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Assessment of Alpha-Tocopheryl Acetate and Metformin Hydrochloride as Independent Agents on Human Dermal Fibroblast Viability: Findings from MTT Assay

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Abstract

Background: Alpha-tocopheryl acetate (Vitamin E) and metformin hydrochloride have been tested as anti-aging compounds at various concentrations. This study aimed to identify the most effective concentrations of alpha-tocopheryl acetate and metformin hydrochloride in promoting the viability of human dermal fibroblasts (HDFs), a primary cell type in skin aging research.

Methods: HDFs were isolated using a mechanical isolation method and cultured under standard conditions. Cells were treated with varying concentrations of alpha-tocopheryl acetate and metformin hydrochloride as independents agents. After 48 hours of incubation, cell viability was measured using the MTT assay.

Results: Alpha-tocopheryl acetate had the highest HDF cell viability (107%) at a concentration of 50 µM. Metformin hydrochloride had the maximum HDF cell viability (158%) at 5 µM. However, the viability response varied across different concentrations for both agents, indicating that optimal dosing was essential for maximizing their effectiveness.

Conclusions: Alpha-tocopheryl acetate at 50 µM and metformin hydrochloride at 50 µM yield the highest viability of HDFs in vitro. These findings suggest potential roles for both agents in anti-aging skin therapies. Further research is recommended to explore their mechanisms of action and to optimize dosing strategies for clinical applications.

Keywords: Alpha-tocopheryl acetate, cell viability, metformin hydrocholride, MTT assay, vitamin E

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Introduction

The aging process is a natural phenomenon that occurs with increasing age. Aging can be defined as a decline in the physiological functions of organs needed for survival over time, including the skin. Skin aging is a complex biological process, influenced by a combination of intrinsic and extrinsic factors. There are several changes in the skin occurred due to aging.[1,2] A crucial component in skin aging is the fibroblast, a cell responsible for maintaining skin structure by producing collagen and other extracellular matrix components.[3] The ability of fibroblasts to undergo cellular contraction is pivotal in the aging process, particularly in wound healing, inflammatory responses, wrinkle formation, scarring, and structural changes in the extracellular matrix.[4]

Anti-aging medicine is an emerging and rapidly evolving field focused on enhancing cell regeneration, inducing autophagy, and modulating gene activity through epigenetic mechanisms. Various compounds, both natural and synthetic, are being investigated for their ability to mitigate age-related processes and potentially extend lifespan or improve skin health.[5]

Alpha-tocopheryl acetate (Vitamin E) and metformin are two compounds with potential

anti-aging agents that have been widely studied. Vitamin E is a fat-soluble antioxidant that protects cells from oxidative damage and has been used in dermatology for over 50 years. The vitamin E group consists of four tocopherols and four tocotrienols.[6,7] Vitamin E provides photoprotective effects by acting as a free radical scavenger, thus reducing the detrimental impacts of solar radiation.[6] Free radicals are molecules with one or more unpaired electrons, rendering them highly reactive. These molecules can damage essential cellular components such as DNA, lipids, and proteins, leading to oxidative stress. Vitamin E mitigates this by donating electrons to free radicals, effectively interrupting chain reactions that damage cells.[8]

Recently, Metformin a well-established anti-diabetic drug approved by the U.S. Food and Drug Administration (FDA) since 1994 for the treatment of type 2 diabetes, has gained attention for its potential role in anti-aging and anti-cancer therapies.[9–11] Beyond glucose control, long-term use of Metformin has been associated with improved clinical outcomes, delayed onset of age-related diseases, and enhanced overall health.[12] Metformin has been shown to improve cell viability in skin fibroblasts subjected to photoaging or high-glucose environments. Its anti-aging mechanisms include activating AMP-activated protein kinase (AMPK), a key enzyme regulating cellular energy homeostasis. AMPK activation increases the production of sirtuin 1 (SIRT1), a protein involved in metabolism and cellular longevity. As AMPK sensitivity declines with age, metformin, as an AMPK activator, represents a promising therapeutic intervention to delay aging processes.[13]

Aging is also associated with mitochondrial dysfunction, leading to increased production of reactive oxygen species (ROS).[14] Metformin inhibits mitochondrial complex I in the electron transport chain, reducing ROS production by disrupting electron flow and minimizing oxidative stress.[11]

