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Gene Expression Analysis in Male Rat Liver After Oral Exposure to Titanium Dioxide Nanoparticles

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ABSTRACT

Nanotechnology is considered a new technology playing an essential role in several science fields. Titanium dioxide nanoparticles (TiO_2 NPs) are extensively utilized inseveral fields like electronics, medicine, agriculture, food additives, paint, and sunscreens. Oral exposure mainly occurs through food products containing TiO_2 NP-additives. Therefore, exposure to TiO_2 NPs orally may have some adverse effects on exposed mammals and thus might be in potential health risk. The current study aims to study the possibility of liver damage in male rats exposed to a single oral dose (250 mg/kg b.w.) of 25 ± 5 TiO_2 NPs. The gene expression of oxidative stress genes (Gpx and Cu-Zn SOD), protein folding gene (Hsp70), apoptosis gene (p53), metal toxicity gene (Mt1) as target genes, and GAPDH as housekeeping genes were determined. The results indicated that significant over-expression of the oxidative stress, apoptosis and protein folding genes was observed in the liver at 7 days post -treatment while there were no significant changes at day 28 as compared with control. Meanwhile, expression of the metal toxicity gene was increased significantly at both 7 and 28 days after exposure.Based on the above-mentioned observation, it should be considered to possible health risks associated with the consumption of food products containing high concentrations of TiO_2 NP-additives.

1. INTRODUCTION

Nanotechnology is the science of manipulating materials at very small scales (1.0 to 100 nm) at the atomic and molecular levels [1].Nanotechnology, could be considered a new technology playing an essential role in several fields like electronics, medicine and agriculture. Nanoparticles that are used in this technology have unique chemical, physical and biological characteristics such as high electrical conductivity, more chemical reactivity, and extraordinary strength [2, 3]. Nano-metal oxides can be used in piezoelectric devices, sensors, fuel cells, anti-corrosion coatings, and encapsulation of tailored nano pesticides and nano fertilizers, and also as catalysts [4].

TiO_2 NPs have a large surface area [5], photocatalytic characteristics, a high redox potential, and heat and magnetism sensitivity[6].Because, of their distinct characteristics nano$TiO_2$are commonly utilized as a dye or sunscreen, applied in paint, food, lotions, and toothpaste. $TiO_2$is applied in clinical medicine as a photosensitizer for photodynamic therapy[7], in the clinical applications as carrier platforms[8], for drug delivery[9], and as a cancer photothermal therapy[10].TiO_2 NPs have the same mechanism as the bulk powder based on the reactive oxygen species (ROS) generation, but the nanoscale nature has several advantages; one of them is the increased surface area which allows for maximum contact with water and oxygen in the environment[11]. The second characteristicis its small size, which enables it to easily penetrate the cell wall and membrane, increasing intracellular oxidative damage[12]. TiO_2 NPs can also infiltrate the nucleus causing DNA damage or altering gene expression[13].

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Several investigations have indicated the effects of TiO_2 NPs on intestine and liver in rats and clarified that TiO_2 NP increased apoptosis, oxidative stress, and cytotoxicity.TiO_2 NPs interact with cytoplasmic proteome and fetch posttranslational modifications by oxidative stress and other mechanisms [14]. They affect the endoplasmic reticulum function, and either enter the nucleus or block nuclear pore and interact with DNA causing the up-regulation of cytokines, oxidative stress, and apoptosis-related genes[12, 15].

TiO_2 nanoparticles up-regulate mRNA expression of oxidative-stress-related genes, induce oxidative DNA damage, and production of intracellular reactive oxygen species [16]. Exposure to TiO_2 NPs changed the antioxidant enzyme activity and gene expressions of GPx, SOD, and CAT. Also, it significantly increased gene expressions of p53, and Bax in exposed rats when compared with the control rats [17, 18].

