Pulmonary Clearance and Lesions in Rats after a Single Inhalation of Ultrafine Metallic Nickel at Dose Levels Comparable to the Threshold Limit Value

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Abstract: This study aimed to (1) determine the deposition and clearance rates of ultrafine metallic nickel (Uf-Ni) in rats after a 5 hours single inhalation exposure, and (2) to histopathologically examine the pulmonary lesions induced at dose levels comparable to the Occupational Exposure Limit recommended in Japan (OEL). The exposure concentrations of Uf-Ni for the 3 groups were 0.15 (Low), 1.14 (Medium), and 2.54 (High) mg/m³. Five rats/group were sacrificed at 0h and 1, 3, 7, 14, and 21 days post exposure. The amount of Ni in the lung accumulated dose-dependently. The half-times for Ni in the lung were estimated as 32 days on average, and were similar to each other regardless of the initial dosage. The histopathologically observed pulmonary lesions induced by a single inhalation of Uf-Ni were, (1) a significant increase in lung weight in the High and Medium groups with time, (2) accumulation of foamy alveolar macrophages (AM), (3) degenerated AM indicating alveolar lipoproteinosis which was aggravated for up to 4 weeks in the High group and (4) acute calcification of the degenerated AM was remarkable. The present results suggest that even a single inhalation of Uf-Ni induces potency of lung lesions at dose levels comparable to the OEL (1 mg/m³ as Ni), or the TWA of ACGIH (1.5 mg/m³ for elemental/metal).

Key words: Ultrafine metallic nickel, Inhalation exposure, Deposition and clearance, Lipoproteinosis, Calcification of alveolar macrophages

Introduction

Many workers⁽¹⁾ in the occupational environment have been exposed to nickel (Ni) in the forms of metal, various alloys and compounds. The Japan Society for Occupational Health has recommended that the Occupational Exposure Limit (OEL) for Ni as 1 mg/m³, except for nickel carbonyl, since 1967⁽²⁾. Many reports^(3,4) on the toxicity of water soluble Ni compounds clearly showed evidence of irritation and injuries to the lungs. These reports showed that the injuries caused by water soluble Ni compounds were more severe than those caused by water insoluble Ni compounds or metallic Ni,

but the injuries recovered quickly due to high clearance of water soluble Ni from the lungs. Recently Kyono *et al.*⁽⁵⁾ showed that exposure to highly water soluble NiCl₂ aerosols at a lower concentration than that of OEL developed bronchiolitis in rats exposed only for 5 h/d for 5 days.

On the other hand, as for water insoluble Ni metal and NiO, it has been reported that in spite of their lower initial effect compared to those caused by water soluble Ni compounds, their higher accumulation in the lungs or longer half-time contributed to the induction of alveolar lipoproteinosis or human lung cancer^(1,6).

In 1998, ACGIH adopted a new TWA for Ni and its compounds. The newly adopted values are 1.5 mg/m³, 0.1 mg/m³ as Ni, and 0.2 mg/m³ as Ni for elemental/metal, soluble

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compounds, and insoluble compounds of Ni, respectively⁷⁾.

Recent new technology has produced various new ultrafine inorganic particles with diameters in the order of nano meters. These particles, such as ultrafine nickel (Uf-Ni), ultrafine cobalt (Uf-Co) and ultrafine titanium dioxide (Uf-Ti), have some specific characteristics, e.g. a high level of surface energy, large surface area and high magnetism, etc. The solubilities of Uf-Co in water and artificial lung fluid⁸⁾ were higher than ordinary cobalt particles micro meters in diameter. Although available reports on the toxic effects of ultrafine metals on the lungs are limited in number, most of them report higher toxicity in the lungs of ultrafine metals than metallic powder of common respirable size⁹⁻¹²⁾.

The present study aimed to 1) determine the deposition and clearance rates of Uf-Ni after a single exposure in rats, and 2) examine the pulmonary lesions at comparable levels to the OEL and/or TWA by histopathological observations.

Materials and Methods

Animals

All rats used in the present experiments were male Wistar-jcl strain purchased from Clea Japan Inc. at 6-weeks of age and they were kept in our animal facility until use. The rats were housed 6 per stainless wire cage and supplied with sterilized food (CE-1) and UV irradiated water *ad libitum*. Rats at 10-weeks of age weighing 270–290 g were moved to an inhalation chamber and a control chamber.