Despite promising evidence, the effective concentration levels of vitamin E and Metformin as anti-aging agents in in vitro studies remains highly variable. For example, in a study investigating tocotrienol-rich fractions (a component of the vitamin E family) used doses ranging from 30 to 240 µg/mL to evaluate effects on human fibroblast growth.[15] Another study comparing the effects of γ-tocotrienol on young and aged fibroblasts utilized 40 µM and 80 µM concentrations, reporting increased cell death at higher doses, though without specifying an optimal concentration.[16]

Similarly, a study on metformin's effects on human gingival fibroblasts (HGF) reported that concentrations of 0.5 mM, 1 mM, and 2 mM exhibited low cytotoxicity, with 0.5 mM yielding the highest cell viability.[17] Another study reported that a 100 mM concentration produced the lowest cytotoxic effects in fibroblasts.[18] These inconsistencies highlight the need for further research to establish optimal concentration ranges for safe and effective use. Therefore, this study aimed to determine the most efficacious dosage or concentration of Vitamin E and metformin hydrochloride using the MTT assay method to assess human dermal fibroblast viability. The findings may provide preliminary insights into the potential anti-aging effects and contribute to the development of optimized concentrations for future therapeutic or cosmetic applications.

Methods

The experimental study was conducted using an in vitro human dermal fibroblast (HDF) model conducted at the Cell Culture and Cytogenic Laboratory, Faculty of Medicine, Universitas Padjadjaran. with ethical approval from the Research Ethics Committee of the Universitas Padjadjaran, Indonesia (No. 1115/UN6.KEP/EC/2023).

Primary cultures of HDF were developed as a model system to mimic human skin. The concentration range for metformin (0.005 to 250 µM) was selected based on previous studies that identified 100 µM as the most effective dose in HDF models.[19] Vitamin E concentrations typically range from 5 µM to 80 µM, depending on the specific type used.[15,16]

The research samples consisted of an HDF culture isolated from the preputium tissue of male children under 16 years old, taken from the Seno Medika Khitan Clinic after consent. In brief, the isolation process was performed using a mechanical separation technique. The preputium tissue was initially washed with sterile PBS, followed by iodine solution, and subsequently rinsed with PBS three times. The dermal layer was separated from the epidermal and adipose layers, washed thoroughly, and minced into small fragments approximately 1–2 mm^2 in size. The tissue fragments were placed into a culture flask containing low glucose-DMEM supplemented with penicillin-streptomycin (100 mg/mL) and incubated at 37°C with 5% CO_2. The culture medium was

replaced every two days.

Cell growth was monitored daily. ImageJ software was employed to measure the area covered by HDF cells in the culture flasks during the growth observation phase. Cell-covered areas were selected using the paintbrush tool, and the polygon selection tool was utilized to calculate the area based on a 0.25 mm bar scale. Once the HDF cells reached approximately 80% confluence, cells were harvested by discarding the medium, rinsing the cells with PBS, and adding 2 mL of trypsin-EDTA (0.25% trypsin). The cells were incubated for approximately three minutes at 37°C with 5% CO_2 until detachment was observed. The reaction was halted by adding 5 mL of complete DMEM medium. The cell suspension was centrifuged at 1,500 g for five minutes, and the resulting cell pellet was resuspended in fresh medium and transferred to 96-well plates at a density of 10,000 cells per well for subsequent experiments.

After overnight incubation, cells were treated with alpha-tocopheryl acetate (Sigma Aldrich, No. 258024) and metformin hydrochloride (Tocris Bioscience, Batch No. 1115-70-4), both dissolved in DMSO and diluted in DMEM medium. The concentrations of alpha-tocopheryl acetate were 250, 100, 50, 5, 0.05, and 0.005 µM, and for metformin hydrochloride were 250, 200, 50, 5, 0.05, and 0.005 µM, administered independently. Each treatment was performed in triplicate. The cultures were incubated for 48 hours.

