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Effects of selenium nanoparticles produced by *Lactobacillus acidophilus* HN23 on lipid deposition in WRL68 cells

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ABSTRACT

Selenium is an essential trace element for most organisms, protecting cells from oxidative damage caused by free radicals and serving as an adjunctive treatment for non-alcoholic fatty liver disease (NAFLD). In this study, We used the lactic acid bacterium *Lactobacillus acidophilus* HN23 to reduce tetra-valent sodium selenite into particulate matter, and analyzed it through inductively coupled plasma mass spectrometry (ICP-MS), scanning electron microscopy (SEM), X-ray diffraction energy dispersive spectrometry (EDS), and Fourier transform infrared spectroscopy (FTIR). We found that it consisted of selenium nanoparticles (SeNPs) with a mass composition of 65.8 % zero-valent selenium and some polysaccharide and polypeptide compounds, with particle sizes ranging from 60 to 300 nm. We also detected that SeNPs were much less toxic to cells than selenite. We further used free fatty acids (FFA)-induced WRL68 fatty liver cell model to study the therapeutic effect of SeNPs on NAFLD. The results show that SeNPs are more effective than selenite in reducing lipid deposition, increasing mitochondrial membrane potential (MMP) and antioxidant capacity of WRL68 cells, which is attributed to the chemical valence state of selenium and organic composition in SeNPs. In conclusion, SeNPs produced by probiotics *L. acidophilus* had the potential to alleviate NAFLD by reducing hepatocyte lipid deposition and oxidative damage. This study may open a new avenue for SeNPs drug development to treat NAFLD.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease characterized by liver inflammation, hepatocellular damage, and fibrosis [1], with hepatic steatosis as the main symptom. The pathogenesis mechanism of NAFLD has not been fully elucidated, but it is currently considered to be a complex systemic metabolic syndrome triggered by multiple stimulating factors, such as the deposition of toxic lipid metabolites, the emergence of oxidative and endoplasmic reticulum stress, tissue hypoxia and the development of sinus endothelial cell dysfunction [2]. NAFLD can also cause multisystem diseases that affect organs other than the liver, such as type 2 diabetes, cardiovascular disease, dyslipidemia, hypertension, and chronic kidney disease [3,4].

Treatments of NAFLD are still being explored. There is currently no FDA-approved treatment method for the disease. At this stage, except for improving diet and combining it with appropriate exercise, some drugs are also recommended for auxiliary treatment of NAFLD, such as insulin sensitizers thiazolidinediones, metformin, and MSDC-0602 K, etc., which can improve liver metabolic function. Since lipid deposition causes oxidative stress in liver, it is also important to combine antioxidant drugs such as pentoxifylline, selenium (Se) and vitamin E during the adjuvant NAFLD treatment [5].

Se is an essential trace element for most organisms. It can prevent oxidative damage through the synthesis of glutathione (GSH) in animal cells. Se supplementation can reduce hepatocellular damage caused by oxidative stress in animals, reduce hepatic lipid deposition, and slow

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down the formation of fatty liver [6]. A large number of studies have shown that Se supplementation can alleviate NAFLD and Se mainly assists the treatment of NAFLD through the following three ways: 1) Se exerts antioxidant effects in the treatment of NAFLD by increasing glutathione peroxidase (GPx) activity, thereby reducing hepatitis and liver fibrosis [7]. 2) Se inhibits inflammatory response to alleviate NAFLD by reducing hepatitis and fibrosis by inhibiting IL-1, IL-6, TNF-α, TGF-β1, as well as other inflammatory pathways (e.g. the NF-Kb pathway) and growth factors involved in the aetiology of NAFLD [8]. Selenium supplementation also promotes seleno-protein biosynthesis to restore circulating selenium levels and inhibits C-reactive protein production, thereby reducing the inflammatory response [9]. 3) Se reduces hepatic fat metabolism to alleviate NAFLD, and supplementing sodium selenite reverses the adverse effects of high-fat diet on blood lipids, and hepatic steatosis in mice [10]. The daily Se intake of the human body (60 kg) is about 50–250 μg. However, due to the limited content of Se in food, the Se-containing compound sodium selenite (Na_2SeO_3) is often used as a food additive to supplement Se. But Na_2SeO_3 is toxic, and excessive use can damage the liver [11–13].

