



Effects of titanium oxide nanoparticles on *Oryzias latipes* embryos and sac-fry under different irradiation conditions

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ABSTRACT

Some phototoxicity of titanium dioxide nanoparticles (TiO₂ NPs) has been reported in recent years in studies with fish embryos or larvae. However, it is necessary to focus on the potential effects of embryonic exposure due to irreversible abnormalities and mortalities observed in sac-fry, and to expand various fish embryos to generate multiple test species. The aim of this study was to evaluate the effects of TiO₂ NPs under different irradiation conditions in exposed *Oryzias latipes* (*O. latipes*) at the embryonic and sac-fry stages. The effects of different irradiation conditions were observed using ultra-violet (UV) and visible light, and the corresponding effects were monitored by determining cumulative mortality and abnormality. *O. latipes* were exposed for 8 d to 0, 1, 5, 10, or 50 mg/L TiO₂ NPs under UV (4,818.86 mW/m² at the bottom of clear vials) or visible light, after which the embryos were transferred to NP-free embryo-rearing solution until 16 days post fertilization (dpf). Abnormalities of embryos and sac-fry increased at high TiO₂ NP concentrations under UV irradiation, compared to control samples treated with visible light or UV irradiation alone. This work provides information regarding the phototoxicity of TiO₂ NPs using *O. latipes* at the embryonic and sac-fry stages.

Keywords: Fish embryo, *Oryzias latipes*, Phototoxicity, TiO₂ NPs, UV irradiation

1. Introduction

Titanium dioxide nanoparticles (TiO₂ NPs) have been used in beauty products, hygienic goods, and textiles [1-3]. Boxall et al. [2] presented modeling data used to estimate the TiO₂ NP concentration in water as 0.245 mg/L, based on a 100% market-penetration scenario of paints and sunscreen, particularly paints containing 5 mg/g TiO₂ NPs and sunscreen containing 50 mg/g TiO₂ NPs. TiO₂ NPs tend to become rapidly distributed throughout the environment because of their small sizes and non-biodegradability [3], and the potential exposure to bio-receptors increases as a consequence.

Several studies have been conducted in recent years to assess the toxicity of TiO₂ NPs. Because the photoreactivity of TiO₂ NPs can cause differing toxicities based on the bio-receptors that are activated [1, 4-5], the phototoxicities of TiO₂ NPs have been tested in various organisms, including fish [1, 4, 6-11], waterfleas [7, 9], algae [12], and bacteria [13]. The phototoxicity of TiO₂ NPs induced increased mortality of *Daphnia magna* [7, 9] and decreased colony formation of *Escherichia coli* and *Bacillus subtilis* [13].

Evidence suggests that TiO₂ NPs in surface water can readily absorb ultra-violet (UV) irradiation and that the absorbed energy can generate an excited state by electron-hole pair production on the surface of particles [4, 7, 8, 12].

Some authors have reported the phototoxicity of TiO₂ NPs using fish embryos or larvae [1, 4, 6-7, 11]. These studies were focused on the effects of *Danio rerio* (*D. rerio*) embryos [1, 4, 11, 14] and larvae [11, 14], *Oryzias latipes* (*O. latipes*) larvae [7, 9], and *Piaractus mesopotamicus* juveniles [6]. Although Ma et al. [14] presented evidence to suggest that yolk-sac larvae were most sensitive to the phototoxicity of TiO₂ NPs, it is still necessary to study their effects on potential embryonic exposure due to irreversible abnormalities and mortalities observed in sac-fry [15-16]. However, limited phototoxicity data exist regarding the effects of TiO₂ NPs on fish embryos, and such studies have mainly focused on *D. rerio*. Therefore, it is necessary to expand various fish embryos for use as multiple test species in order to assess the phototoxicity of TiO₂ NPs in water. *O. latipes* is recommended as a test fish species by Organization for Economic Co-operation and Development (OECD) No. 212 [17].



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The aim of this study was to evaluate the effects of TiO₂ NPs under different irradiation conditions in exposed *O. latipes* at the embryonic and sac-fry stages. The effects of different irradiation levels were observed using UV and visible light, and the corresponding effects were monitored by measuring cumulative mortality and abnormality. *O. latipes* was chosen as the test species due to the lack of available phototoxicity data. To the best of our knowledge, this is the first study to evaluate phototoxicity of TiO₂ NPs using *O. latipes* embryos.