The MTT assay was used to evaluate HDF cell viability. After 48 hours, the treatment medium was discarded and cells were washed with PBS to eliminate residual compounds. Subsequently, 10 µL of MTT reagent (5 mg/mL) and 90 µL of DMEM were added to each well and incubated at 37°C for three hours. After incubation, 100 µL of DMSO was added to dissolve the formazan crystals. The ratio of MTT to DMSO was 1:1. The absorbance was measured using a microplate reader at a wavelength of 595 nm.[20]

Data analysis was performed using Microsoft Excel and GraphPad. Absorbance data from the MTT assay were converted to percentages of viable cells. The mean value of triplicate measurements was used for further analysis.

Results

Primary culture of HDF was successfully conducted prior to testing with vitamin E and Metformin. The cultured cells exhibited a typical spindle-shaped morphology characteristic of fibroblast cells. These spindle-

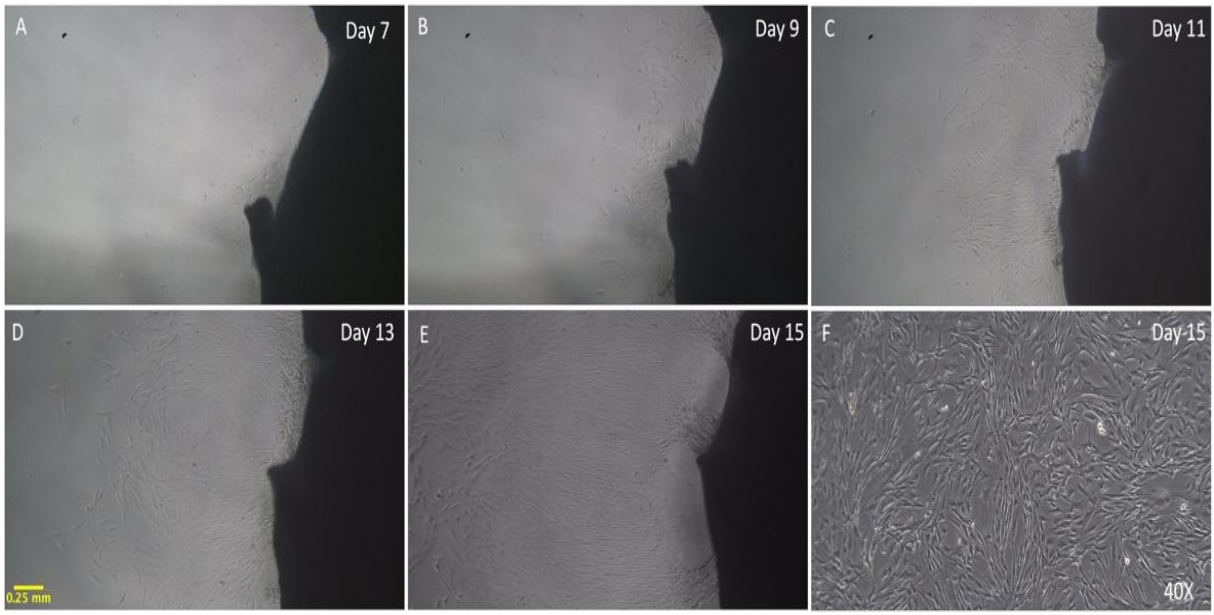

Figure 1 Primary Culture of Human Dermal Fibroblast (HDF) Cells

Note: Inverted microscopy images showing the growth progression of fibroblast cells derived from skin biopsies using the explant method. The images depict fibroblast cultures on (A) day 7, (B) day 9, (C) day 11, (D) day 13, and (E) day 15, (F) 80% confluence on day 15. The cells display typical spindle-shaped fibroblast morphology and adhere to the surface of the tissue culture flask. Images were captured using an inverted microscope at 40× magnification; scale bar=0.25 mm.