Using probiotics to reduce Na_2SeO_3 to Se^0 can effectively reduce its toxicity. Indeed, a large number of studies have found that microorganisms such as *Escherichia coli, Bacillus*, and *Lactobacillus* can reduce highly soluble tetra-valent selenite (SeO_3^{2-}) and *hexa*-valent selenate (SeO_4^{2-}) ions to red zero-valent Se^0 nanoparticles (SeNPs) [14]. Compared to SeO_3^{2-} and SeO_4^{2-}, SeNPs have been shown to exhibit higher biological activity but lower cyto-toxicity [14].

Lactobacillus is an important probiotic that can improve the function of intestinal microbiota in animals and is often used as an auxiliary treatment for metabolic diseases, such as colitis, fatty liver, hypertension, etc. Previous studies have shown that *Lactobacillus* can alleviate NAFLD by reducing cholesterol and steatosis [15]. Given the fact that the biological activity of *Lactobacillus* itself can also reduce SeO_3^{2-} and SeO_4^{2-} into SeNPs with higher biological activity and lower cytotoxicity, we have used *Lactobacillus acidophilus* HN23 to prepare SeNPs, characterized them, and further explored their potential in the treatment of NAFLD using a fatty liver cell model.

2. Materials and methods

2.1. Preparation of SeNPs using L. acidophilus

We first cultured *L. acidophilus* HN23 at 37 °C to the logarithmic phase ($OD_{600} = 0.8$), and inoculated it at a ratio of 1 % to a MRS broth medium (10.0 g of caseinase digest, 10.0 g of beef extract powder, 4.0 g of yeast extract powder, 2.0 g of triammonium citrate, 5.0 g of sodium acetate, 0.2 g of magnesium sulfate ($MgSO_4$ $7H_2O$), and 0.2 g of manganese sulfate ($MnSO_4$ $4H_2O$), pH 6.2 ± 0.2) with 0, 2, 4, 6, 8 or 10 mM Na_2SeO_3, and incubated at 37 °C for 96 h.

To determine the reduction rate of Na_2SeO_3, we added 3,3′-diaminobenzidine (DAB) to the *L. acidophilus* HN23 fermentation supernatant to reduce residual Na_2SeO_3 and generate DAB-Se compounds, and then measured the absorbance at 420 nm by UV spectrophotometry to obtain the total Na_2SeO_3 reduction rate [16].

We then centrifuged the *L. acidophilus* culture at 12,500 × *g* for 10 min at 4 °C, collected the pellet, and washed three times with 10 mM phosphate buffer saline (PBS, pH 7.4) by centrifugation. Then, we placed the pellet in a mortar and ground it with liquid nitrogen. After that, we collected the powder and washed it 3 times with ultrapure water and further sonicated the powder in an ice bath for 15 min and washed 3 times with 1.5 M Tris-HCl (pH 8.3) containing 1 % SDS and washed it again with ultrapure water. The subsequent purification method was referred to the previous description [17], and it was freeze-dried to obtain a red lyophilized powder [18].

2.2. Particle size observation and qualitative element analysis of SeNPs

We observed the morphologies of *L. acidophilus* HN23 and SeNPs using a scanning electron microscope (SEM, SU8100, Hitachi, Japan) and analyzed the elemental composition of SeNPs using an X-ray diffraction energy dispersive spectrometer (EDS, Ultimmax 100, Oxford, Britain). We placed the processed sample on the conductive double-sided carbon film tape, put it on the sample stage of the ion sputtering instrument for about 30 s, and then observed it with SEM, while scanning the selected area with EDS [17].