2. Materials and Methods

2.1. Characterization and Preparation of Nanoparticles

TiO₂ NPs (particle size 21 nm, P25, white powder form) were purchased from Evonik Degussa (Germany). A field emission transmission electron microscope (FE-TEM, JEM2200FS, JEOL, Japan) was used to observe the morphology of the NPs. Specific surface area of the TiO₂ NPs was analyzed by particle size analyzer (UPA-150, Microtrac, USA). An electrophoretic light scattering spectrophotometer (ELS-8000, Otuska Electronics Co., Japan) was used to measure particle size distributions of hydrodynamic diameters and zeta potential of 10 mg/L TiO₂ NPs dispersed in deionized water (DW) and embryonic rearing solution (ERS). UV absorption spectra of 10 mg/L TiO₂ NPs suspended in ERS was measured by a UV/Vis spectrophotometer (Libra S32 PC, Biochrom Ltd., Cambridge, UK) at 10-min intervals for 1 h, to verify aggregation of TiO₂ NPs. ERS included NaCl (1 g/L), KCl (0.03 g/L), CaCl₂·2H₂O (0.04 g/L), MgSO₄·7H₂O (0.163 g/L), and methylene blue (1.0 mg/L) to prevent fungal growth [18]. When UV spectra analysis, Methylene blue was eliminated from the ERS due to photodegradation [18]. A stock solution of 100 mg/L TiO₂ NPs was prepared by dispersing the NPs in ERS with sonication for 10 min in a water bath sonicator (40 kHz frequency, Powersonic 420, Hwashin Technology, Korea). A series of exposure concentrations of TiO₂ NPs (0, 1, 5, 10, and 50 mg/L) were diluted with ERS.

2.2. Test Species and Pre-culture Conditions

O. latipes (Japanese medaka) was received from the National Academy of Agricultural Science (NAAS, Suwon, Korea). Adult orange-red type *O. latipes* for broodstock were kept in dechlorinated tap water (pH 7.0 ± 0.2; temp. 25 ± 2°C; light:dark = 16:8 h), with a diet of commercial Tetra Min (Tetra Werke, Germany) and brine shrimp (OSI PRO80™, USA) twice per day. On the day of the experiment, newly fertilized and healthy eggs of *O. latipes* were collected from the broodstock.

2.3. Phototoxicity Assay Using Fish Embryo and Sac-fry

The fish embryo and sac-fry assay was performed based on the OECD guidelines (No. 212) for chemical testing [10, 14, 15, 19]. Embryos (9-10 stages) were transferred into 2 mL clear vials with screw caps (Wheaton), one embryo per vial and one mL exposure solution per vial, and there were 10-15 replicates prepared. All experiments were repeated 1 to 4 times for treatments and 3 to 4 times for controls. The capped vials were placed upside down

in a photoreactor (model LZC-4, Luzchem Research Inc., Ottawa, ON, Canada) under a UV lamp for UV irradiation and in the laboratory under standard fluorescent lamps for visible light irradiation, before hatching. The photoreactor has one UV lamp (spectral distribution of 316 nm to 400 nm), positioned 18 cm above the test units. The light intensity was measured by a spectroradiometer (SPR 4001, Luzchem Research Inc., Ottawa, ON, Canada) and its values were 6,198.03 mW/m² at the top of the clear vials and 4,818.86 mW/m² at the bottom of the clear vials. At 8 days post fertilization (8 dpf, when the control embryos hatched), exposure solutions were changed with NP-free embryo-rearing solution. UV irradiated- or visible light irradiated-vials were maintained in the incubator under pre-culture conditions (without oxygen and food supply in both the control and exposure groups). Fish embryos and sac-fry were observed daily under a dissection microscope based on cumulative mortality and various abnormalities; abnormal embryos and sac-fry: Heart damage (e.g., tube heart, blood circulation disorder, pericardial necrosis, pericardial edema, and hemostasis), whole body necrosis, malformation of the pericardium and peritoneum, gallbladder edema, small eyes, tail deformity, swim bladder deformity (e.g., undeveloped, over-inflated, and deflated swim bladder), and erratic swimming activity. The dead embryos or sac-fry were immediately eliminated from the test units. The detailed experimental design of fish embryo and sac-fry assay is shown in Fig. 1. The effect of Ti ions was not measured because TiO₂ NPs are insoluble in water [3].