Metallothioneins (MTs) belong to an intracellular cysteine-rich group as cadmium-binding proteins, isolated from horse kidney, and is found in microorganisms, plants, vertebrates, and invertebrates [19, 20, 21]. Metallothioneins (MTs) play a key role in heavy metal detoxification and essential metal ion balance. MTs may provide protection against metal toxicity and oxidative stress [22].

Food products containing nanoTiO_2-additives are the most common source of oral exposure. [23]. Therefore, oral exposure to TiO_2 NPs may have some adverse effects on mammals and thus the safety of their use needs to be evaluated.

In the present study, changes of the expression of genes related to oxidative stress (SOD and GPx), protein folding (Hsp70), apoptosis (p53), and metal toxicity (Mt1) were determined in male rat liver exposed orally to 250 mg 25 ±5 nm TiO_2 NPs/ kg b.w.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

TiO_2NPs were purchased from NanoTech, Egypt. All reagents and kits were supplied by Thermo Fisher Scientific, USA.

2.2. Transmission electron microscopy Analysis and X-ray diffractionPattern of TiO_2 NPs

The commercial TiO_2 NPs used in this study were suspended in de-ionized water, sonicated for 20 minutes, and then placed on carbon-coated copper TEM grids, which were subsequently dried before measurement. A transmission electron microscope (TEM) Model JEM - 2100, JEOL, Japan, was used to examine the nanoparticles morphology and size, whereas an X-ray diffractometer was used to study the NPs crystalline pattern and phase structure.

2.3. Animals and Treatments

Adult male Wistar rats *Rattusnorvegicus* averaging 120-140 g were procured from the National Research Center's animal house in Giza, Egypt, and were given two weeks to acclimate before being treated.Rats were housed in plastic cages and kept in animal house (24°C, 12:12 h, light:dark cycle). Animals were distributed into two groups,12 rats each. The first group of rats received a single dose (250 mg/kg b.w.) of TiO_2 NPs in 0.5 ml corn oil by oral gavage. The second group served as control and the animals received only 0.5 ml corn oil. The animals' behaviors and symptoms were carefully observed daily for 28 days post-exposure. At days 7 and 28 after exposure, rats were anaesthetized via inhalation using diethyl-ether,then the liver was removed, frozen in liquid nitrogen and subjected to RNA extraction and determination.

2.3.1. RNA extraction and determination

Total RNA was extracted from about 30 mg frozen liver tissue using GeneJET RNA Purification Kit (Thermo Fisher Scientific, concentrations were measured using Nabi UV/Vis nano Spectrophotometer (Nano Drop) and the purity was estimated by the OD_{260}/OD_{280} absorption ratio.

cDNA synthesis via reverse transcription was carried out according to the instruction of cDNA synthesis kit [24].cDNA was reverse transcribed from500 ng total RNA combined with 1µl of random hexamer primer and Water nuclease-free to 12µl final volume. The mixture was centrifuged briefly and incubated at 65°C for 5 min to denature secondary structures then chilled on ice while adding 4 µl of 5X reaction buffer, 1µl of RNase inhibitor (20 U/µl),2µl of 10 mMdNTP and 1µlof

Reverse Transcriptase enzyme (200 U/µl) (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, USA).The RT mixture was briefly centrifuged before being incubated for 5 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 70°C.

2.3.2. Real Time PCR and Data Analysis

Gpx, Cu-ZnSOD, Hsp70, p53, Mt1as target genes, and GAPDH as housekeeping gene were amplified with PCR in a Rotor-Gene real-time fluorescence thermal cycler (Corbett Ltd., Australia) with a heated lid (105°C) using the programs presented in Table 1. All primers were purchased from Jena Bioscience, Germany. Three µl of cDNA, 1 µl of specific primers, 5 µl of DNase-free water, and 10 µl of SYBER Green master mix were used for amplification[25].For verifying the amplification specificity and distinguishing any artifacts from the specific amplicons, melting curves were generated by denaturing the PCR products from (55-99°C). Optical data were collected through the duration of temperature, increase gradual drop in fluorescence seen when the strands reannealed. The ΔΔCt method was used to calculate the relative expression of real-time PCR products as well as the fold change in target genes[26].The gene expression was expressed as$2^{-(\Delta\Delta Ct)}$ where: ΔCt is the difference in Ct (Crossing threshold) values for the tested gene and the housekeeping gene (GADPH); and ΔΔCt = ΔCt of the tested gene - ΔCt of the control.