2.3. Determination of Se content

Se content was detected using an inductively coupled plasma mass spectrometer (ICP-MS, NexlON 300X, Perkim Elmer, USA). We first dissolved 0.01 g of lyophilized powder in 50 mL of ultrapure water and then performed microwave digestion in a closed vessel using a solution containing 1 mL of concentrated HNO_3 and 0.5 mL of 30 % (v/v) H_2O_2 according to the method used by Martínez et al. [19]. After cooling the digestion solution and diluting it 10 000 times, we took 50 μL of the diluted solution for Se content measurement by ICP-MS.

2.4. Analysis of organic compounds in SeNPs

We took spectrally pure crystalline potassium bromide (KBr), ground it into a fine powder in an agate mortar, and immediately dried it in a far-infrared dryer. We then took 1–2 mg of freeze-dried SeNPs powder and about 100 mg of dry KBr (particle size 200 mesh) and mixed them evenly to make 5 mm flakes. We further used a Fourier transform infrared spectrometer (FTIR, Fisher Nicolet iS10, ThermoFisher, USA) to conduct spectral scanning tests, and qualitatively grouped and analyzed the obtained spectral data [17].

2.5. WRL68 cell culture

We first used Dulbecco's modified Eagle's medium (DMEM, 11965–092, Gibco, USA) containing 1 % antibiotics and 10 % fetal bovine serum (ThermoFisher, USA) to culture the WRL68 human embryonic hepatocyte cell line (obtained from Wuhan Procell Life Science & Technology Co., Ltd. China) at 37 °C in a humidified atmosphere of 5 % CO_2. We observed the growth of the cells under a microscope and conducted subsequent experiments when the confluence reached 80 %.

2.6. Cytotoxicity assay

We seeded WRL68 cells in a 96-well plate at a concentration of 5×10^4 cells per well with the medium described above and adhered them to the wells. Subsequently, we treated WRL68 cells with Na_2SeO_3 or SeNPs at different concentrations (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 μM) for 48 h and further cultured them for 12, 24, and 48 h. After that we added 10 μL of CCK-8 and serum-free DMEM (40 μL) to each well, and then incubated them at 37 °C for 2 h to observe the effects on cell viability. Finally, we measured the absorbance at 450 nm using a microplate reader and expressed the results as a percentage of the treatment group compared to the blank group [17].

2.7. Construction of free fatty acids (FFA)-induced fatty liver cell model

We first cultured WRL68 cells using DMEM containing 1 % antibiotics and 10 % fetal bovine serum (ThermoFisher, USA), and then added Na_2SeO_3 or SeNPs (Se concentrations were 0.5, 1, 2, 4, 6, 8 and 10 μM, respectively) [20], followed by transferring FFA (2:1 ratio of oleic acid to palmitic acid in isopropanol at 1 mM concentration, Supplementary Table S1) to 1 % skim bovine serum albumin (BSA) solution and mixing them thoroughly before adding to cell culture medium [21].

2.8. Staining with Oil red O to observe intracellular lipid deposition

WRL68 cells treated in different ways (Supplementary Table S1) were fixed with 10 % formalin for 1 h and then stained with Oil red O working solution at room temperature for 30 min. The intracellular lipid deposition was then observed under a microscope [22].

2.9. Detection of lipid deposition and antioxidant indexes

We first washed WRL68 cells treated in different ways (Supplementary Table S1) in 6-well plates twice with 10 mM PBS (pH 7.4), digested them with 300 μL trypsin for 2 min, and then added 700 μL DMEM to terminate the digestion process. Afterwards, we took 100 μL of cell suspension to determine the protein concentration with a Bicinchoninic acid (BCA) detection kit (P0010S, Beyotime Biotechnology, Shanghai, China) [23]. We also used Triglyceride (TG) detection kit (70902, Biobase, Shandong, China) and Non-esterified fatty acid (NEFA) detection kit (702110, Biobase, Shandong, China) to detect various lipid deposition indicators [23–25].