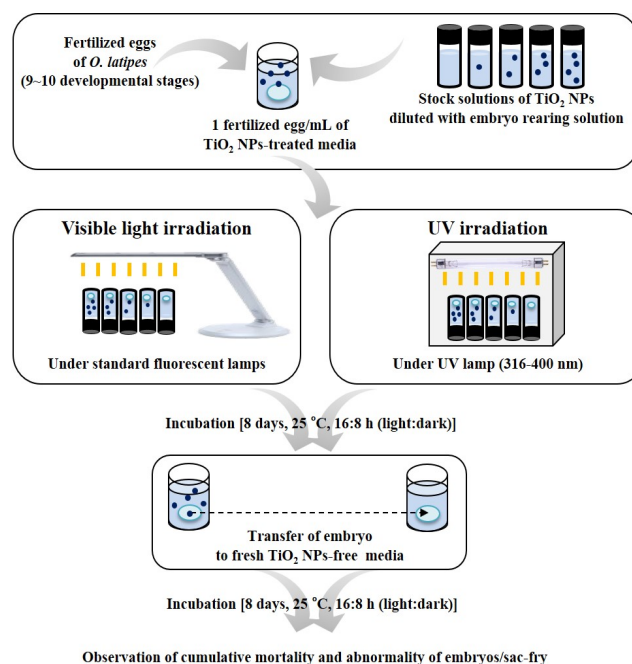


Fig. 1. Experimental study design. Fertilized eggs of *Oryzias latipes* were exposed to TiO₂ NPs in embryo rearing solution until 8 days post fertilization (dpf), under visible light or UV irradiation. At 8 dpf, the exposure solution was replaced with TiO₂ NP-free embryo rearing solution. All tests were assessed with respect to cumulative mortality and abnormalities of embryos and sac-fry until 16 dpf (modified from Shin et al. [10, 15]).

2.4. Statistical Analysis

The results were reported as the sum of cumulative mortalities and abnormalities of embryos and sac-fry. If abnormal embryo was counted, sac-fry developed from an abnormal embryo was excluded from the calculation (even if the abnormal sac-fry manifested). The data were presented as mean \pm standard error. Means were compared using one-way analysis of variance (ANOVA) (Origin 8, OriginLab Corp.). Multiple comparisons were conducted using Fisher test and differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Physicochemical Characterization and Dissolution of TiO₂ NPs

Fig. 2 shows physicochemical characterization of TiO₂ NPs. Morphological TEM image of TiO₂ NPs is shown in Fig. 2(a). Fig. 2(b) shows distributions of the hydrodynamic diameter of TiO₂ NPs suspended in DW and ERS. According to Table 1, mean hydrodynamic diameter of TiO₂ NPs dispersed in ERS was estimated to be $1,218 \pm 109$ nm, which is 3.4-fold greater than the TiO₂ NPs dispersed in DW (354 ± 15 nm). The zeta potential of TiO₂ NPs was estimated to be -7.0 ± 2.4 mV (in ERS) and -2.0 ± 1.4 mV (in DW). This indicates that TiO₂ NPs are rapidly coagulated or flocculated in solution. The specific surface area of TiO₂ NPs analyzed using the Brunauer-Emmett-Teller (BET) method was estimated to be 55 ± 0.4 m²/g. The UV absorption spectra of 10 mg/L TiO₂ NPs suspended in ERS were determined at 10-min intervals for 1 h (Fig. 2(c)). TiO₂ NPs absorbed UVA light (320-400 nm) and peaked at 330-340 nm for TiO₂ NPs. The absorbance

Table 1. Glossary of Terms and Equations Used to Analyze Chlorophyll Fluorescence Transients

Properties	TiO ₂ NPs
Manufacturer	Evonik Degussa
Crystal structure ^a	Anatase 72.6%, Rutile 18.4%, Amorphous 9%
Surface coating ^a	No
Mean hydrodynamic diameter (nm) ^b	
Distilled water	354 ± 15
Embryo rearing solution	$1,218 \pm 109$
Zeta potential (mV) ^b	
Distilled water	-2.0 ± 1.4
Embryo rearing solution	-7.0 ± 2.4
Specific surface water (m ² /g) ^c	55 ± 0.4

^a supplied from the manufacturer.

