Title

Evaluation of the *in vivo* antioxidative activity of redox nanoparticles by using a developing chicken egg as an alternative animal model

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Abstracts

Antioxidants have been demonstrated to exert beneficial effects as pharmacotherapies for cardiovascular diseases. The *in vitro* systems generally employed to evaluate antioxidants, however, are limited by having no appreciable *in vivo* redox status of the antioxidants. Therefore, we used our developing chicken egg model to evaluate the *in vivo* antioxidative activity of a redox nanoparticle possessing 2,2,6,6-tetramethylpiperidine-1-oxyl (RNP⁰). The 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) elicited strong oxidative stress and its LD₅₀ value for chick embryos was 3.5 ± 0.9 mg/egg. The low molecular weight nitroxide compound, 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), which is known to have the highest level of antioxidant activity, showed no significant protective effect against

AAPH-induced embryo lethality. On the contrary, RNP^O had potent protective effects against AAPH-induced embryo lethality. Moreover, RNP^O could significantly suppress the production of lipid peroxides in chick serum induced by hydrocortisone. Since RNP^O has a longer retention time in blood than TEMPOL, RNP^O may protect the embryo against lethal oxidative stress by suppressing lipid peroxidation. The validity of *in vivo* experiments using developing chicken eggs was supported by our data, where RNP^O was determined to elicit strong antioxidative activity *in vivo*, irrespective of the lack of a significant difference in the *in vitro* activity between low-molecular weight TEMPOL and RNP^O. Our results support the use of the developing chicken egg model to evaluate potential *in vivo* antioxidative activity of RNP^O.

Keyword

Developing chicken egg, Redox nanoparticle, Tempol, *In vivo* antioxidative activity.

1. Introduction

Reactive oxygen species (ROS) such as superoxide and hydroxyl radical play an important role in the living body. Excessive amounts of ROS are usually eliminated by endogenous antioxidants such as superoxide dismutase and catalase. Sometimes, high levels of ROS are not completely scavenged by endogenous reducing systems. These excessive levels of ROS often cause severe damage to various biomolecules, such as lipids, proteins, nucleic acids, and carbohydrates, and can lead to life-threatening oxidative stress [1]. ROS are believed to underlie a number of diseases such as Parkinson's disease, cataracts, chronic obstructive pulmonary disease (COPD), myocardial infarction, diabetes, allergy, and rheumatoid arthritis [1-3]. Since antioxidants are useful for preventing oxidative injury, various antioxidant agents have been developed worldwide. Edaravone (Radicut) is now marketed for medical use as a free-radical scavenging drug that prevents neurodegeneration following an acute cerebral infarction [4,5]. However, antioxidative drugs, including edaravone, are very unstable *in vivo* and their therapeutic effect is currently controversial. Therefore, the development of effective antioxidative drugs remains a challenge.

The development of effective antioxidative drugs requires evaluation of their antioxidative activity *in vivo*. Although the oxygen concentration in *in vitro* systems is usually about 21% O₂ at normal atmospheric pressure, it is far higher than that in the living body, where many naturally occurring antioxidants have been identified. The reductive environment *in vivo* accelerates the production of reaction sites for antioxidants [6]. Although animal models such as mice and rats are widely employed

for the evaluation of *in vivo* antioxidative activity [7,8], the regulations governing the use of animals for research as well as ethical concerns caused us to hesitate to use rodent models as a primary screening system for many candidate compounds. Therefore, we investigated the use of the developing chicken egg as an alternative *in vivo* animal model to evaluate antioxidative activity. The Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health state defines chick embryo as an experimental animal. OLAW has ruled that birds become regulated animals once they have hatched from the egg [9,10]. Since the developing chicken egg can be used without any regulation of animal experimentation until it has hatched (about 21 days), researchers can utilize the developing chicken egg in the laboratory without ethical considerations. In this developing chicken egg model, intravenous administration of an agent is possible, similar to that which is done using rodent experimental models. Thus, the *in vivo* activity reflecting the pharmacokinetics of candidate compounds may be evaluable using this developing chicken egg model [11]. We have already succeeded in evaluating the *in vivo* activity of an antiangiogenic agent and a radiosensitizing agent by using this model [12-15].