Referring to the above cell treatment method, we used GSH (A006-2–1, Nanjing Jiancheng Biotechnology, Nanjing, China), glutathione peroxidase (GSH-PX, A005-1, Nanjing Jiancheng Biotechnology, Nanjing, China) and total antioxidant capacity (*T*-AOC, A015-1, Nanjing Jiancheng Biotechnology, Nanjing, China) to detect the corresponding antioxidant indexes of GSH, GSH-PX, and *T*-AOC, respectively.

2.10. Detection of inflammatory factors IL-1 and IL-6

The enzyme-linked immunosorbent assay kits (Meilian Bio, Shanghai, China) were used to measure the production of IL-1 and IL-6 in samples (25 μL serum) according to the manufacturer's recommendations. The absorbance was measured at 450 nm in a MK3 enzyme calibration (Shanghai Thermoelectric Instrument Co., Ltd.).

2.11. Detection of cellular reactive oxygen species (ROS)

We cultured the WRL68 cells according to the method in the Section 2.7, and then transferred cells to serum-free medium with DCFH-DA (final concentration 10 mM, S0063, Beyotime Biotechnology, Shanghai, China) and Hoechst reagent (final concentration 5 μg/ml, 14533, Sigma-Aldrich, USA) and incubated WRL68 cells at 37 °C for 20 min. We then centrifuged and washed the cells three times with serum-free medium. Finally, we examined and collected images using a fluorescence microscope (XZ-10, MSHOT, Guangdong, China) [17].

2.12. Determination of mitochondrial membrane potential (ΔΨm)

We cultured cells according to the method in Section 2.7 above, and analyzed the changes in ΔΨm of WRL68 cells using JC-1 staining assay kit (C2006, Beyotime Biotechnology, Shanghai, China). We first added 500 μL of DMEM medium to a 12-well plate, then washed the cells with PBS, and stained them with JC-1 (20 μg/ml) for 30 min at 37 °C in the dark. We then centrifuged it to remove supernatant, and washed the pellet twice with JC-1 staining buffer. Finally, we used a microscope slide scanner (SensoScope, Pannoramic Scan, 3Dhistech, Hungary) to observe the intensity of fluorescent staining of cells to determine changes in ΔΨm [17].

2.13. Statistical analysis

All our experiments were performed in 3 biological replicates, and values were expressed as mean ± standard deviation. We used GraphPad Prism 8.0 for 2D graphical analysis and IBM SPSS Statistics 25 for one-way analysis of data variation. A p-value < 0.05 was considered significant.

3. Results

3.1. Reduction of Na_2SeO_3 by L. acidophilus HN23

As can be seen from Supplementary Fig. S1A, *L. acidophilus* HN23 shows a strong ability to reduce Na_2SeO_3. The reduction efficiency seemed to decrease with increasing substrate concentration; simultaneously, the increasing rate of reduction efficiency decreased with the incubation time, and the reduction efficiency reached its peak at 96 h of cultivation.

To increase the total yield of reductive particle matters, it is necessary to compromise substrate concentration and reduction efficiency. As shown in Supplementary Fig. S1B, when 6 mM Na_2SeO_3 was added to the culture medium, the reduction efficiency was 74.3 %, which was not the highest, but the yield of Se^0 was the highest, reaching 4.46 mM. Based on this, we adopted this condition to prepare Se^0 in subsequent experiments.

3.2. Characterization of SeNPs produced by L. acidophilus HN23

It can be seen from Fig. 1A, *L. acidophilus* HN23 produced particle matters after 48 h of culture in a medium containing 6 mM Na_2SeO_3. We then performed EDS analysis on intracellular particles (see the red square in Fig. 1A), and found that the particle matters contain Se and other elements (Fig. 1B), indicating that these may be SeNP particles comprising Se and biomolecules.

Further enlarged SEM observation of the purified particles reveals that the particle matter sizes range from 60 to 300 nm (Fig. 2A) and compose mainly of Se (Fig. 2B), thus the particles are SeNPs.