^b measured by electrophoretic light scattering spectrophotometer (ELS).

^c measured by particle size analyzer.

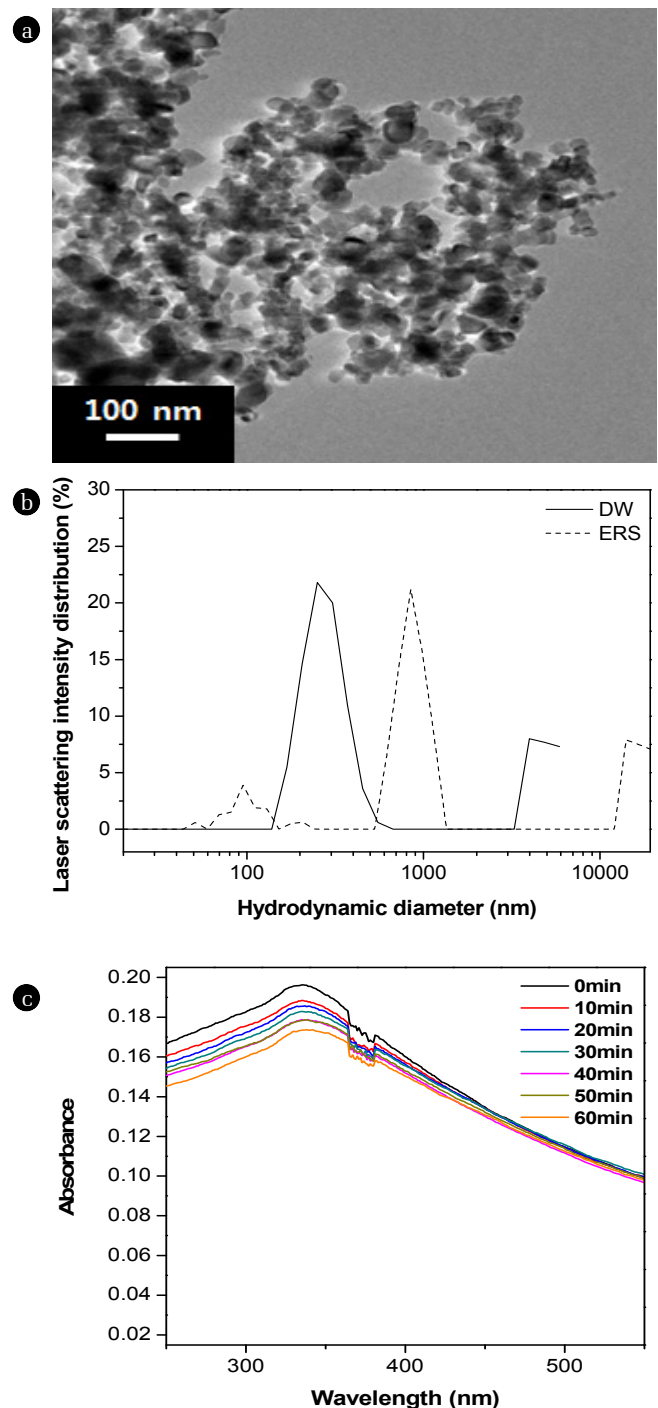


Fig. 2. Transmission electron microscope image (a), particle size distributions of hydrodynamic diameters (b), and absorption spectra (c) of TiO₂ NPs.

of TiO₂ NPs decreased as a function of increased irradiation time. This is possibly due to aggregation of NPs. When TiO₂ NPs were re-sonicated for 10 min, the absorbance of TiO₂ NPs increased (data not shown). In a previous study, Lee and An [12] reported the absorbance of TiO₂ NPs as a function of time until 1 h. They

reported the absorbance of TiO₂ dispersed in OECD algal medium decreased as a function of increased irradiation time, similar to the results obtained in this study.