Recently, nanoparticles such as liposomes and polymeric micelles have gained worldwide attention as a new medical technology [16], because they alter the biodistribution of drugs, thereby improving their therapeutic effects. In particular, a redox nanoparticle possessing 2,2,6,6-tetramethylpiperidiny-1-oxyls (TEMPOs) (RNP^O, Fig. 1), which is a self-assembling polymeric micelle with a diameter of 40 nm and is prepared using poly(ethylene glycol)-*b*-poly[4-(2,2,6,6-tetramethylpiperidine-1-oxyl)oxymethylstyrene] (PEG-*b*-PMOT) diblock copolymer in aqueous media, has been developed as a nanosized ROS scavenger [17]. Since RNP^O covered with PEG outer shell displays prolonged blood circulation time *in vivo* because of its nanometer-sized and high biocompatible structure it, effectively scavenges ROS [18]. However, results obtained from *in vitro* experiments using RNP^O do not always reflect *in vivo* therapeutic effects. Pharmacokinetic characteristics are one of most important factors for evaluating nanoparticle therapeutics, and the *in vitro* experiments do not support the evaluation of the pharmacokinetics of such nanotechnologies.

Here, we used the developing chicken egg model to evaluate the *in vivo* antioxidative activity of RNP^O under conditions of severe oxidative stress induced by AAPH or hydrocortisone. We sought to verify the efficacy of RNP^O as an antioxidant drug, and the potential of the developing chicken egg model as an alternative animal experiment method.

Fig. 1. Chemical structure of RNP^O used in this study.

2. Material and methods

2.1. Chemicals

Concentrated sulfuric acid, phosphoric acid, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH), *N,N*-dimethylformamide and liquid paraffin were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-Hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), hydrocortisone hemi succinate sodium salt (HChs-Na), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), phosphotungstic acid hydrate, and 1-butanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thiobarbituric acid (TBA) and butylated hydroxytoluene (BHT) were purchased from Kanto Chemical (Tokyo, Japan).

2.2. Preparation of RNP^O

RNP^O was prepared by the self-assembly of an amphiphilic block copolymer (MeO-PEG-*b*-PMOT) composed of the hydrophilic PEG segment and the hydrophobic poly(4-methylstyrene) segment possessing nitroxide radicals as side chains via ether linkages, according to our previous study [17]. Briefly, poly(ethylene glycol)-*b*-poly(chloromethylstyrene) (MeO-PEG-*b*-PCMS) was synthesized by the radical telomerization of chloromethylstyrene using MeO-PEG-SH (M_n = 5000; NOF Corporation, Tokyo, Japan) as a telogen. The PCMS segment possessed 14 repeating units, as determined by using ¹H NMR data. The chloromethyl groups were converted to TEMPOs via a Williamson ether synthesis of benzyl chloride in the MeO-PEG-*b*-PCMS block copolymer with an alkoxide of TEMPOL. After purification of the obtained MeO-PEG-*b*-PMOT, the substitution ratio of the modified TEMPO moieties per repeating unit of PCMS was 89.5%, as determined by electron spin resonance (ESR) with a standard curve generated from TEMPOL (Molecular weight of MeO-PEG-*b*-PMOT = 8,800). To prepare RNP^O, the MeO-PEG-*b*-PMOT was dissolved in *N,N*-dimethylformamide, and transferred into a dialysis membrane tube (SpectraPor,

molecular weight cut-off size: 3,500 Da, Spectrum Laboratories Inc., Savannah, GA, USA) and then dialyzed for 24 h against 2 L of distilled water, which was changed after 2, 4, 8, 12, and 20 h. Following dialysis, the diameter of the particles obtained was determined using a dynamic light scattering measurement (Zetasizer Nanoseries ZEN3600, Malvern Instruments Ltd., Worcestershire, UK).