FTIR detection results show that the purified SeNPs are composed of substances containing functional groups such as –NH, –OH, C–O–C, COO– and amide (Supplementary Fig. S2, Table S2). C–O–C and –OH are commonly found in polysaccharides, while the existence of –NH, –OH and amides indicate the presence of proteins or peptides.

We also examined the Se content in lyophilized purified SeNPs using EDS and ICP-MS. The results show that the average Se concentration in the re-suspended diluted SeNPs is 1.67 mM (Supplementary Table S3), and the Se content in the purified SeNPs is 65.8 % (Supplementary Table S4).

3.3. Cytotoxicity of Na_2SeO_3 and SeNPs to WRL68 cells

As shown in Fig. 3A, after adding 4 μM Na_2SeO_3 to the culture medium, we found cell viability was significantly decreased ($p < 0.01$), but adding low concentration of SeNPs did not seem to significantly inhibit cell viability until the SeNPs concentration reached 10 μM ($p < 0.05$). In addition to concentration, incubation time also had a strong impact on cell viability. As we can see from Fig. 3B, cell viability was not affected when cultured in a lower concentration of 2 μM Na_2SeO_3 for 24 h, but it was significantly reduced after 48 h ($p < 0.05$). Similarly, cell viability was not affected after cultured for 24 h at a safe concentration of 8 μM SeNPs, while it was significantly reduced after cultured for 48 h ($p < 0.05$). Therefore, 1 μM Na_2SeO_3 and 6 μM SeNPs were considered acceptable and safe concentrations for cells.

The viability of FFA-induced WRL68 cells (model group, Mod) was significantly lower than that of the control group (Con) ($p < 0.01$). However, after treatment with 1 μM Na_2SeO_3 and 1 μM SeNPs, WRL68 cell viability was increased significantly ($p < 0.01$), and there was a significant difference between the SeNPs and the Na_2SeO_3 treated groups ($p < 0.05$) (Fig. 3C).

3.4. SeNPs reduced lipid deposition in adipose WRL68 fatty liver cells

We found that WRL68 cells in the Con group showed no lipid deposition (Fig. 4A), but in the Mod group, they showed substantial lipid deposition (Fig. 4B). Both Na_2SeO_3 (TRE 1) and SeNPs (TRE 2)

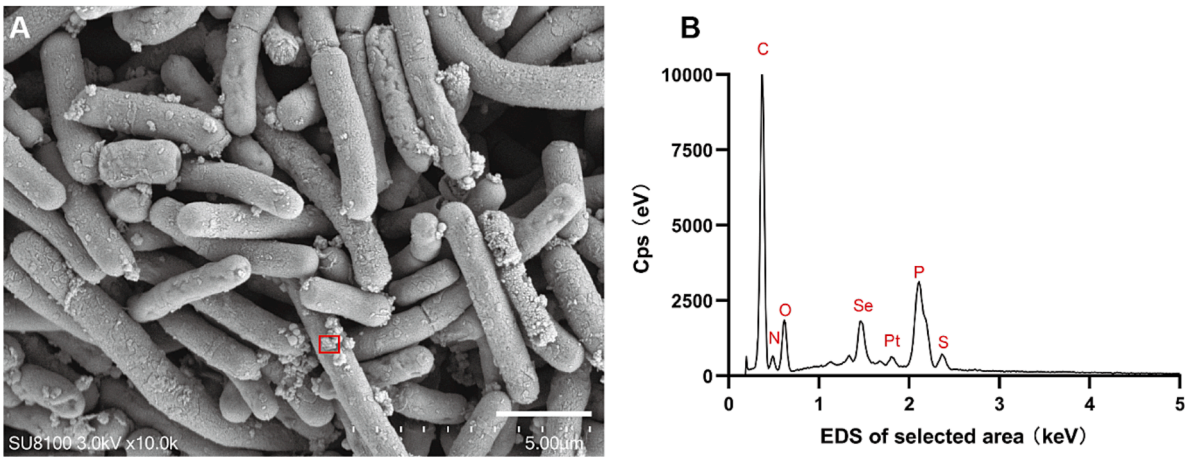

Fig. 1. SEM image and EDS analysis of particle matter produced by *L. acidophilus* HN23. A. The SEM image of *L. acidophilus* HN23 and particle matters. The red square shows the area scanned by EDS analysis. B. EDS analysis results of the selected area. Detected elements are indicated in red capital letters. The white scale is 2 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