2.4. Data analysis

All experimental values were compared to their corresponding control values, and data were given as mean ± SD. SPSS version 24 for Windows (IBM, Armonk, NY, USA) was used to determine differences in mean values using one-way ANOVA. $P < 0.01$wasconsidered to be statistically significant.

Table (1): Primers sequences and PCR program

Gene		Sequence 5' – 3'	Annealing temp (˚C)	Size (bp)	Reference
Gpx	F	CTCTCCGCGGTGGCACAGT	58	290	[27]
	R	CCACCACCGGGTCGGACATAC			
Cu-ZnSOD	F	GCAGAAGGCAAGCGGTGAAC	58	447	[27]
	R	TAGCAGGACAGCAGATGAGT			
p53	F	CTACTAAGGTCGTGAGACGCTGCC	60	106	[28]
	R	TCAGCATACAGGTTTCCTTCCACC			
Hsp70	F	ATGCGCTCGAGTCCTACGCCTT	59	71	[29]
	R	GCTGATCTTGCCCTTGAGACCCTC			
Mt1	F	CACCGTTGCTCCAGATTCAC	60	238	[30]
	R	GCAGCAGCACTGTTCGTCAC			
GAPDH	F	CACCCTGTTGCTGTAGCCATATTC	57	204	[31]
	R	GACATCAAGAAGGTGGTGAAGCAG			

3. RESULTS

3.1. Transmission Electron Microscopy Analysis and X-ray diffraction Pattern of TiO_2 NPs

As shown in Figure 1, the TEM image revealed that the major entity of the examined $TiO_2$was 25± 5 nm and had a reasonable narrow size distribution with no aggregation.

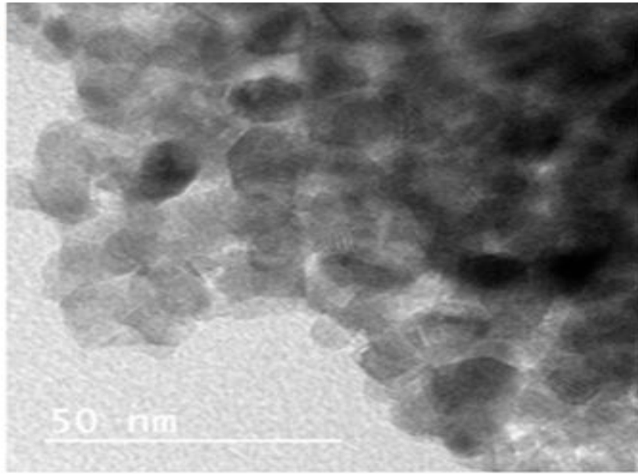

Fig. (1): Transmission electron microscope image of TiO_2NPs.