Subject material and exposure system

Uf-Ni of the impurity less than 0.1% as metal and average particle diameter of 20 nm was purchased from Vacuum Metallurgical Co., Japan. The exposure system comprised an ultrasonic nebulizer, an aerosol dryer, an exposure chamber and a clean air chamber¹³⁾. Aerosols of Uf-Ni were generated with an ultrasonic nebulizer. Passing the mist, generated by the ultrasonic nebulizer from a suspension of 1 g Uf-Ni in 1 liter of distilled water, through an aerosol dryer, the exposure aerosol in an agglomeration of Uf-Ni was generated¹³⁾. The mass median aerodynamic diameter (MMAD) of agglomerated Uf-Ni, measured by means of an Andersen sampler, was 1.3 μm with a geometric standard deviation (σ_g) of 1.54. The capacity of both chambers, inhalation and control, was 1.2 m³ accommodating 12 wire net cages, and they were ventilated 20 times per hour.

Solubility of Uf-Ni

The solubilities of Uf-Ni in de-ionized water and Gamble's

Table 1. Experimental design

Groups		Number of rats	Postexposure [day]								
			0	1	3	7	14	21	28	56	84
High	Exp.	32	5	5	5	5	5	5	2	–	–
	Cont.	30	5	5	5	5	5	5	–	–	–
Medium	Exp.	30	5	5	5	5	5	5	–	–	–
	Cont.	30	5	5	5	5	5	5	–	–	–
Low	Exp.	40	5	5	5	5	5	–	5	5	5
	Cont.	40	5	5	5	5	5	–	5	5	5

Exp.: Uf-Ni inhalation, Cont.: Clean air control.

physiological solution¹⁴⁾ were measured as follows; 1) three 60 mg samples of Uf-Ni, suspended in 60 ml of de-ionized water and in Gamble's solution in 100 ml polyethylene bottles, were shaken for 48 hours in a water bath at 37°C, 2) the supernatant solutions were obtained after centrifugation at 3000 rpm for 20 min, and 3) the concentrations of Ni ion in the supernatant of water or Gamble's solution were determined five times for each bottle by Inductivity Coupled Plasma Atomic Emission Spectrometry (ICP-AES).

Experimental design

The inhalation experiments were done at three concentration levels of Ni: $2.54 \pm 0.27 \text{ mg/m}^3$ (High), $1.14 \pm 0.19 \text{ mg/m}^3$ (Medium) and $0.15 \pm 0.04 \text{ mg/m}^3$ (Low). The numbers of rats used in the High, Medium and Low groups were 62, 60 and 80, respectively. Half of the rats in each group were exposed whole-body to Uf-Ni aerosol for 5 hours, and the rest were put for 5 hours in a chamber which was supplied with filtered clean air as the control. During exposure, 5 or 6 rats were accommodated in a wire net cage which was separated into 6 cells to prevent the rats contacting each other, and drinking water and food were withheld. After a single 5 hours exposure of to Uf-Ni aerosol, 5 rats/group in the High and Medium groups were sacrificed immediately (0 hour), and after 1, 3, 7, 14 and 21 days. At 28 days postexposure two rats in the High group were sacrificed for histopathological observations. 5 rats/group in the Low group were sacrificed immediately (0 hour), and after 1, 3, 7, 14, 28, 56 and 84 days postexposure, as shown in Table 1.

Analysis of Ni contents in the lungs and deposition ratio of Ni

The right lungs obtained from the experimental rats were digested with concentrated nitric acid and a hydrogen peroxide in Kjeldahl flasks on a hot plate. The digestants dispersed in distilled water were analyzed for nickel content by atomic absorption spectrometry (Hitachi Zeeman Atomic

Absorption Spectrophotometer, Hitachi Co. Japan).

The ratio of deposition of Ni was expressed as the ratio of the Ni content in whole lung (Ni in right lung $\times$ whole lung weight/right lung weight) against the total amount of Ni inhaled. The total amount of Ni inhaled was calculated by the Ni concentration in exposed air $\times$ total volume of exposed air during the exposure periods. The total volume of inhaled air was calculated according to the reported data¹⁵⁾ for rats of the same strain and the same age (46 ± 2 ml/min/100 g body weight was used as the ventilation value for one minute).