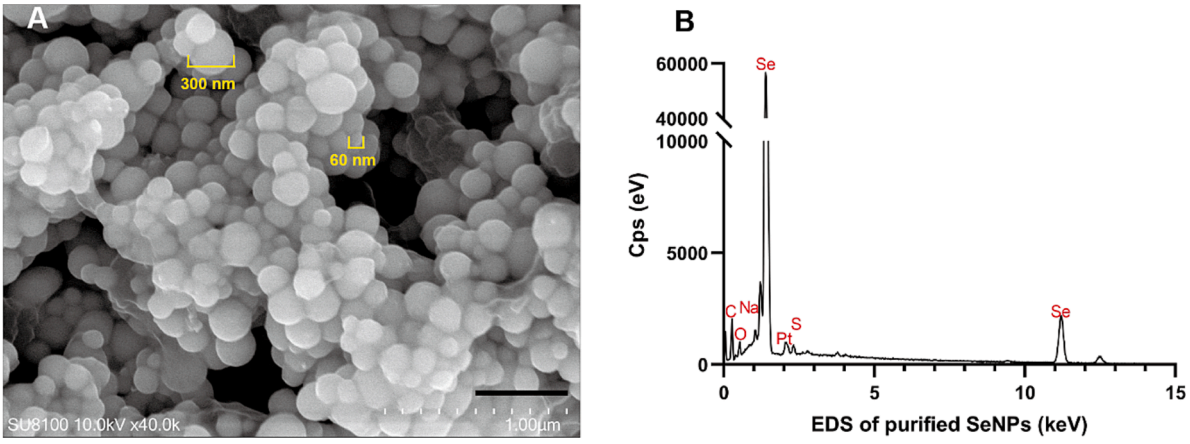

Fig. 2. SEM image and EDS analysis of purified SeNPs. A. SEM image of purified SeNPs. The distribution marked in yellow are the largest (300 nm) and smallest (60 nm) particle matters. B. EDS analysis results of purified SeNPs. The black scale is 500 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

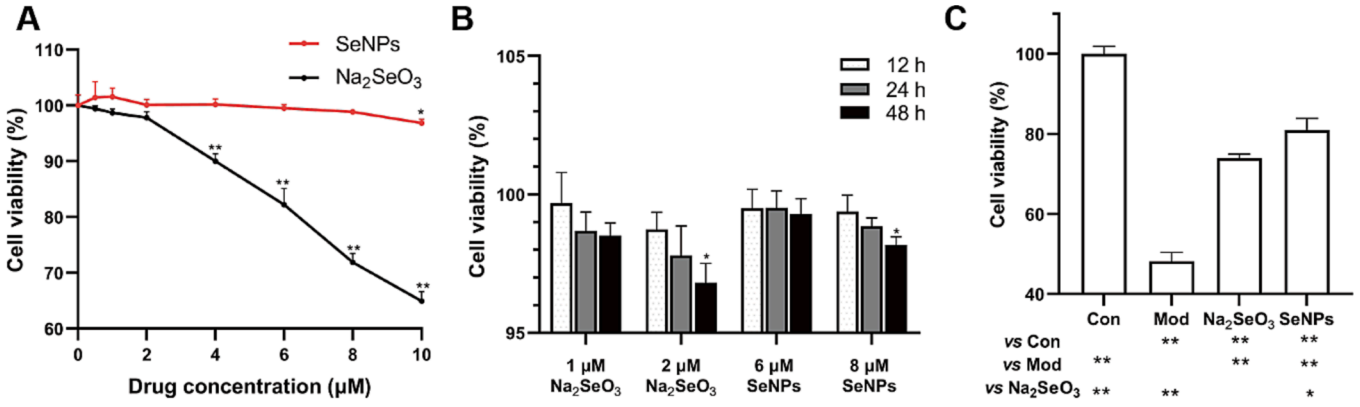

Fig. 3. Cytotoxicity of Na_2SeO_3 and SeNPs on WRL68 cells. A. Effects of Na_2SeO_3 and SeNP concentrations on cell viability. B. Effects of concentrations and treatment time of Na_2SeO_3 and SeNPs on cell viability. C. Effects of different treatments on cell viability. Values are expressed as mean ± standard deviation using one-way analysis of variance. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