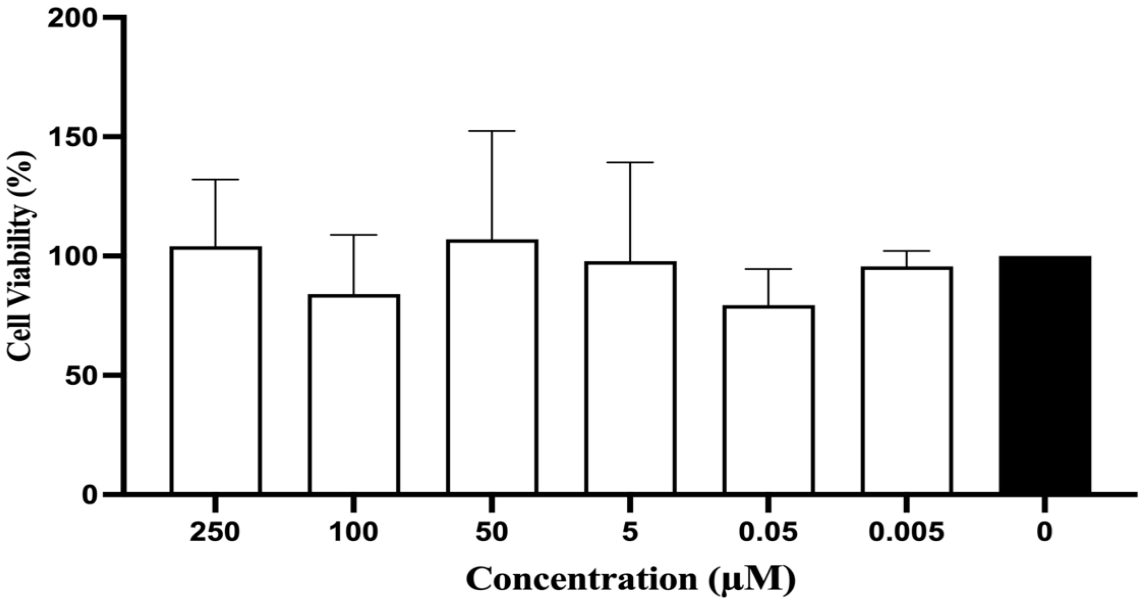

Figure 2 Effect of Vitamin E on HDF Cell Viability

Note: Cell viability was assessed using the MTT assay after a 48-hour incubation of human dermal fibroblasts (HDF) with various concentrations of vitamin E. Error bars represent the mean ± standard deviation from three independent experiments

shaped cells began to show active growth from the first day of culture, with a steady increase in proliferation observed daily until the cells reached approximately 80% confluence on day fifteen (Figure 1).

The area covered by HDF cells was measured using ImageJ software from day seven to day fifteen. The analysis revealed that the area covered by fibroblasts increased substantially during this period. Between the seventh and ninth day of culture, the area expanded by 643.53%, followed by an increase of 165.82% from the ninth to the eleventh day. From the eleventh to the thirteenth day, the growth rate continued with an increase of 70.83%, and finally, between the thirteenth and fifteenth day, the area expanded by 70.98%.

The MTT assay was used to assess HDF cell viability after treatment with varying concentrations of vitamin E and Metformin. The results were expressed as the percentage of viable HDF cells compared to the untreated control group.

The administration of vitamin E at different concentrations, ranging from 0.005 µM to 250 µM, yielded varied effects on cell viability. At a concentration of 0.005 µM, cell viability was slightly reduced to 95.7% compared to the control. A further decrease was observed at 0.05 µM, with viability dropping to 79.5%. However, as the concentration increased to 5 µM, cell viability improved significantly to 97.9%, and further enhancement was observed at 50 µM, reaching 107%. At 100 µM, a notable decrease occurred, with viability dropping to 84.1%. Interestingly, at the highest concentration of 250 µM, cell viability increased again to 104.1%. The control group-maintained cell viability at 100%. Based on these findings, the concentration of 50 µM emerged as the most effective dose of vitamin E for enhancing HDF cell viability in this experiment (Figure 2).

A similar trend was observed with Metformin administration across the concentration range of 0.005 µM to 250 µM. At the lowest concentration of 0.005 µM, cell viability increased slightly to 103.6%. A substantial rise was observed at 0.05 µM, where cell viability reached 147.4%. The highest enhancement was recorded at 5 µM, with viability increasing to 158%. However, a significant decline occurred at 50 µM, where viability decreased to 98.8%. The viability improved again at 100 µM, reaching 129.5%, but experienced a slight reduction to 122% at the highest concentration of 250 µM. The control group maintained 100% viability. These results indicated that a concentration of 5 µM Metformin was the most effective dose for enhancing HDF cell viability in this study (Figure 3).