Tissue samples

Each rat of 5 rats/group was anesthetized by intraperitoneal injection of pentobarbital sodium and the whole trachea-lung and heart were taken out en bloc after exsanguination by cutting the abdominal aorta and vein. The total weight of lungs and heart (A) was found, and then the hilum of the left lung was ligated and 10% buffered neutral formalin was instilled into the right lung via the trachea. The right lung was cut out and prepared for histopathological observation. The left lung was cut out and analyzed to find the amount of Ni in the lung. The other organs including the left lung, heart and trachea were weighed, and their total weight was subtracted from total weight (A) to find the weight of the right lung.

Histopathological examinations

After fixation of the left lobes for one week, three longitudinal tissue slices including the main intrapulmonary bronchus were prepared according to the routine method, and embedded in paraffin. 3 μ m sections were cut and stained with hematoxylin and eosin (HE), and/or Alcian blue (pH. 2.5) and Periodic acid Schiff (AB/PAS).

Table 2. Solubility of Uf-Ni in water and Gamble's solution

De-ionized water	9.93 ± 0.16	[μ g/ml]
Gamble's solution	106.23 ± 10.18	[μ g/ml]

Table 3. Deposition ratio of Uf-Ni in the lungs

Experimental groups		High	Mid	Low
Concentration of Ni exposed	[mg/m ³]	2.54 ± 0.27	1.14 ± 0.19	0.15 ± 0.04
Weight of lungs	[mg]	1090.8 ± 90.2	950.0 ± 51.7	1041.8 ± 23.1
Content of Ni in the lungs	[μ g]	33.5 ± 3.8	10.1 ± 1.1	1.4 ± 0.2
Total amount of Ni inhaled*	[μ g]	98.8 ± 2.1	43.0 ± 1.8	5.7 ± 0.2
Deposition ratio	[%]	33.9	23.4	23.5

Values for lung weight, Ni content in the lungs, and estimated total amount of Ni were the mean $\pm$ SD. n=5.

*The total amount of inhaled Ni in each animal was calculated as follows: minutes ventilation (46 ml/min/100 g) $\times$ body weight/100 $\times$ Ni concentrations (2.54, 1.14 or 0.45 mg/m³) $\times$ inhalation period (300 min).

Fig. 1. Clearance of nickel in the lungs after exposure.

The lines represent the data obtained from high (◆), medium (■), and low (▲) dosage groups, respectively. n=5. The Ni content in the lungs of the control rats are less than 7 ng/lung, and not shown in the figure.

Statistical analysis

All results are expressed as the mean $\pm$ standard deviation (SD). Statistical analysis was carried out with a software package Statview. The significant difference between control and experimental animals was calculated by analysis of variance (ANOVA). The criterion of significance was set at 5 %.

Results

Solubility of Uf-Ni

The solubilities of Uf-Ni in de-ionized water and Gamble's solution were 9.93 ± 0.16 μ g/ml, and 106.23 ± 10.18 μ g/ml, respectively (Table 2).

Deposition and clearance of Uf-Ni in the lungs

The ratios of deposition of Uf-Ni in the lungs of the three experimental groups are shown on Table 3. The values for the High, Medium and Low groups were 33.9%, 23.4%,

Fig. 2. Changes in the body weight of rats after a single exposure to Uf-Ni.

The data obtained from the control (open), and exposed rats (dot meshed). Figures represent, from top to bottom, high, medium, and low dosage, respectively. Values are the mean $\pm$ SD. $n=5$. *: Statistical significance level set at 5% vs control by ANOVA.

and 23.5%, respectively. Fig. 1 shows decreases in Ni deposited in the lungs with the time-course clearance at the three doses. Although the amounts of Ni deposited in the lungs immediately after the exposure were different dose-dependently, their half-times were all similar for the three different dosages at 28 to 39 days.

Body weight and lung weight

There was no difference between control and exposed

Fig. 3. Changes in the wet lung weight of rats after a single exposure to Uf-Ni.

