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Protection of Radiation-Induced DNA Damage by Functional Cosmeceutical Poly-Gamma-Glutamate

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This study compared the radioprotective effects of high-molecular-weight poly-gamma-glutamate (γ-PGA, average molecular mass 3,000 kDa) and a reduced form of glutathione (GSH, a known radioprotector) on calf thymus DNA damage. The radiation-induced DNA damage was measured on the basis of the decreased fluorescence intensity after binding the DNA with ethidium bromide. All the experiments used ^{60}Co gamma radiation at 1,252 Gy, representing 50% DNA damage. When increasing the concentration of γ-PGA from 0.33 to 1.65 μM, the DNA protection from radiation-induced damage also increased, with a maximum of 87% protection. Meanwhile, the maximal DNA protection when increasing the concentration of GSH was only 70%. Therefore, γ-PGA exhibited significant radioprotective effects against gamma irradiation.

Keywords: Poly-gamma-glutamate, radioprotective material, gamma radiation, DNA damage, DNA protection

Introduction

With the Fukushima nuclear accident in Japan in 2011 and increasing use of nuclear facilities and radiation therapies, public concern about radiation exposure has also strengthened interest in radioprotective agents [1, 2]. These agents prevent or reduce radiation exposure by removing or inactivating the free radicals produced by radiation [3]. In experimental and clinical studies, radioprotective agents have already been introduced as chemical or molecular therapeutic agents, such as amifostine, antioxidants (glutathione, genistein, *etc*.), palifermin, and cysteine [4, 5].

Radiation-induced DNA damage includes both direct and indirect actions. Direct action is when the radiation energy is directly absorbed by organic molecules (DNA, *etc*.) and causes changes (point mutations, DNA strand breaks, DNA crosslinks, and chromosome aberrations) [6, 7]. If not repaired, these changes can result in cellular damage or death [5]. Indirect action is when the water in cells is ionized and produces free radicals and peroxides that interact with the surrounding cellular components, causing metabolic disorders [6, 7]. All organisms consist of 70–85% water. Thus, exposing organisms or cells to radiation results in primary ionization of the water molecules, known as radiolysis, and the formation of free radicals, such as hydroxyl radicals, sub-excitation electrons, hydrogen peroxide, hydrogen atoms, hydrated electrons, and superoxides [8]. In particular, hydroxyl radicals produce the most DNA damage [9, 10]. Additionally, the DNA damage caused by free radicals generated by radiation includes biologically important cellular lesions (single-strand breaks, double-strand breaks, multiply damaged sites, base modifications, and adduct formation) [11, 12]. Moreover, when an organism is exposed to radiation, both direct and indirect actions are thought to occur at the same time, where direct action accounts for about 25%, while the remaining 75% is attributed to indirect action. Thus, the DNA damage caused by the free radicals and peroxides generated by the ionization of water molecules due to the indirect action of radiation is much more significant than the direct action of radiation, as the portion of intracellular DNA is very small [13].

Radioprotective agents are defined as substances that are capable of modifying harmful biological responses to radiation [14]. The reduced form of glutathione (GSH) is the tripeptide thiol, which consists of L-glutamine, cysteine,

and glycine and is an endogenous scavenger of antioxidants. GSH acts as a hydrogen donor to hydroxyl radicals to prevent DNA strand breaks [15, 16]. Amifostine is the only radioprotective agent that has been approved for clinical use by the US FDA. Amifostine is a thiol derivative that acts as a free radical scavenger for radiation protection. However, the side effects of amifostine include acute hypotension, nausea, vomiting, and allergic reactions [17, 18]. Therefore, there is an important need to identify more effective radiation protection materials with fewer side effects.

Poly-gamma-glutamic acid (γ-PGA) is an anionic polypeptide in which D- and/or L-glutamate is polymerized via γ-amide linkages between the α-amino and γ-carboxylic acid functional groups [19, 20]. γ-PGA is also a very promising biodegradable polymer produced by *Bacillus subtilis* (Chungkookjang) [21]. As a microorganism, *Bacillus subtilis* is generally regarded as safe (GRAS) and is widely consumed as a food ingredient [22]. Moreover, in terms of attractive properties, γ-PGA is water soluble, anionic, biodegradable, and edible, which has resulted in a variety of applications, including cosmetics/skin care, bone care, nanoparticle for drug delivery systems, hydrogels, immune-stimulating agents, and pharmaceuticals [23–25].