treatments reduced lipid deposition in WRL68 fatty liver cells (Fig. 4C, 4D). The detection of intracellular TG and FFA showed that SeNPs effectively counteracted the abnormal increase of intracellular TG caused by FFA ($p < 0.01$, Fig. 4E, 4F). Moreover, the effect of SeNPs was more significant than that of Na_2SeO_3 ($p < 0.05$, Fig. 4E, 4F).

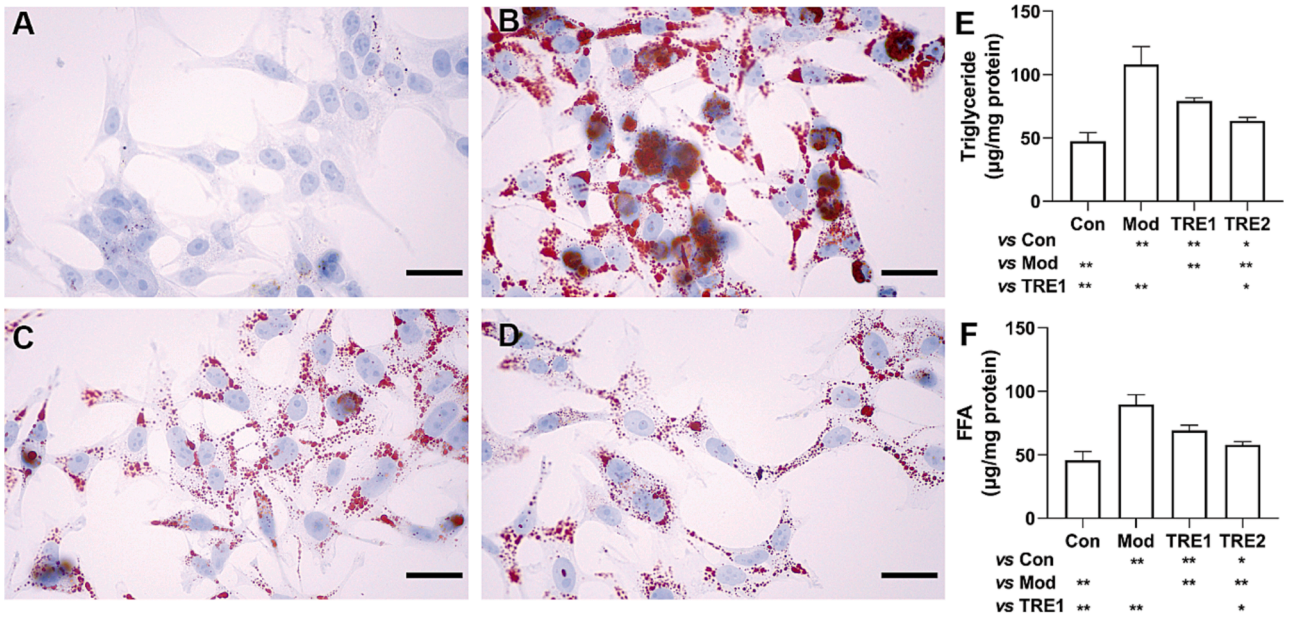

Fig. 4. Observation of intracellular lipid deposition in each group. Con is normal WRL68 cells; Mod is WRL68 adipose liver cell model; TRE 1 and TRE 2 are WRL68 adipose liver cells treated with 1 μM Na_2SeO_3 or 1 μM SeNPs, respectively. A. Oil-red-O staining of Con cells (400 X); B. Oil-red-O staining of FFA-induced cells (400 X); C. Oil-red-O staining of cells treated with 1 μM Na_2SeO_3 (400 X); D. Oil-red-O staining of cells treated with 1 μM SeNPs (400 X); E. Cell TG content; F. Cell FFA content. The black scale is 50 μm. Values are expressed as mean ± standard deviation using one-way analysis of variance. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. SeNPs significantly reduced the levels of inflammatory factors IL-1 and IL-6