3.2. Effects of TiO₂ NPs on the Embryos and Sac-fry of *O. latipes*

Fig. 3-5 show embryos and sac-fry at 16 dpf of exposure to TiO₂ NPs, under either visible light or UV irradiation. The abnormalities of embryos and sac-fry were increased at 10 and 50 mg/L NPs under UV irradiation, compared to the control under visible light irradiation or UV irradiation ($p < 0.05$) (Fig. 3). Under UV irradiation, compared to the control, at 50 mg/L TiO₂ NPs, some TiO₂ NP-treated embryos developed but exhibited abnormalities ($p < 0.05$). Tube heart, pericardial edema, and undeveloped or over-inflated swim bladder deformity were primarily induced as symptom of embryo and some exhibited hemostasis, malformation of pericardium, whole-body necrosis, pericardial edema, pericardial necrosis, and tail deformity (Fig. 4). In particular, the effect of TiO₂ NPs during the embryonic stages of some fish (10% for 1 mg/L

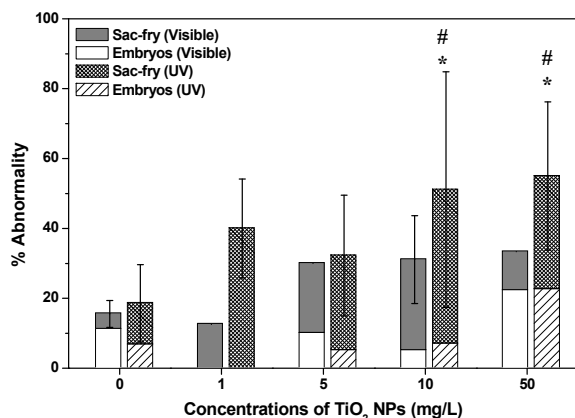


Fig. 3. Cumulative abnormality of TiO₂ NPs under visible light or UV irradiation on *Oryzias latipes*. Asterisks (*) indicate significant differences from controls under visible light irradiation ($p < 0.05$). Crosshatches (#) indicate significant differences from controls under UV light irradiation ($p < 0.05$).

and 10% for 50 mg/L) died as sac-fry, and embryonic exposure generated irreversible developmental impairment. Under visible light irradiation, some TiO₂ NP-treated embryos developed but exhibited abnormalities [e.g., deflated yolk, undeveloped or over-inflated swim bladder deformity, blood circulation disorder] at 50 mg/L TiO₂ NPs. The abnormalities shown by sac-fry included undeveloped or over-inflated swim bladder deformity and hemostasis. However, there was no significant increase of abnormality under visible light irradiation, compared to control ($p < 0.05$). There was no significant difference of abnormality between UV and visible light at the same exposure concentrations in the range of tested exposure concentrations ($p < 0.05$). No significant mortality of TiO₂ NP-treated sac-fry was observed under UV irradiation and visible light irradiation ($p < 0.05$) (Fig. 5).

3.3. Comparison of TiO₂ NPs on the Embryos and Sac-fry of *O. latipes* under UV and Visible Light

We studied the phototoxicity of TiO₂ NPs under UV and visible light using *O. latipes* embryos and sac-fry, and found that there are no significant differences at low exposure concentrations (0-50 mg/L). Likewise, Bar-Ilan et al. [4] studied the phototoxicity of anatase/rutile TiO₂ NPs using zebrafish embryos. They reported that there are no significant differences in the survival of embryos under UV or visible light at TiO₂ NP concentrations of 0, 1, and 10 mg/L. However, their significant differences of survival are observed at 100, 500, and 1,000 mg/L. Clemente et al. [1] studied the phototoxicity of anatase and anatase/rutile TiO₂ NPs using zebrafish eggs and larvae. They reported that there are no significant differences in the survival of eggs and larvae under UV or visible light at TiO₂ NP concentrations of 0, 1, and 10 mg/L. However, their significant differences of survival are observed at 100 mg/L. Meanwhile, Faria et al. [5] studied the phototoxicity of rutile and anatase/rutile TiO₂ NPs using zebrafish embryos. They reported that rutile TiO₂ NPs did not cause significant mortality at a concentration of 1 mg/mL under solar radiation. However, anatase/rutile TiO₂ NPs caused significant mortality at the same concentration and radiation. Felix et al. [11] studied the phototoxicity of anatase/rutile and anatase poly (acrylic acid)-coated TiO₂ NPs using