Accordingly, this study demonstrates the radioprotective functions of high-molecular-weight γ-PGA (average molecular mass 3,000 kDa). When increasing the concentration of γ-PGA, the fluorescence intensity of an EtBr–DNA solution increased when compared with the DNA-irradiated control, indicating protection against radiation damage [16]. Thus, the results indicate that γ-PGA is a radioprotective agent and potential functional cosmeceutical material against gamma irradiation.

Materials and Methods

Materials

Calf thymus DNA (CT-DNA), a reduced form of GSH, and D-glutamic acid were all purchased from Sigma-Aldrich (USA). Ethidium bromide (EtBr) was purchased from Amresco (USA). L-Glutamic acid was purchased from Samchun (Korea). A BPE buffer (6 mM $NaHPO_4$, 2 mM NaH_2PO_4, and 1 mM ethylenediaminetetraacetic acid disodium salt dehydrate, pH 7.0) was used for the experiments [26]. All other chemicals and reagents were of analytical grade.

Preparation of DNA Solution

The CT-DNA (20 mg) was dissolved in the BPE buffer (10 ml) and kept overnight in a refrigerator to obtain a homogenous DNA solution and avert thermal degradation [16]. A molar absorption coefficient of 6,600 M^{-1} cm^{-1} at 260 nm was estimated for the DNA concentration, which was expressed in base pairs using a spectrophotometer [27]. The concentration of the prepared stock CT-DNA solution was 6.86×10^{-3} (M) and the final concentration was 10^{-5} (M). Moreover, the 260/280 ratio of the CT-DNA was 1.8, indicating that the DNA was free of any contaminating proteins [16].

Preparation of GSH Solution

A stock solution of the reduced form of glutathione (GSH) at a concentration of 5.0×10^{-3} (M) was prepared in the BPE buffer, and then 10–60 μM of GSH was added to the CT-DNA to create a final GSH concentration of 10^{-5} M. CT-DNA damage was induced by gamma radiation to determine the damage protection by GSH [16].

Preparation of Ethidium Bromide Solution

A stock solution of 1.0×10^{-3} M ethidium bromide (EtBr) was dissolved in the BPE buffer, and 60 μM of the final EtBr concentration was added for maximal DNA binding [16].

Preparation of D/L-Glutamate Solution

1.36×10^{-1} M D-glutamate and 1.36×10^{-1} M L-glutamate were dissolved in the BPE buffer as stock solutions and titrated to pH 6.8, respectively. A D/L-glutamate solution was then prepared by mixing equal portions and titrated to pH 6.8.

Preparation of Poly-Gamma-Glutamate

The 3,000 kDa γ-PGA (BioLeaders Corporation, Korea) was dissolved in the BPE buffer to make a stock solution of 3.33×10^{-6} M and titrated to pH 6.8. The average molecular mass of γ-PGA is 3,000 kDa and its polydispersity is 4.6.

Gamma Irradiation

The gamma irradiation dose rate was 3,756 Gy/h up to a total dose of 3,756 Gy using a ^{60}Co gamma-irradiation facility (point source AECL, IR-79; MDS Nordion International Co. Ltd., Canada) at the Korea Atomic Energy Research Institute (Korea).

Fluorescence Spectrometry

The fluorescence emission intensity of the samples was measured using a FS-2 fluorescence spectrometer (Scinco, Japan). Radiolyzed DNA damage produced a decreased fluorescence binding intensity with EtBr. Thus, the samples including radioprotector agents showed an increased fluorescence intensity when compared with the samples without radioprotector agents. Three different samples containing 60 μM CT-DNA in 1.5 ml polypropylene tubes were exposed to gamma irradiation. One sample without a radioprotector and the other two samples with an added radioprotector (10–60 μM GSH and 0.33, 0.66, 0.99, 1.33, or 1.65 μM γ-PGA) in the BPE buffer were irradiated by the gamma ^{60}Co source using a total

dose of 3,756 Gy and 1,252 Gy. The fluorescence intensity analyses were then performed immediately. A 1 mM EtBr fluorophore solution was added to the different samples, which were then incubated for 30 min at 37°C for maximal binding with the CT-DNA. Thereafter, the fluorescence spectra were obtained by emission excitation at 500 nm and scanning from 510 to 800 nm.