2.3. Developing Chicken egg assay

Fertilized chicken eggs (Goto Hatchery, Inc. Gifu, Japan) were incubated in a humidified incubator at 37.6°C for 15 days. In the case of intravenous (i.v.) administration, an egg was candled with halogen light to mark the crossing of two large blood vessels on its chorioallantoic membrane (CAM) on day 15. A 0.5 × 2 cm rectangular window was marked around the blood vessel, and the eggshell was cut with a grinder. After the eggshell was removed with a pair of tweezers, liquid paraffin was dropped onto the eggshell membrane to visualize the blood vessels under the membrane. The AAPH and TEMPOL were dissolved in physiological saline and administered i.v. with a 30-G needle. At 1 min after i.v. administration of TEMPOL or RNP^O (dissolved in physiological saline and containing 1% Intralipos, Otsuka Pharmaceutical, Tokyo, Japan), AAPH was administered to another blood vessel on the CAM with a 30-G needle. In the case of air sac administration, a small hole was made with a drill at the blunt end of the egg on day 15. HChs-Na was administered into the air sac through a small hole, which was subsequently closed using a surgical adhesive film, Opsite (Smith & Nephew, Kingston upon Hull, UK). Chick embryos that survived the treatment were checked for heart rate by using a digital egg monitor, Buddy (Avitronics, Truro, UK).

2.4. TBARS assay

The level of lipid peroxidation in chick serum was determined by measuring malondialdehyde (MDA) using a TBARS (thiobarbituric acid reactive substances) assay [19]. Blood (300 µL) was collected from chick vein with a 25-G needle. For plasma preparation, blood was transferred to a tube containing 0.3 mg of EDTA and centrifuged at 3,000 rpm for 10 min at 4°C. Alternatively, for serum preparation, blood was chilled on ice for 30 min and centrifuged at 3,000 rpm for 10 min at 4°C. Serum or homogenates of liver or brain (100 µL) were mixed with 1/12 mol/L H₂SO₄ (4 mL) and 10% (w/v) phosphotungstic acid (500 µL). The mixture was centrifuged at 3,000 rpm for 10 min at 4°C. The sediment was suspended in a solution containing 1/12 mol/L H₂SO₄ (2 mL) and 10% (w/v) phosphotungstic acid (300 µL). The mixture was centrifuged at 3,000 rpm for 10 min at 4°C. The sediment was resuspended in a solution

containing 1% phosphoric acid (1 mL), 10 mM BHT (50 μ L), and 0.67 % (w/v) TBA (1 mL). The mixture was incubated at 100°C for 30 min. After cooling on ice, 1-butanol (1 mL) was added, vortexed for 30 s, and centrifuged at 3,000 rpm for 10 min at 4°C. The layer of butanol was collected and measured at 532 nm using a spectrophotometer (HITACHI U-2000, Tokyo, Japan). The concentration of TBARS was calculated using the Beer-Lambert law (molar extinction coefficient: 1.56×10^5).

2.5. X-band ~~electron-spin-resonance (ESR)~~ measurement

The blood circulation of nitroxide radicals in the RNP^O was evaluated using ESR. RNPO (300 μ g) or TEMPOL (80 μ g) was administered i.v. to the egg. The concentrations of nitroxide radicals in all drugs were identical. Blood samples were collected from chick vein by using a syringe with a 25-G needle at 0.16, 0.5, 1, 3, 12, and 24 h after administration, followed by transfer into a tube with 0.3 mg of EDTA. Plasma samples were then obtained by centrifuging the blood samples (3,000 rpm, 4°C, for 10 min). To determine the ESR intensities at 0 min, 30 μ L of RNP^O solution (10 mg/mL) was added to the blood (270 μ L), followed by centrifugation of the blood (3,000 rpm, 4°C, for 10 min). The ESR measurement was conducted assuming that the total blood volume in the egg was 50 mL/kg. Generally, nitroxide radicals are reduced to hydroxylamine by an in vivo reducing agent such as ascorbic acid, which is not detected using ESR. Since hydroxylamine also possesses ROS-scavenging capabilities, the total drug concentrations (nitroxide radicals + hydroxylamines) in the blood was determined by the addition of potassium ferricyanide ($K_3[Fe(CN)_6]$) (200 mM, 10 μ L) to plasma (100 μ L) to re-oxidize the hydroxylamines to nitroxide radicals. Using an X-band ESR spectrometer (JES-TE25X; JEOL, Tokyo, Japan), the ESR measurements were carried out under the following conditions: frequency, 9.41 GHz; power, 8.00 mW; field, 333.8 ± 5 mT; sweep time, 1.0 min; modulation, 0.1 mT; and time constant, 0.1 s.