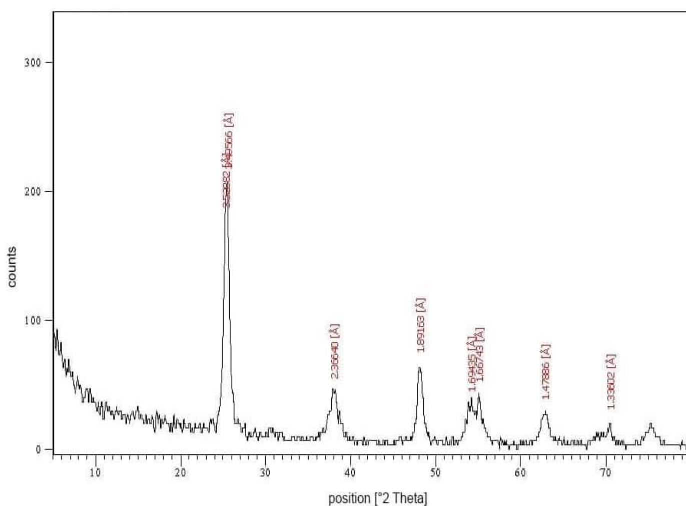

Fig. (2): X-ray diffraction pattern of TiO_2NPs

Figure 2 shows the XRD pattern of TiO_2 NPs. There were no impurity peaks. The positions of all the diffraction peaks were harmonious with the crystalline pattern of titanium dioxide

3.2. Gene expression analysis

Gene expression changes of the oxidative stress genes (Cu-Zn SOD and Gpx), Protein folding gene (Hsp70), apoptosis gene (p53), and metal toxicity gene (Mt1)as target genes, and Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) as housekeeping gene were determined in male rat liver samples by Real-time PCR. The rats were exposed orally to 250 mg 25 ±5 nm TiO_2 NPs/ kg b.w. and the gene expression was recorded at 2 times post-exposure.

The data was expressed as $2^{-\Delta\Delta Ct}$ and presented in Fig. 3. The controls were resulted 2^0=1 as the ΔCt of the control conditions was subtracted from those of the treated conditions. The obtained results showed a significant up-regulation of Cu-Zn SOD; Gpx; Hsp70; and p53 genes in the liver of exposed male rats 7days post exposure while gene expression profiles at 28 days did not differ significantly from the controls (Fig. 3 A-D). The increase in expression was more pronounced with Gpx followed by Hsp 70, P53 and then SOD. On the other hand, expression of the metal toxicity gene (Mt1) was up-regulated significantly in the male rat liver at 7 and 28 days after exposure (Fig. 3-E).