Results

Estimation of Radiation-Induced Damage to Calf Thymus DNA

Free radicals generated by radiation cause DNA damage, resulting in a decreased fluorescence intensity due to reduced binding with EtBr-DNA. Thus, a decreased fluorescence intensity indicates DNA damage by radiation. Several forms of DNA damage can contribute to a decreased fluorescence intensity, including strand breaks, base liberation, and base oxidation [28].

The CT-DNA irradiated by the gamma source at a dose of 3,756 Gy/h up to a total dose of 3,756 Gy was bound with EtBr and the fluorescence emission spectra were measured at 624 nm. The control was DNA-EtBr exposed to 0 Gy radiation. The fluorescence intensity decreased gradually when increasing the radiation dose (Fig. 1). The residual quantity of double-strand DNA following radiation exposure was measured using a dose-effect curve (Fig. 2), where $(I–I_a)/(I_0–I_a)$ represents the radiation-induced DNA damage, I_a represents the fluorescence intensity of EtBr, I_0 is the control (*i.e.*, the fluorescence intensity of the DNA-EtBr exposed to 0 Gy radiation), and I is the fluorescence intensity of the EtBr–DNA exposed to radiation [29]. The dose effect curve in Fig. 2 is almost linear, indicating the DNA strand breakage induced by the radiation.

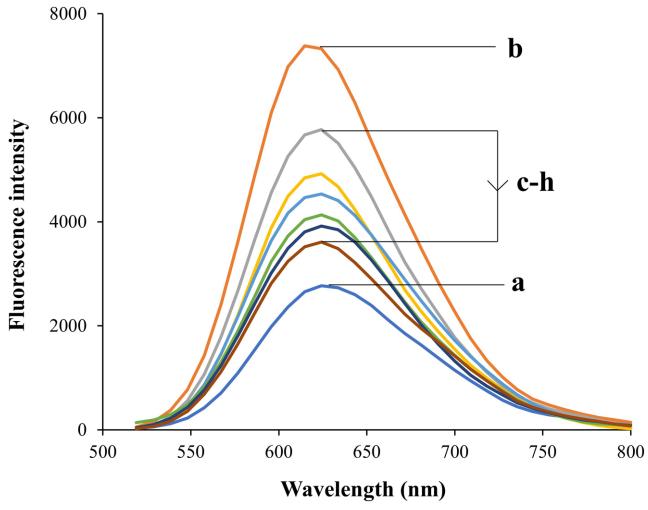

Fig. 1. Fluorescence spectra of the EtBr–DNA complex when increasing the radiation dosage.
a [EtBr] = 60.0 μM, **b** [EtBr] = 60.0 μM + [DNA] = 60.0 μM, and **c–h** EtBr–DNA when gradually increasing gamma radiation in increments of 626 Gy up to total dose of 3,756 Gy.

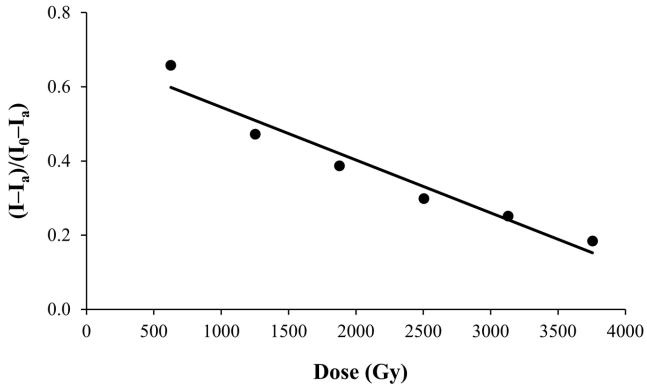

Fig. 2. Dose–response relationship of DNA strand breaks induced by gamma irradiation with a total dose of 3,756 Gy.
I_a: EtBr fluorescence intensity, I_0: EtBr–DNA control fluorescence intensity; and I: EtBr–DNA irradiated sample fluorescence intensity.