The data obtained from the control (open), and exposed rats (dot meshed). Figures represent, from top to bottom, high, medium, and low dosage, respectively. Values are the mean $\pm$ SD. $n=5$. *, **, ***: Statistical significance levels set at 5%, 1% and 0.1% vs control by ANOVA.

groups in body weight throughout the periods of observation in the High and Medium dose groups, but the average body weight of the Low group was significantly lower than that of the control group at day 28 (Fig. 2). During the first 3 days after exposure to Uf-Ni, no difference was found between the control and the exposed groups in the wet weight of the whole lungs. After 7 days, the lung weight increased significantly with time in the exposed groups as compared to the corresponding control groups (Fig. 3). The increment

Table 4. Ratio of lung weight (exposed/control)

groups \ days	0	1	3	7	14	21	28
High	1.04	0.98	1.05	1.24***	1.34***	1.38***	–
Medium	1.00	1.03	1.12	1.11*	1.23*	1.30**	–
Low	1.01	0.98	1.05	1.02	1.01	–	0.93*

Fig. 4. Light photomicrographs of the airways from a control rat.

(A) Pulmonary large (axial) bronchiole, (B) medium-size bronchiole, and (C) the terminal bronchiole. The airway epithelium of both ciliated and non-ciliated show their normal morphology, and there is no infiltration of inflammatory cells in the submucosa or peri-vascular connective tissues. Alveolar structures appear normal. Stained with HE.

ratios for the weight of lungs (exposed/control) were dependent to the dose of Ni (Table 4).

Histopathological observation

In the control groups, morphology of the epithelium in the intrapulmonary airways, the parenchymal structure, and the size of alveolar macrophages (AM) appeared normal throughout the postexposure periods (Fig. 4). Meanwhile in High group immediately after the exposure, sporadic foci of sparse infiltration of polymorphonuclear leukocytes (PMN) were found at the peri-vascular interstitia of the small arterioles associated with the terminal airways (Figure not shown). The morphology of AMs with phagocytized Uf-Ni appeared within the normal range (Fig. 5A), but at day 3 postexposure, sporadic foci of the injured airway epithelium were noticed, where the mucus layer was covered with the

debris of particle-laden AM (Fig. 5B). Initial lesions in the epithelium were characterized by damage to cilia and influx of inflammatory cells into the intercellular space in the airway epithelium. Focal hypertrophy and hyperplasia of the airway epithelium were most prominent in the medium size bronchioles (M-br) (Fig. 6A). At one week postexposure, epithelial hyperplasia, goblet cell hyperplasia, and submucosal infiltration of PMN were most prominent at the bifurcation of the medium sized to small bronchioles (S-br) (Fig. 6B). Epithelial hypertrophy had also progressed to the intrapulmonary large bronchus (L-br), but the cilia of the ciliated cells were well preserved in this level of the bronchus, and a mild goblet cell hyperplasia was observed in all the rats (Fig. 7).

Slight damage to the Ni-laden AMs was first noticed at one week postexposure, these were enlarged foamy

Fig. 5. Light photomicrographs from rats which received high dose exposure to Uf-Ni. (A) A terminal airway and adjacent alveoli showing normal structure just after the termination of exposure. Arrows indicate alveolar macrophages (AM) with phagocytized Uf-Ni particles. T: terminal airway. (B) A small bronchiole 3 days after exposure. Slightly hypertrophic epithelia and loss of cilia (arrow-heads) indicate the initial lesion in the bronchiole. Some of the particle-laden AMs are decomposing and covering the surface of epithelia intermingled with mucus (arrow). SL: small bronchiolar lumen. Stained with HE.

Fig. 6. Light photomicrographs from rats which received high dose exposure to Uf-Ni. (A) A medium-size bronchiole (M-br) 3 days after exposure. Hypertrophy and hyperplasia of the epithelium are more progressed in the M-br level (compare with Fig. 5-B). Infiltration of inflammatory cells in the submucosal connective tissue is prominent. ML: M-br lumen. (B) A medium-size bronchiole (ML: M-br lumen) one week after exposure. At the bronchial bifurcation towards the small bronchioles (S), submucosal infiltration of inflammatory cells continues (arrow). Stained with HE.

Fig. 7. Photomicrographs of two continuous sections from an intrapulmonary large bronchiole at one week after exposure to high dosage of Uf-Ni.