2.6. Ethical issues

Animal experiments were approved by the animal ethics committee of the University of Tokushima (approval #10020) and carried out in accordance with international care regulations.

2.7. Statistical analysis

Data are indicated as mean $\pm$ S.D. For the fatality test, the significance of the differences between the results of the independent experiments was analyzed using the Chi-square test. In the case of TBARS, the statistical significance was analyzed using

Student's *t*-test. A *p* value of less than 0.05 was considered to be significant. A Q rejection test was performed for individual values of TBARS.

3. Results

3.1. Fatal toxicity of AAPH for the developing chicken egg

We evaluated the correlation between AAPH dosage and fatality rate of the chick embryo to determine the LD₅₀ value of AAPH. The oxidative damaging agent AAPH was administered i.v. to the developing chicken egg at 15 d, and the viability of chick embryos was evaluated by heart pulse measurement. Fatality induced by administration of AAPH typically occurred within 24 h after i.v. administration, with the fatality rate remaining constant from 48 to 72 h. The fatality rates of AAPH (i.v.) at 0, 3.0, 3.5, 4.0, 4.5, and 5.0 mg/egg at 72 h after administration were 8.7, 24.7, 33.3, 66.7, 89.0, and 97.7% respectively. Consequently, the LD₅₀ of AAPH was 3.5 ± 0.9 mg (Fig. 2).

Fig. 2. Fatal toxicity of AAPH in the developing chicken egg. AAPH was administered i.v. to the developing chicken egg at 15 d, and the case fatality rate was evaluated 72 h later by heart pulse measurement. At each dose, 14–17 eggs were used. The experiment was repeated three times. Values are mean $\pm$ S.D.

3.2. Protective effect of RNP^O against AAPH-induced lethal oxidative injury

Since AAPH induced lethal toxicity in chick embryos, we investigated whether the RNP^O could protect chick embryos from a fatality caused by AAPH administration. The fatality rates of the control, AAPH, RNP^O, and AAPH+RNP^O groups 72 h after AAPH administration were 0, 57, 0, and 15% respectively. The AAPH+RNP^O group showed a significantly lower fatality rate than the AAPH group (Fig. 3A). On the other hand, the fatality rates for the control, AAPH, TEMPOL, and AAPH+TEMPOL groups were 0, 92, 7, and 100%, respectively. Pre-administration of TEMPOL did not decrease fatality

rates (Fig. 3B), which is in sharp contrast to the results from *in vitro* experiments, in that low molecular weight TEMPOL had a fairly greater antioxidative effect *in vitro* [20].

Fig. 3. The protective effects of RNP^O and TEMPOL against AAPH-induced lethal oxidative injury in chick embryos. The administration of AAPH (i.v.) was performed 1 min after administration of RNP^O and TEMPOL, and surviving chick embryos were checked after 72 h (**p* < 0.05, ****p* < 0.001). A: The protective effect of RNP^O (57 µg/egg) against AAPH (4.0 mg/egg)-induced lethal oxidative injury of chick embryo. The number of eggs (surviving eggs/total eggs) was as follows: control, (14/14); AAPH, (6/14); RNP^O, (13/13); and AAPH+ RNP^O, (11/13). B: The protective effect of TEMPOL (8.6 µg/egg) against AAPH (4.0 mg/egg)-induced lethal oxidative injury of chick embryo. The number of eggs (surviving eggs/total eggs) was as follows: control, (15/15); AAPH, (1/13); TEMPOL, (13/14); and AAPH+ TEMPOL (0/13).

