®, as determined by HPLC. (C) ROS production by HaCaT cells in the presence of ascorbic acid, with or without Aptamin C®, using the CAA assay. The values are presented as mean ± SD (n = 3).
***, P-value < 0.001 (compared to other groups and as indicated).

### Effect on the Inflammatory Response Identified through Analysis of NO Production and TNF-α Production

NO production was significantly reduced in a dose-dependent manner, with 4.37%, 11.12%, 15.05%, 23.47%, and 24.19% at concentrations of 0.01 μg/mL + 0.5 μg/mL, 0.1 μg/mL + 5 μg/mL, 0.25 μg/mL + 12.5 μg/mL, 0.5 μg/mL + 25 μg/mL, and 1 μg/mL + 50 μg/mL ($p < 0.05$, Table 1, Figure 3A).

TNF-α production was significantly reduced in a dose-dependent manner, with 2.99%, 9.41%, 10.04%, 19.79%, and 23.54% at concentrations of 0.01 μg/mL + 0.5 μg/mL, 0.1 μg/mL + 5 μg/mL, 0.25 μg/mL + 12.5 μg/mL, 0.5 μg/mL + 25 μg/mL, and 1 μg/mL + 50 μg/mL ($p < 0.05$, Table 2, Figure 3B).
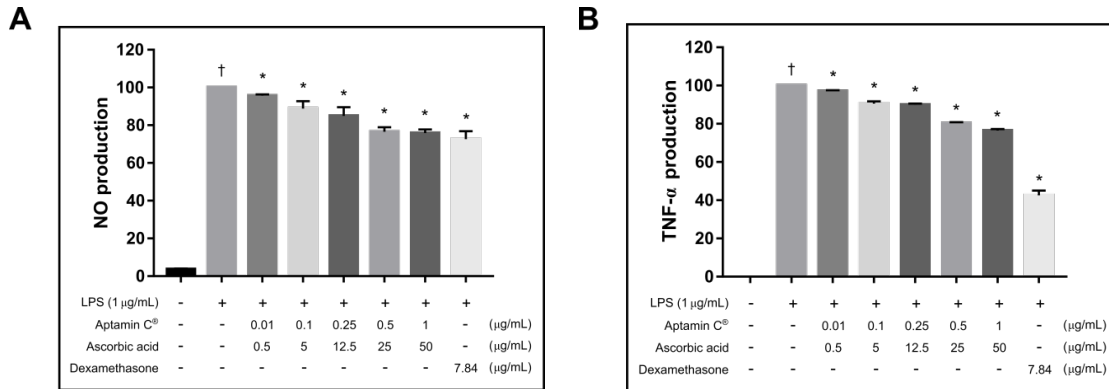


**Figure 3:** Anti-inflammatory effect of ascorbic acid with and without Aptamin C®. (A) NO production in RAW 264.7 cells after treatment. (B) TNF-α production in RAW 264.7 cells after treatment. The data are expressed as % of control. The values are presented as mean ± SD (n = 3).
† P-value < 0.05 (Ascorbic acid with Aptamin C® vs control without LPS).
* P-value < 0.05 (Ascorbic acid with Aptamin C® vs control with LPS).

| Material | LPS | Dose (μg/mL) | | Mean (%) | SD | p-value[2] | Change from solvent control (%) |
|---|---|---|---|---|---|---|---|
| | | Aptamin C® | Ascorbic acid | | | | |
| Ascorbic acid with Aptamin C® | - | 0 | 0 | 3.64 | 0 | - | - |
| | + | 0 | 0 | 100 | 0 | 0.000† | - |
| | | 0.01 | 0.5 | 95.63 | 1 | 0.000* | 4.37▼ |
| | | 0.1 | 5 | 88.88 | 4 | 0.008* | 11.12▼ |
| | | 0.25 | 12.5 | 84.95 | 5 | 0.005* | 15.05▼ |
| | | 0.5 | 25 | 76.53 | 2 | 0.000* | 23.47▼ |
| | | 1 | 50 | 75.81 | 2 | 0.000* | 24.19▼ |
| Dexamethasone[1] | | 7.84 | | 72.68 | 4 | 0.000* | 27.32▼ |
| [1] Positive control | | | | | | | |
| [2] Significant difference at $p<0.05$ compared to solvent control without LPS(†) and with LPS(*) | | | | | | | |

**Table 1:** NO production in RAW 264.7 cells after treatment (n=3).

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| Material | LPS | Dose (µg/mL) | | Mean (%) | SD | *p*-value[2] | Change from solvent control (%) |
| | | Aptamin C® | Ascorbic acid | | | | |
|---|---|---|---|---|---|---|---|
| Ascorbic acid with Aptamin C® | - | 0 | 0 | - | 0 | - | - |
| | + | 0 | 0 | 100 | 0 | 0.000† | - |
| | | 0.01 | 0.5 | 97.01 | 1 | 0.001* | 2.99▼ |
| | | 0.1 | 5 | 90.59 | 1 | 0.000* | 9.41▼ |
| | | 0.25 | 12.5 | 89.96 | 1 | 0.000* | 10.04▼ |
| | | 0.5 | 25 | 80.21 | 1 | 0.000* | 19.79▼ |
| | | 1 | 50 | 76.46 | 1 | 0.000* | 23.54▼ |
| Dexamethasone[1] | | 7.84 | | 42.56 | 3 | 0.000* | 57.44▼ |
| [1] Positive control | | | | | | | |
| [2] Significant difference at *p*<0.05 compared to solvent control without LPS(†) and with LPS(*) | | | | | | | |

**Table 2:** TNF-α production in RAW 264.7 cells after treatment (n=3).

## Conclusion

Aptamin C® delays the oxidation of vitamin C and maximizes its antioxidant properties, suggesting that the Aptamin C® and vitamin C complex is effective against ROS and ROS-associated diseases. In this study, we demonstrated that the Aptamin C® and vitamin C complex removes intracellular ROS. Besides, based on previous studies showing that ROS causes inflammation, we used HaCaT cells to confirm that Aptamin C® and vitamin C have anti-inflammatory effects. Overall, our results suggest that Aptamin C® could be used as a treatment for oxidative stress-associated diseases, particularly in skin and scalp care.

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