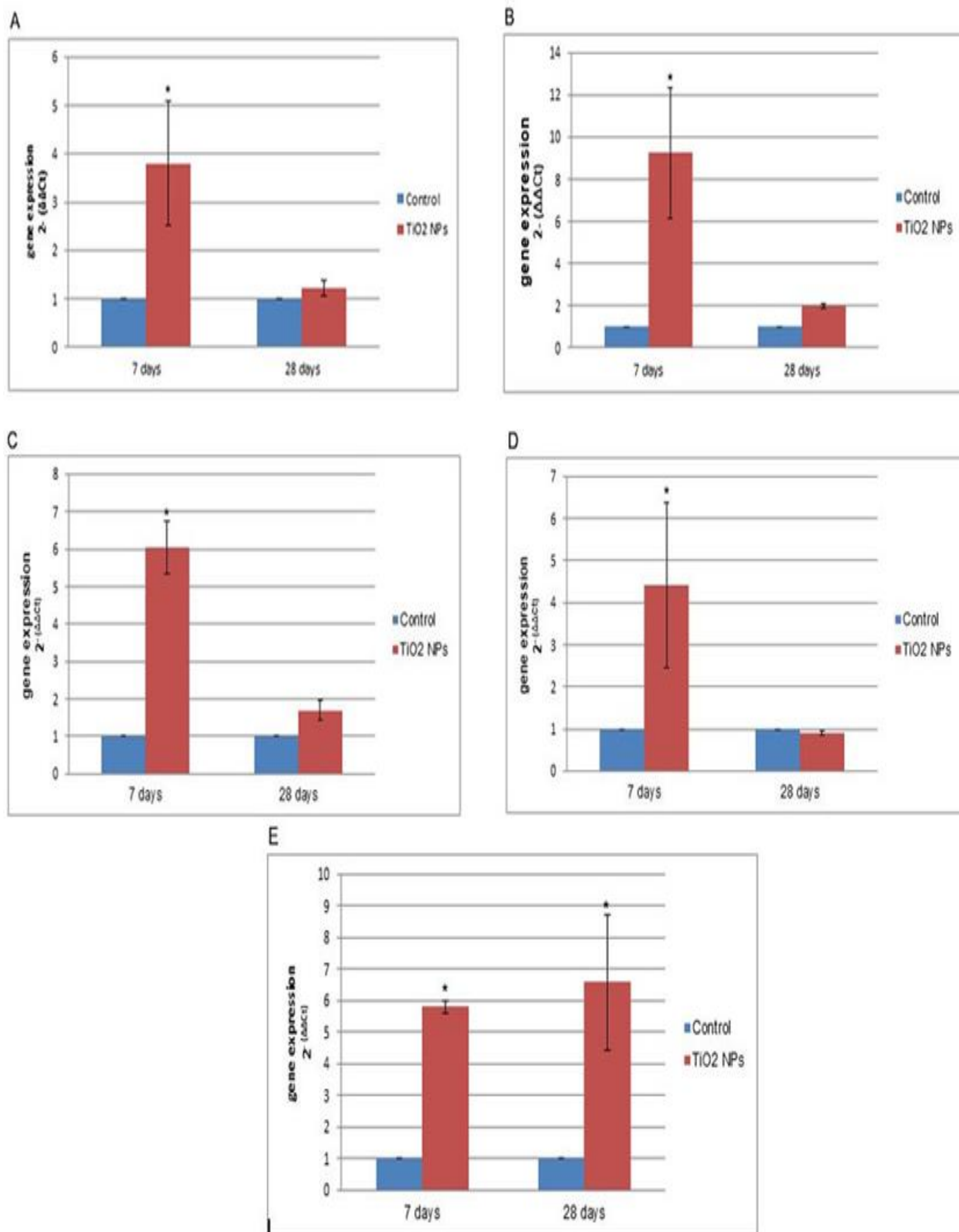

Fig. (3): Expression of A: Cu-Zn SOD ; B:Gpx;C: Hsp70; D: p53; andE;Mt1 genes in liver of treated and control male rats. Rats were exposed to a single oral dose (250 mg/kg b.w.) 25 ± 5nmTiO_2NPs in corn oil. Control rats received only corn oil. Liver was examined after 7 and 28 days, post-exposure. Data were expressed as $2^{-(\Delta\Delta Ct)}$ and presented as Mean ± SD (p<0.01).

4. DISCUSSION

Oral exposure mainly occurs through food products containing TiO_2 NP-additives [32,33]. Therefore, exposure to TiO_2NPs orally may have some adverse effects on exposed mammals and thus might be in a potential health risk. According to WHO (1970) [34], the median lethal dose (LD_{50}value) of oral ingestion of TiO_2 in rats is more than 12 g/kg b.w.

In the present study, the expression of Cu-Zn SOD, Gpx, Hsp70, p53, and Mt1genes were evaluated in male rats exposed orally to a single dose of 250 mg 25 ± 5 nm TiO_2 NPs /kg b.w. after 7 and 28 days post treatment. Acute toxicity describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short period of time To be described as acute toxicity, the adverse effects should occur within 14 days of the administration of the substance [**35**]. Several studies have examined the acute toxicity of the TiO_2 nanoparticles within 14 days after treatment [**36,37]**

In the present study gene expression changes after 7 days post exposure indicate the potential acute toxicity, and gene expression at 28 days after exposure may indicate recovery from the early gene expression changes caused by TiO_2 NPs possibly due to repair mechanisms or elimination of titanium from the liver, the present method is according to **Coccini*et al.*, 2014[38].**When compared to untreated controls, the treated male rats showed no death, normal behaviors, and no toxicity symptoms**.**