3.3. Induction of oxidative injury to liver, brain, and serum by hydrocortisone

Nishigori *et al.* has reported that lipid peroxidation occurs in the blood of developing chicken eggs by administration of HChs-Na [21]. Therefore, we used the TBARS assay to evaluate the degree of oxidative injury 48 h after the administration of HChs-Na into the air sac. The TBARS values in the brain were 169.9 ± 14.7 for the control group and 167.6 ± 32.1 nM for HChs-Na group, while the TBARS values for the liver were 130.8 ± 20.2 and 153.7 ± 32.6 nM, respectively (Fig. 4). Although HChs-Na treatment did not cause oxidative injury in the chick brain and liver, a significant increase in oxidative damage was observed in chick serum. The TBARS values in serum were 176.3 ± 48.1 for control and 386.4 ± 83.0 nM for HChs-Na. Fatal toxicity following HChs-Na treatment was not observed in this experiment.

3.4. Protective effect of RNP^O against hydrocortisone-induced lipid peroxidation in the developing chick embryo

Since the amount of TBARS in the blood of a developing chicken egg increased following treatment with HChs-Na (300 µg), the inhibitory effect of the antioxidants on HChs-Na was investigated. Following administration of RNP^O (300 µg), the TBARS value of the control, HChs-Na, RNP^O, and HChs-Na+RNP^O groups were 281.0 ± 109.1, 462.5 ± 122.0, 170.9 ± 50.1, and 352.6 ± 70.2 nM, respectively. Significant reduction of the TBARS value was seen in the HChs-Na+RNP^O group (Fig. 5A, *p* < 0.05). In the case of TEMPOL (45.2 µg), the TBARS values in the control, HChs-Na, TEMPOL, and HChs-Na+TEMPOL groups were 167.5 ± 68.7, 370.4 ± 43.7, 186.8 ± 42.1, and 400.2 ± 70.9 nM, respectively. TEMPOL did not show any significant protective effect against HChs-Na-induced lipid peroxidation (Fig. 5B).

Fig. 4. Induction of oxidative injury to liver, brain, and serum by hydrocortisone. PBS (white bar) or HChs-Na (300 µg, black bar) was administered into the air sac of a developing chicken egg at 15 d. Brain, liver, and serum were extracted 48 h afterward, and the TBARS assay was performed (****p* < 0.001). The TBARS values for liver and brain were presented as the value per mg protein, and the TBARS value in serum was presented as the value per 100 µL. The number of eggs was as follows: PBS (6) and HChs-Na (8). Values are mean ± S.D.

Fig. 5. Protective effect of RNP^O and TEMPOL against hydrocortisone-induced lipid peroxidation in the blood of the developing chick embryo. HChs-Na (300 μ g) was administered into the air sac of a developing chicken egg at 15 d, and RNP^O (300 μ g) or TEMPOL (45.2 μ g) were administered i.v. 15 h later. Blood was extracted after 48 h, and a TBARS assay was performed using serum. A: Protective effect of RNP^O against hydrocortisone-induced lipid peroxidation (* p < 0.05). The number of eggs was as follows: control (6), HChs-Na (7), RNP^O (6), and HChs-Na+RNP^O (8). Values are mean $\pm$ S.D. B: Protective effect of TEMPOL against hydrocortisone-induced lipid peroxidation (***) p < 0.001). The number of eggs was as follows: control (8), HChs-Na (7), TEMPOL (6), and HChs-Na+TEMPOL (6).

3.5. Evaluation of pharmacokinetic properties of RNP^O and TEMPOL using the developing chicken egg

Because only RNP^O showed antioxidative activity in the developing chicken embryo, the time course of RNP^O and TEMPOL concentrations in blood was measured after i.v. administration of RNP^O and TEMPOL to the developing chicken egg at 15 d. While the intensity of the ESR signal was very low in blood 10 min after TEMPOL administration, 30% of ESR signals from the nitroxide radical in blood following RNP^O administration remained detectable at 10 min as shown in Fig. 6A. About 10% of the ESR signal from RNP^O was still detected in blood after 24 h. The ESR spectra of nitroxide radicals in RNP^O can provide information on morphological changes *in vivo*. Generally, the ESR signal of TEMPO derivatives shows a sharp triplet because of an interaction between the ¹⁴N nuclei and the unpaired electron in the dilute solution. Clear triplet signals were observed in the blood at 10 min after the i.v. administration of TEMPOL to the egg, as shown in Fig. 6B. In contrast, the ESR signal of RNP^O in the blood stream showed a broad spectrum (Fig. 6C). This result indicates the confinement of the nitroxide radicals

1 into the solid core of RNP^O [17].