The D_{50}, representing the radiation dose that caused 50% DNA damage, was also measured using the dose-effect curve (Fig. 2). In this study, D_{50} was a single dose of

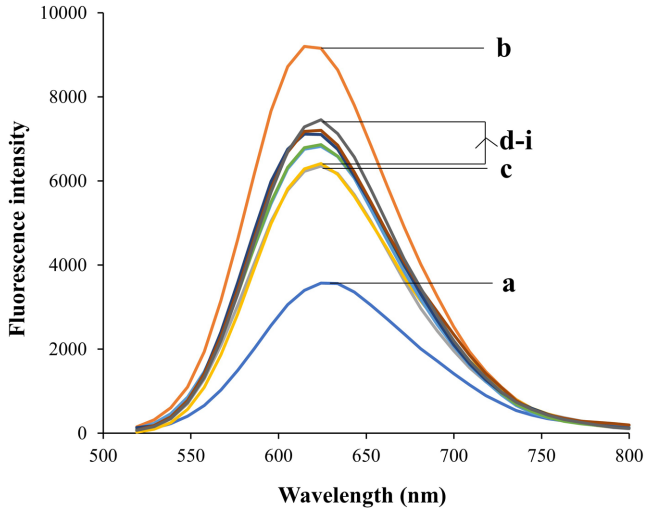

Fig. 3. Fluorescence emission spectra of the EtBr–DNA complex when increasing the amount of GSH with a total gamma-irradiation dosage of 1,252 Gy.
a [EtBr] = 60.0 μM only, **b** [EtBr] = 60.0 μM + [DNA] = 60.0 μM, **c** [EtBr] = 60.0 μM + [DNA] = 60.0 μM; with total gamma-irradiation dosage of 1,252 Gy, **d** 10.0 μM GSH, **e** 20.0 μM GSH, **f** 30.0 μM GSH, **g** 40.0 μM GSH, **h** 50.0 μM GSH, and **i** 60.0 μM GSH.

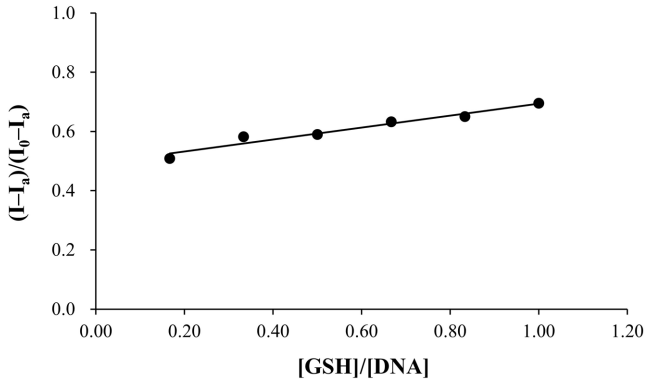

Fig. 4. Plot of DNA protection vs. [GSH]/[DNA].

1,252 Gy, as the exposed DNA-EtBr showed a drastically reduced fluorescence intensity when compared with the control DNA-EtBr exposed to 0 Gy radiation [16].

Estimation of Radiation-Induced DNA Damage Protection by GSH

To protect the DNA from radiation-induced damage, 10–60 μM of a reduced form of GSH was added to the DNA solution prior to the radiation exposure. GSH was selected as it has already been confirmed as a radioprotective agent. When increasing the concentration of GSH, the fluorescence intensity of the EtBr–DNA solution increased when compared with the control DNA exposed to 0 Gy, thereby indicating protection against DNA damage (Fig. 3). Fig. 4 shows a graph of $(I–I_a)/(I_0–I_a)$ vs. [GSH]/[CT-DNA]. The damage protection increased gradually when increasing the amount of GSH with 1,252 Gy. As a result, GSH was calculated to provide 70% protection from gamma radiation-induced DNA damage when compared with the control exposed to 0 Gy. Table 1 shows the gamma radiation-induced DNA damage protection by GSH and the molar ratio of [GSH]/[CT-DNA] [16].

Table 1. GSH protection of CT-DNA from gamma radiation-induced damage.