Our Data showed significant up-regulation of the tested oxidative-stress, protein folding, and apoptosis genes in the liver of exposed male rats tested 7days after administration. The gene expression profiles were not statistically different from the controls at 28 days following treatment. This result may reflect repair and elimination processes. Geraets *et al.* (2014)[39] mentioned that the half-life of TiO_2 NPs for liver was depending on TiO_2 NPs used and rout of administration. An accumulation and retention of some amounts of TiO_2 NPs in the liver 30 days after treatment have been reported[40]. On the other hand, expression of the metal toxicity gene (Mt1) was increased significantly in the male rat liver at 7 and 28 days after oral exposure.

Up-regulation of SOD and Gpx expression at day 7 after exposure could be a defense role performed by the liver to prevent the harm action of reactive oxygen species (ROS) generated by TiO2 NPs. These genes encoding enzymes are linked to metabolic pathways. SOD enzyme is the most powerful antioxidant in the cell and the first detoxification enzyme[41]. Gpx enzymes convert lipid hydroperoxides to alcohols and convert the free hydrogen peroxide to water [42]. Several investigators have reported that nano-TiO_2 up-regulate mRNA expression of oxidative-stress-related genes, induce oxidative DNA damage and thus, production of intracellular reactive oxygen species in various cell types was recorded [16,18, 43].It has been concluded that the large surface area of nanoparticles causes an increase in the production of ROS, leading to an imbalance between oxidation and anti-oxidation, which causes oxidative stress, genotoxicity, and hepatotoxicity[23,44].

Our results indicate that TiO_2 NPs induces up-regulation of the Hsp70 gene in male rats at 7 days after exposure to the tested single oral dose. Hsps are proteins produced by cells in response to their exposure to stressful conditions when exposed to high temperature, extreme pH, heavy metals, or nanoparticles. The alteration in the content of these proteins in exposed cell is depending on the strength and time of exposure [45]. HSP70 has an important role in the immune reaction[46]. Up-regulated by a wide range of cytotoxic stimulations[47], it appears to be a suitable biomarker for inflammation[48].

Our finding that the apoptotic gene p53 expression was upregulated in the liver significantly after 7 days and insignificantly at 28 days post exposure is supported by other previous studies which confirm increases of liver apoptosis through the activation of apoptotic gene expression as a result of exposure to TiO_2 NPs[12,15,49].Apoptosis is a crucial physiological process for maintaining equilibrium between cell division and death [50]. Toxicity of nanoparticles by apoptosis may be due to pathways regulated by mitochondria or death receptors [51].It has been reported that exposure to nano TiO_2 resulted in DNA damage, mutations, and alteration in expression of the apoptosis p53 gene[18, 52].

In present study, expression of the metal toxicity gene (Mt1)was increased significantly in the male rat liver at 7 and 28 days after oral exposure. Different investigations revealed that metallic NPs caused a high up-regulation of MTs in various cell types[53, 54].Metallothioneins (MTs) play a key role in heavy metal detoxification and essential metal ion balance. MTs may provide protection against metal toxicity and oxidative stress. Several studies reported that the synthesis of MT was elevated during the oxidative stress to protect the cells against cytotoxicity[55, 56]. Mt1overexpression may be potential biomarkers of toxicity following exposure to TiO_2 NPs [57]

5. CONCLUSION

This study indicated that 250 mg 25 ± 5 nm TiO_2 NPs /kg b.w. induced over-expression of five genes in the liver of orally exposed male rats. The induced genes expression changes are important in titanium toxicity mechanisms such as oxidative stress, protein folding, apoptosis and metallothionein genes. The incidence ofsignificant up-regulation of the metal toxicity gene expression caused by the tested dose given orally should be considered in possible health risks associated with consumption of food products containing high concentrations of TiO_2 NP-additives. Furthermore, a metabolomics analytical approach could be applied to study sub chronic and chronic effects of oral exposure to nanoTiO_2.

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