(A) In a L-br, a mild goblet cell hyperplasia is noticed (arrows). V: blood vessel. Stained with AB (pH. 2.5) and PAS. (B) The continuous section stained with HE, showing a slightly hypertrophic epithelium, but cilia are well preserved. V: blood vessel.

Fig. 8. Photomicrographs of alveoli from rats which received high dose exposure to Uf-Ni.

(A) The terminal airway (T) and alveoli after 2 weeks. Epithelium of the terminal airway has returned to normal. No change in the number of AMs (arrows). Stained with HE. (B) At 3 weeks postexposure, increased number of larger size AMs (big arrows), and decomposed debris of AMs (small arrows) are observed together with inhaled Uf-Ni particles. Stained with HE.

Fig. 9. Photomicrographs showing calcification of AMs 4 weeks after exposure to a high dose.

(A) At 4 weeks postexposure, the epithelium of M-br (ML) and S-br (SL) return to normal appearance, but in the alveoli calcification of decomposed AMs becomes clear and only a living AMs (small arrow) are few found. Stained with HE. (B) A focus of advanced calcification (mineralization) of AM characterized by the appearance of flakes of broken shells (big arrows), that show strong positive staining by Hematoxylin or PAS. A small number of both normal and foamy AMs (small arrows) are also seen. Stained with HE.

macrophages or degenerated macrophages, and the lesions became more severe with time during the postexposure period until 4 weeks (Fig. 8). At 4 weeks postexposure, most of the alveoli were filled with foamy AM and debris of burst AM. Calcification (or mineralization) started at 4 weeks in some of the burst residues of AMs - those located predominantly in the alveolar ducts (Fig. 9), and they stained a clear red color with PAS (Fig. 9B). The pathology of the lungs at 4 weeks after Uf-Ni exposure corresponded to alveolar lipoproteinosis¹⁶⁾, most of the peripheral alveoli and the subpleural zone of the lung being occupied by foamy AM (Fig. 10). Nevertheless, the airway epithelium from the proximal to the distal bronchial tree completely recovered after one week and there was no lesion noticed at all.

Fig. 10. A focus of alveolar lipoproteinosis developed after 4 weeks in the High group.

At 4 weeks postexposure, there appear foci of alveolar lipoproteinosis predominantly in peripheral and subpleural regions. In those foci, accumulation of small round cells in the perivascular area is also prominent (arrow). Most of the AM occupying the alveolar lumen are foamy or binucleated. Stained with HE.

Fig. 11. Photomicrographs from rats which received exposure to Uf-Ni at medium or low doses.

(A) A representative area of alveoli from a rat which received a medium dosage of Uf-Ni. At 3 weeks postexposure, a few foamy AMs with inhaled particles are observed (big arrow). Debris of decomposed AMs with inhaled particles are also seen (small arrows). Stained with HE. (B) A representative area of alveoli from a rat which received a low dosage of Uf-Ni. At one week postexposure, there is no remarkable change observed. Airway epithelium both in the M-br (M) and S-br (S) looks normal and no inflammatory cells are seen. Stained with HE.

Pathological observations on the Medium group revealed a far milder but the same type of initial lesions as found in the High group at 3 weeks postexposure (Fig. 11A), but no remarkable change had occurred in the Low group throughout the postexposure periods (Fig. 11B).

Discussions

The observed deposition ratios showed that the ratio for the High dose group of 33.9% was 1.5 times higher than those of the Medium and Low groups. No comparable data for the initial deposition on Uf-Ni have been available, but the initial deposition of the inhaled ^{63}NiO in rats with comparable particle diameter to those in the present study was 4.3% immediately after the 70 min exposure to $9.9 \text{ mg } ^{63}\text{NiO}/\text{m}^3$ (MMAD=1.39 μm ; $\sigma_g=2.74$). The half-time for ^{63}NiO was reported as 120 days^{17,18}. The half-time for inhaled nickel fumes, composed of 97 to 99% NiO and the remainder of Ni_2O_3 ¹⁹, was about half a year in our previous study. Since the half-times of the deposited Uf-Ni from the lung in our study were between 28 and 39 days, Uf-Ni would have a higher deposition ratio and a shorter half-time than NiO.