GSH concentration (μM)	DNA concentration (μM)	[GSH]/[DNA] (μM/μM)	DNA protection (%)
10	60	0.17	51
20		0.33	58
30		0.50	59
40		0.67	63
50		0.83	65
60		1.00	70

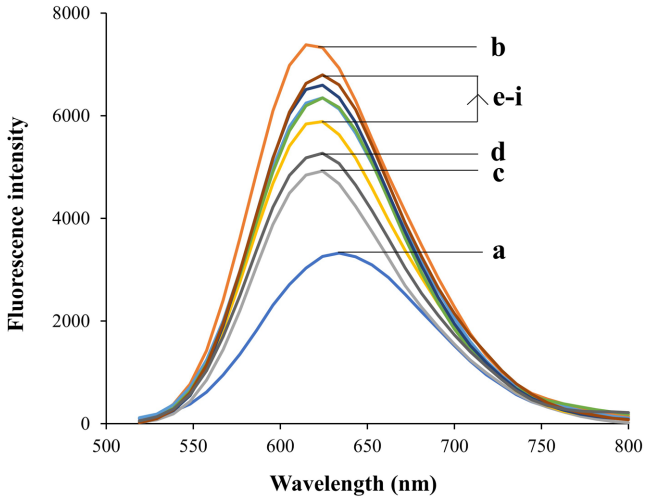

Fig. 5. Fluorescence emission spectra of the EtBr–DNA complex when increasing the amount of γ-PGA with a total gamma-irradiation dosage of 1,252 Gy.
a [EtBr] = 60.0 μM only, **b** [EtBr] = 60.0 μM + [DNA] = 60.0 μM, **c** [EtBr] = 60.0 μM + [DNA] = 60.0 μM; with a total gamma-irradiation dosage of 1,252 Gy, **d** 1.65 μM D/L-glutamate, **e** 0.33 μM γ-PGA, **f** 0.66 μM γ-PGA, **g** 0.99 μM γ-PGA, **h** 1.33 μM γ-PGA, and **i** 1.65 μM γ-PGA.

Estimation of Radiation-Induced DNA Damage Protection by Poly-Gamma-Glutamate

To protect the DNA from radiation-induced damage, 0.33, 0.66, 0.99, 1.33, or 1.65 μM of γ-PGA was added to the DNA solution prior to the radiation exposure. When increasing the concentration of γ-PGA, the fluorescence intensity of the EtBr-DNA solution increased when compared with the control DNA exposed to 0 Gy, indicating DNA damage protection, whereas D/L-glutamate, a monomer of γ-PGA, showed no radioprotective effects (Fig. 5). Fig. 6 shows a graph of $(I–I_a)/(I_0–I_a)$ vs. [γ-PGA]/[CT-DNA]. The

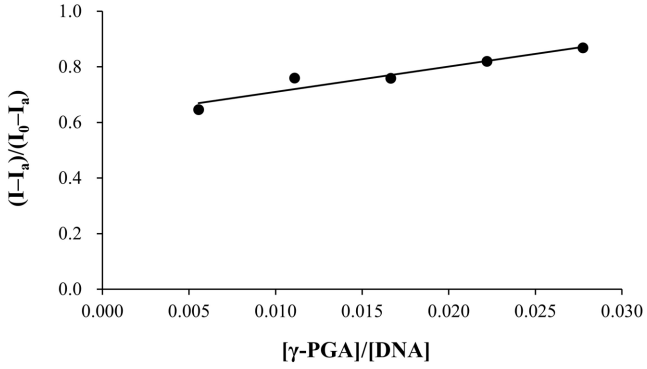

Fig. 6. Plot of DNA protection vs. [γ-PGA]/[DNA].

Table 2. γ-PGA protection of CT-DNA from gamma radiation-induced damage.

γ-PGA concentration (μM)	DNA concentration (μM)	[γ-PGA]/[DNA] (μM/μM)	DNA protection (%)
0.33	60	0.006	65
0.66		0.011	76
0.99		0.017	76
1.33		0.022	82
1.65		0.028	87

damage protection increased gradually when increasing the amount of γ-PGA with 1,252 Gy. As a result, γ-PGA was calculated to provide 87% protection from gamma radiation-induced DNA damage when compared with the control exposed to 0 Gy. Table 2 shows the gamma radiation-induced DNA damage protection by γ-PGA and the molar ratio of [γ-PGA]/[CT-DNA].