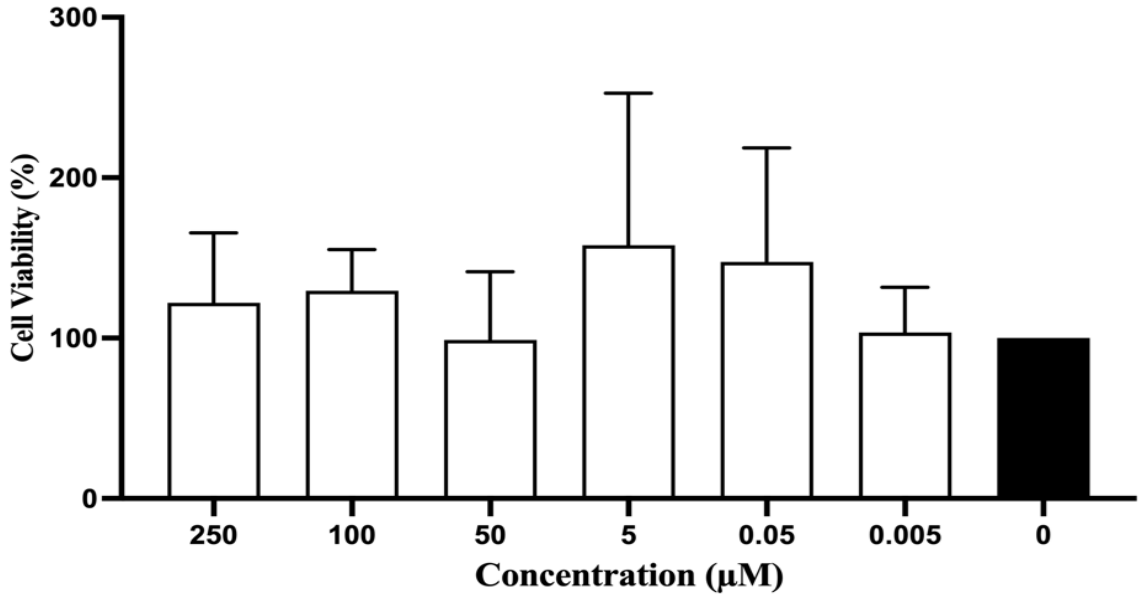

Figure 3 Effect of Metformin on HDF Cell Viability

Note: Cell viability was assessed using the MTT assay after a 48-hour incubation of human dermal fibroblasts (HDF) with various concentrations of vitamin E. Error bars represent the mean ± standard deviation from three independent experiments

Discussion

This study primarily emphasized identifying safe dose ranges for vitamin E and metformin as a crucial initial step in understanding their biological activities. Evaluation of optimal doses is essential for determining the safety and feasibility of using these bioactive compounds in subsequent anti-aging research.

This study successfully cultured HDF from preputium samples. HDF cells play an essential role in synthesizing collagen and elastin, proteins responsible for skin firmness and elasticity. The general characteristics of HDF include rapid proliferation, responsiveness to hormonal and growth factor stimuli, and the ability to synthesize various components of the extracellular matrix.[21,22] HDF cells are widely used in anti-aging research in vitro due to their representativeness in simulating human skin.

The ideal concentrations of vitamin E and metformin, based on this study, were 50 µM for vitamin E, which enhanced cell viability to 107%, and 5 µM for metformin, which showed the highest cell viability at 158%.Several studies evaluating fibroblast viability with vitamin E and metformin across various concentrations have reported variability in optimal doses.[15–20,23–26] The optimal concentration of γ-tocotrienol to enhance viability in MRC-5 human lung fibroblasts was reported at 150 µM, while higher concentrations reduced cell viability.20 Variations in optimal doses may be influenced by the type of vitamin E used (tocopherol vs. tocotrienol) and the presence of other active ingredients affecting cell response.[6,7,16]

Interestingly, a study using human gingival fibroblast cells (HGF1-PI) reported no significant differences in cell viability at 100 and 1,000 µM α-tocopherol, but significant increases were observed at 5,000 and 10,000 µM.[23] Another study using human diploid fibroblasts showed an optimal concentration of tocotrienol-rich fraction (TRF) at 50 µg/mL,[24] consistent with our findings on primary foreskin-derived HDF.