Each analytical electron microscopic observation of the Uf-Ni revealed that the Uf-Ni particle consisted of metallic Ni covered by a very thin surface layer of NiO (Kohyama, unpublished data). Sakabe and Kohyama *et al.*²⁰ reported that the amorphous surface layers covering ground quartz particles under 1 μm in diameter were quickly dissolved in Gamble's solution and their crystalline cores appeared on the surface. Andersen *et al.*²¹ reported that metallic Ni was soluble in physiological solutions under certain conditions, whereas NiO seemed to be almost insoluble. The shorter half-time of Uf-Ni than NiO from the lungs may be due to their higher solubility in the physiological solution (Table 2).

Remarkable increases in lung weight both in the High and Medium groups were noticed after 7 days, though there was no significant change in body weight after the exposure in the two experimental groups. The one significant body weight found in the Low group at day 28 may have chanced to be due to the lower mean body weight of this group. The increases in lung weight progressed with time even after 7 days and the ratios of exposed/control were larger in the High group than those in the Medium group. In the case of water soluble NiCl_2 , the increase in lung weight were induced

shortly after inhalation⁵). For exposure to NiO, the lung weight had been increased when observed after 2 months exposure¹⁸).

Not a lung weight increase but comparative acute toxicity evaluated by some biochemical markers showed that such toxicity appeared in the rats within a week after exposure to Ni compounds (NiCl₂, NiSO₄, and Ni₃S₂) but not for NiO. These results relate to the solubility of these compounds^{22, 23}. These phenomena imply that the half-times and solubility of Ni compounds strongly relate to the toxicity of tissue injuries.

Inhalation of low concentrations of metallic Ni dust (0.1–1 mg/m³) for 1 to 8 months, or NiCl₂ (0.3 mg/m³ as Ni) for about one month induced in rabbits the following common toxic effects: 1) increased number of AM with engulfed phospholipid indicating alveolar lipoproteinosis, and 2) swelling of type 2 epithelium accompanying surfactant overproduction^{24–27}. Mild progression of chronic alveolitis and AM hyperplasia were due to inhalation of NiO by the rats for several months^{18, 28}. The present results showed that hypertrophy and hyperplasia of bronchial epithelium accompanied with goblet cell hyperplasia were induced by 7 days, and recovered at around 2 weeks postexposure. On the other hand, the initial change in alveolar lipoproteinosis, observed at 3 weeks postexposure, gradually progressed with time after a single inhalation of Uf-Ni. The pathological character of alveolar lipoproteinosis was the same as those caused by chronic inhalation of the several kinds of Ni compounds mentioned above. One of the most remarkable pathological changes seen in the present study was that some Uf-Ni-laden AMs decomposed in the alveoli and accumulated in situ as calcified debris by 4 weeks postexposure. A large calcified flake is sporadically found in normal rat alveoli, but there has been no mention of calcification of burst AMs at 4 weeks after a single inhalation of Ni compounds as far as we know. There is no direct evidence that this acute calcification progressed for 3 weeks, but this might be related to a disturbance of membranous Ca²⁺ ion transport by solubilized Ni²⁺ ion²⁹). Because both calcified debris of AMs and subpleural accumulation of foamy AMs may restrict the pulmonary ventilation to some extent, these lesions should not be neglected when assessing the toxicity of Uf-Ni in the lungs.

The concentration of exposed Uf-Ni in the High group was of the same level as the TWA by ACGIH in 1998⁷). Nevertheless, we observed that the pulmonary lesions progressed with time after exposure, and the weight of the lungs had increased at least day 21 postexposure. Therefore, the results for the High group indicate that even a single

exposure to Uf-Ni at the level of TWA by ACGHI is not always a non-effective level in the rat. We also found a milder but rather similar tendency in the Medium group, which received less than half the amount of Uf-Ni that the High group received. In the Low group, no remarkable effect was observed under the present experimental conditions, but the exposed concentration in the Low group was only about 6% of that in the High group. Considering the half-time of inhaled Uf-Ni, i.e. about 30 days, even the dose in the Low group might not always be a non-effective level when exposure was repeated. If half the amount of Uf-Ni deposited in the lungs in the Low group were carried over to the next day after a single exposure, the total amount of deposition after daily exposure for about 30 days would exceed the amount for the High group. Therefore, the present results suggest potent adverse health effects of Uf-Ni on workers who handle these materials at around the permissible TWA levels in the workplace.

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