2
3 Fig. 6. The time course of RNP^O and TEMPOL concentration in the blood of developing
4 chicken egg. A: The RNP^O (open square) or TEMPOL (open circle) were administered
5 i.v. to the developing chicken egg at 15 d, and blood (300 μ L) was collected after 0.16,
6 0.5, 1, 3, 12, and 24 h. The plasma (100 μ L) was obtained by centrifugation of the blood
7 samples in preparation for ESR analysis. For comparison of the ESR intensity at each
8 time, the ESR intensity at 0 min was set to 100%. Three to four eggs were used at each
9 time point. Values are represented as mean $\pm$ S.D. B and C: The ESR spectrum of
10 TEMPOL (B) and RNP^O (C) in the blood 10 min after administration. Arrows indicate
11 the signal of manganese marker.

12 4. Discussion

13 We have previously reported that in mice, the nanoparticle-based antioxidant, RNP^O,
14 shows a remarkable increase in blood circulation compared to the low-molecular weight
15 TEMPOL [17]. Here, we demonstrated that the developing chicken egg is a useful
16 model for the *in vivo* evaluation of antioxidative activity, similar to mouse and rat
17 animal models. Wang *et al.* previously demonstrated that the developing chicken egg
18 could be used to evaluate the oxidative damage of butenolide and the antioxidative
19 effect of endogenous antioxidants, where they could measure the amount of thiol group,
20 TBARS, and GPx (glutathione peroxidase) activity in the liver and kidney [22]. We
21 investigated the suppressive effects of antioxidants against the lethal toxicity of AAPH
22 by using a chick embryo. Our method of evaluation has several advantages, including
23 the fact that it represents a rapid and easy method to evaluate antioxidative activity,
24 which is applicable for high-throughput screening and pharmacokinetic analysis, both of
25 which are important steps of pre-clinical research. It is very useful, particularly in the
26 case of nanoparticles because that it is hard to evaluate their pharmacokinetic effects by
27

in vitro experiments, though there are several limitations as follows: i) In the case of chicken embryos, since it is difficult to standardize size of each eggshell, the standard deviation may become larger than that of conventional animal experiments using rodent animals. However, it is possible to increase number of eggs in each group due to its cost; ii) Since the growth speed and drug susceptibility change by week-old of parent chickens, we have to adjust the age of parent chicken; (iii) The evaluation period is restricted to a short period because experiments using chick embryo must be finished before the chicks hatch on 21 days.

We hypothesized from these data that RNP^O can suppress AAPH-induced lethal toxicity in chick embryos, based on its ability to decrease the lipid peroxidation in blood. Rhyu *et al.* reported that when mice (n = 7) were injected i.p with 80 mg/kg body weight of AAPH, two mice died within 15 min [23]. These data were in good agreement with our data, because the dose of 4 mg/egg was equivalent to 67 mg/kg in mouse, bearing a similar sensitivity between the mouse and chick embryo to AAPH. Terao *et al.* reported that the most striking structural changes by AAPH were degeneration, swelling, and disruption of the endothelial lining of cells of the capillaries in various organs, the death of lymphocytes in lymphoid tissues, and fatty degeneration of the liver and kidneys [24]. Water-soluble chain-breaking antioxidants such as 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (vitamin E analogue), uric acid, cysteine, and glutathione suppressed this damage, whereas vitamin C was ineffective. Therefore, we proved that RNP^O was also effective the chain-breaking antioxidants in the blood.

Arteriosclerosis has been characterized as a vascular disease caused by oxidative stress [25,26]. The oxidized low-density lipoprotein serves as a trigger of arteriosclerosis. We are now investigating the protective effect of RNP^O against vascular diseases, including arteriosclerosis that is caused by oxidative stress.

5. Conclusion

We demonstrated in this study that the potent *in vivo* antioxidative activity of the redox nanoparticle RNP^O against AAPH-induced oxidative injury could be successfully evaluated by using a developing chicken egg as an alternative animal model. This technique can be one of the reliable methods for new drug with different pharmacokinetics from conventional low molecular weight drugs.

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