Hence, the current results showed that γ-PGA produced a much greater increase of fluorescence intensity than GSH, indicating that γ-PGA also has a greater ability to protect against radiation-induced DNA damage.

Discussion

Many of the chemical changes in biomolecules (especially in DNA) are caused by free radicals, which are generated by mutagenic substances, including ionizing radiation, where the modifier produced by the radiation reaction has various biological effects [4, 5].

GSH as a single agent has already been shown to affect DNA damage and repair, redox regulation, and multiple cell signaling pathways. Additionally, as a major thiol compound in cells that scavenges OH• radicals, GSH has also been shown to play an important role in the conversion of DNA-derived peroxyl radicals into strand breaks [30]. Moreover, glutathione has been associated with preventing oxidative damage to the skin, and its role as a skin whitener was discovered as a side effect of large doses of glutathione [31].

γ-PGA is a biopolymer produced during the fermentation process by *Bacillus subtilis*, which is fundamental to the production of fermented soy sauce, such as natto (a traditional Japanese fermented food) and chonggukjang (a traditional Korean fermented food). Since these foods have been consumed for centuries, this is strong evidence supporting the safety of γ-PGA [21].

γ-PGA is an anionic, water-soluble, safe, and edible biomaterial naturally synthesized by *Bacillus subtilis*, in which the α-amino and γ-carboxy groups of glutamic acid are polymerized by a γ-amide linkage [32]. Moreover, the multi-functionalities of γ-PGA, such as its biodegradability, nontoxicity, compatibility, and edibility, have made it a promising biopolymer for use as a health food, thickener, osteoporosis-preventing factor, stabilizer in the food industry, moisturizer in cosmetics, and in various biomedical product applications [20, 32, 33].

In this study, the radioprotective effects of γ-PGA on DNA damage and the inhibition of damage after irradiation with a ^{60}Co gamma source were characterized by fluorescence emission intensity measurements [16].

Free radicals generated by radiation cause DNA damage, resulting in a decreased fluorescence intensity due to reduced binding of EtBr–DNA. Thus, a decrease in the fluorescence intensity indicates DNA damage by radiation [28].

D_{50}, representing the dose of radiation that damages 50% of the DNA, was also measured using a dose-effect curve (Fig. 2). In this study, a single dose of 1,252 Gy was determined as the D_{50}, which drastically decreased the fluorescence intensity of the radiation-exposed DNA–EtBr compared with the control DNA–EtBr exposed to 0 Gy radiation.

When increasing the concentration of GSH, the DNA damage was gradually protected up to 70% owing to the presence of a thiol group, plus the fluorescence intensity of the EtBr–DNA solution increased compared with that of the control DNA exposed to 0 Gy (Fig. 3).

Meanwhile, Fig. 5 shows that increasing the concentration of γ-PGA also protected against DNA damage, as indicated by the increased fluorescence intensity of the EtBr–DNA solution when compared with that of the control DNA exposed to 0 Gy. Additionally, γ-PGA was calculated to provide 87% protection from gamma radiation-induced DNA damage when compared with the control exposed to 0 Gy. Thus, the high-molecular-weight 3,000 kDa γ-PGA produced a much greater increase of fluorescence intensity than GSH, indicating that γ-PGA also has a greater radioprotective efficiency against radiation-induced DNA damage [16].

This study also proposes the protection mechanism of poly-gamma-glutamate against radiation-induced DNA damage. Hydrogel is a semi-rigid jelly-like colloid, and most hydrogels contain more than 90% water by volume. The build-up of intramolecular bridges occurs for many reasons, including irradiation, repetitive freezing, and chemical cross-linkage. When exposed to gamma irradiation,

water disintegrates and free radicals occurr in the γ-PGA solution. These free radicals correspond to the hydrogen in the main chain of the polymer, thereby providing reactive centers [32]. Thus, it is hypothesized that the hydroxyl radicals formed by gamma irradiation are the mechanism of a crosslink formation that captures the hydrogen in γ-PGA [34].

In summary, the current in vitro results showed that γ-PGA exhibited significant radioprotective effects against gamma irradiation. Thus, it is hoped that this protective ability of γ-PGA against DNA damage can be used for the development of new functional cosmeceutical materials.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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