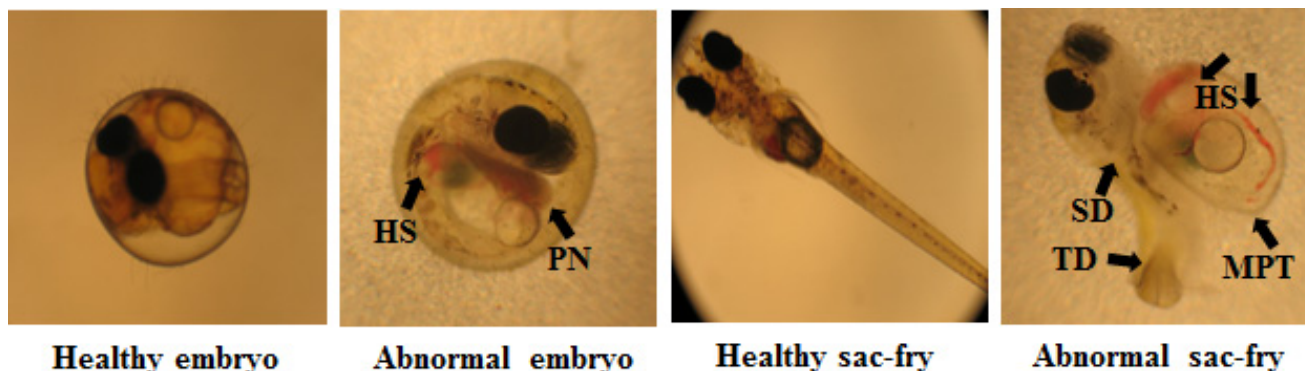


Fig. 4. Morphological appearance of *Oryzias latipes* exposed to TiO₂ NPs. HS: hemostasis, PN: pericardial necrosis, SD: swim bladder deformity, TD: tail deformity, and MPT: malformation of the pericardium and peritoneum.

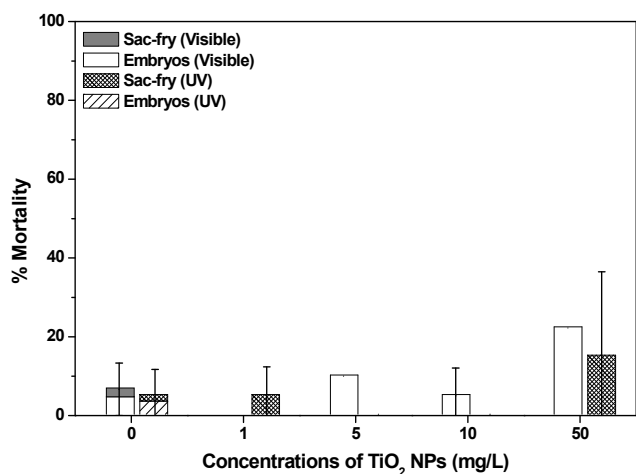


Fig. 5. Cumulative mortality of TiO₂ NPs under visible light or UV irradiation on *Oryzias latipes*.

zebrafish embryos and larvae. They reported that 10 mg/L uncoated TiO₂ NPs induced hydroxyl radicals, production of lipid peroxidation, and increase of total glutathione level under UV illumination, unlike poly (acrylic acid)-coated TiO₂ NPs, due to surface coating. It has been suggested that phototoxicity is due to the production of reaction oxygen species (ROS) that are generated by the exposure of TiO₂ NPs to UV light and damage the receptors [4, 7-8, 12]. However, the results showed that lethal phototoxicity occurs only at relatively high exposure concentrations (100-1,000 mg/L).

4. Conclusions

This study showed that TiO₂ NPs under UV or visible light cause abnormalities to *O. latipes* embryos and sac-fry at tested concentrations (10 and 50 mg/L), while the mortality of TiO₂ NP-treated sac-fry was not significant under UV or visible light. Additionally, no significant differences were identified under UV or visible light at the same low exposure concentrations (0-50 mg/L) of bare TiO₂ NPs. Therefore, further research is needed to assess the effects of higher exposure concentrations of TiO₂ NPs than those tested in this study and the effects of different surface coating of nanoparticles. Further research is also needed to evaluate the toxicity of NPs in natural surface waters for investigating their potential photo-activation and risk involved in biotic partitioning of NPs.

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