In our study, metformin showed peak viability at a low concentration of 5 µM, aligned with earlier study using human gingival fibroblasts (HGFs).[17] Conversely, cell viability declined at 50 µM, similar to findings in fibroblast-like synoviocytes (FLSs).[25] Interestingly, a secondary increase in viability was observed at 100 µM in our study. Extending the concentration to 250 µM still maintained relatively high viability (122%). In contrast, other studies reported that concentrations of 2,000 and 4,000 µM significantly inhibited fibroblast proliferation.[26,28] These discrepancies underline the importance of

Table 1 Summary of Studies Evaluating the Effects of Vitamin E and Metformin On Fibroblast Cell Viability At Various Concentrations

Test Compound	Object	Range of Tested Concentrations (µM)	Results	Reference Number
γ-tocotrienol and δ-tocotrienol	MRC-5 Human Lung Fibroblasts	150, 200, 300	Highest cell viability at 150 µM; viability decreases at concentrations above 300 µM.	20
α -tocopherol	Human Gingival Fibroblast Cell Line HGF1-PI	100, 1,000, 5,000, 10,000	No significant difference at 100 and 1000 µM; significant increase at 5000 and 10,000 µM.	23
Alpha- tocopheryl phosphate (α-TP)	Human Gingival Fibroblasts (HGFs)	0.05, 0.5, 5, 50, 500, 5,000	Well tolerated up to 5,000 µM; highest viability at 500 µM; significant drop in viability at the highest dose.	27
Tocotrienol Rich Fraction (TRF)	Human Diploid Fibroblasts	0, 30, 50, 70, 100, 300, 500, 700 µg/mL	Increased viability at 30, 50, and 100 µg/mL; optimal viability at 50 µg/mL.	24
Metformin	Human Gingival Fibroblasts (HGFs)	0.5, 1 , 2	Highest cell viability at 0.5 µM.	17
Metformin	Fibroblast-like Synoviocytes (FLSs)	0 , 5 , 20 , 40 , 60	Significant inhibition at 60 µM (55%); viability decreases in a dose- and time-dependent manner.	25
Metformin	Human Dermal Fibroblasts	0, 0.25, 0.5, 1, 2, 4	Treatment with 1 µM Metformin increases cell viability.	28
Metformin	Human Gingival Fibroblasts (HGFs)	100, 500, 1,000, 2,000, 4,000	0.1, 0.5, and 1 mM concentrations are not toxic; 2 and 4 mM significantly inhibit cell proliferation.	26

dose precision when considering therapeutic applications of Metformin in skin fibroblasts.

Previous studies indicated that vitamin E began enhancing fibroblast viability at 0.05 µM and that significant increases occurred at very high concentrations, such as 10,000 µM.[23,27] Despite the wide range of dosages tested, these studies consistently suggest that vitamin E is generally safe for fibroblasts.

For metformin, the lowest tested concentration in literature was 0.25 µM, while the highest reached 4000 µM.[26,28] The MTT assay confirmed that 1 µM metformin increased viability, while doses of 100 to 1,000 µM did not exhibit toxicity to human gingival fibroblasts.[17,26] However, at 2,000 to 4,000 µM, proliferation was significantly inhibited, suggesting cytotoxicity at these levels.

Methodological variations across studies, including solvent use, culture conditions, and fibroblast source, likely influence the determination of optimal concentrations.[27] Furthermore, the choice of fibroblast type, whether HDFs or other lines, significantly affects outcomes. Our experiment used primary fibroblast cells derived from human foreskin tissue likely offers more realistic data on physiological responses, but donor variability could still influence results, necessitating further studies on broader

human dermal fibroblast populations.

This study has several limitations, including a focus limited to cell viability without exploring the underlying molecular mechanisms of action, which could provide a more comprehensive understanding of these compound effects. Additionally, method variations can influence the result and even the definition of optimal concentrations, leading to inconsistent findings, and the results may not directly apply to all contexts.

In conclusion, this study demonstrate that 50µM alpha-tocopheryl acetate and 50µM Metformin are the optimal doses for enhancing HDF viability. The literature review highlights significant variability in effective dosing across studies, driven by differences in experimental design, cell types, and donor variability. Further research is needed to validate these doses under different conditions and to investigate the molecular mechanisms underlying their effects on skin aging and cell viability.

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