According to the results shown in Fig. 5, after FFA treatment, the levels of IL-1 and IL-6 in the cells were significantly increased ($p < 0.01$), while Na_2SeO_3 and SeNPs significantly reduced the levels of IL-1 and IL-6 in FFA-treated cells ($p < 0.01$). Among them, the content of IL-1 in cells of TRE2 group treated with SeNPs was significantly lower than that of cells of TRE1 group treated with Na_2SeO_3 ($p < 0.05$), and the content of IL-6 in cells of TRE2 group was lower than that of TRE1 group. In short, SeNPs could significantly decrease the levels of the inflammatory factors.

3.6. SeNPs inhibited the △Ψm reduction induced by FFA

The results in Fig. 6 show that FFA treatment led to a significant decrease in J-aggregates ($p < 0.05$) and a significant increase in J-monomer ($p < 0.01$) in Mod cells, indicating that △Ψm in Mod cells was decreased after FFA treatment. J-aggregates were significantly increased in TRE 1 ($p < 0.05$), while J-monomer was not significantly different. After treatment with 1 μM SeNPs for 24 h, J-monomers in TRE 2 were significantly reduced compared with the Mod and TRE 1 ($p < 0.01$), while J-aggregates in TRE 2 were not significantly different from TRE 1 ($p > 0.05$). The results show that both Na_2SeO_3 and SeNPs can inhibit the △Ψm reduction induced by FFA, and SeNPs is better than Na_2SeO_3.

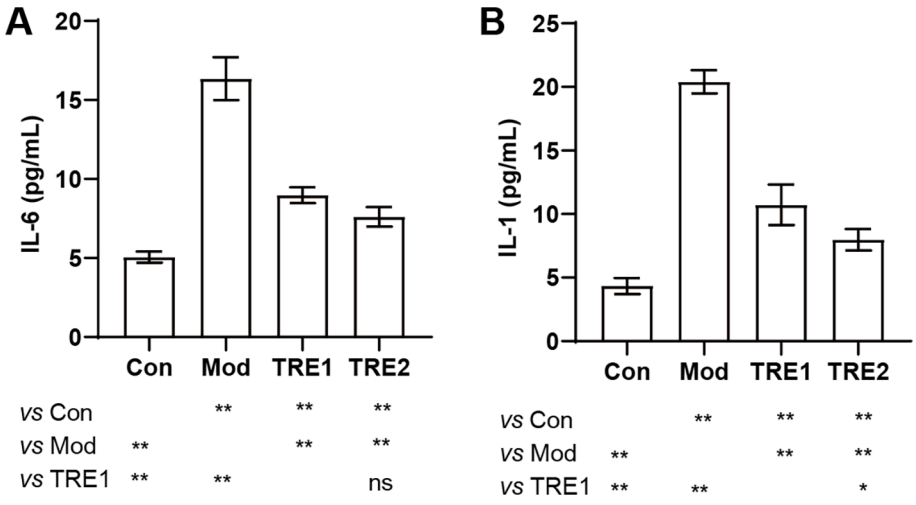

Fig. 5. Expression of Inflammatory factor. Con is normal WRL68 cells; Mod is WRL68 fatty liver cells; TRE 1 and TRE 2 are WRL68 adipose fatty liver cells treated with 1 μM Na_2SeO_3 and 1 μM SeNPs, respectively. A. IL-1 content in each group of cells; B. IL-6 content in each group of cells. Values are expressed as mean ± standard deviation, using one-way analysis of variance. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

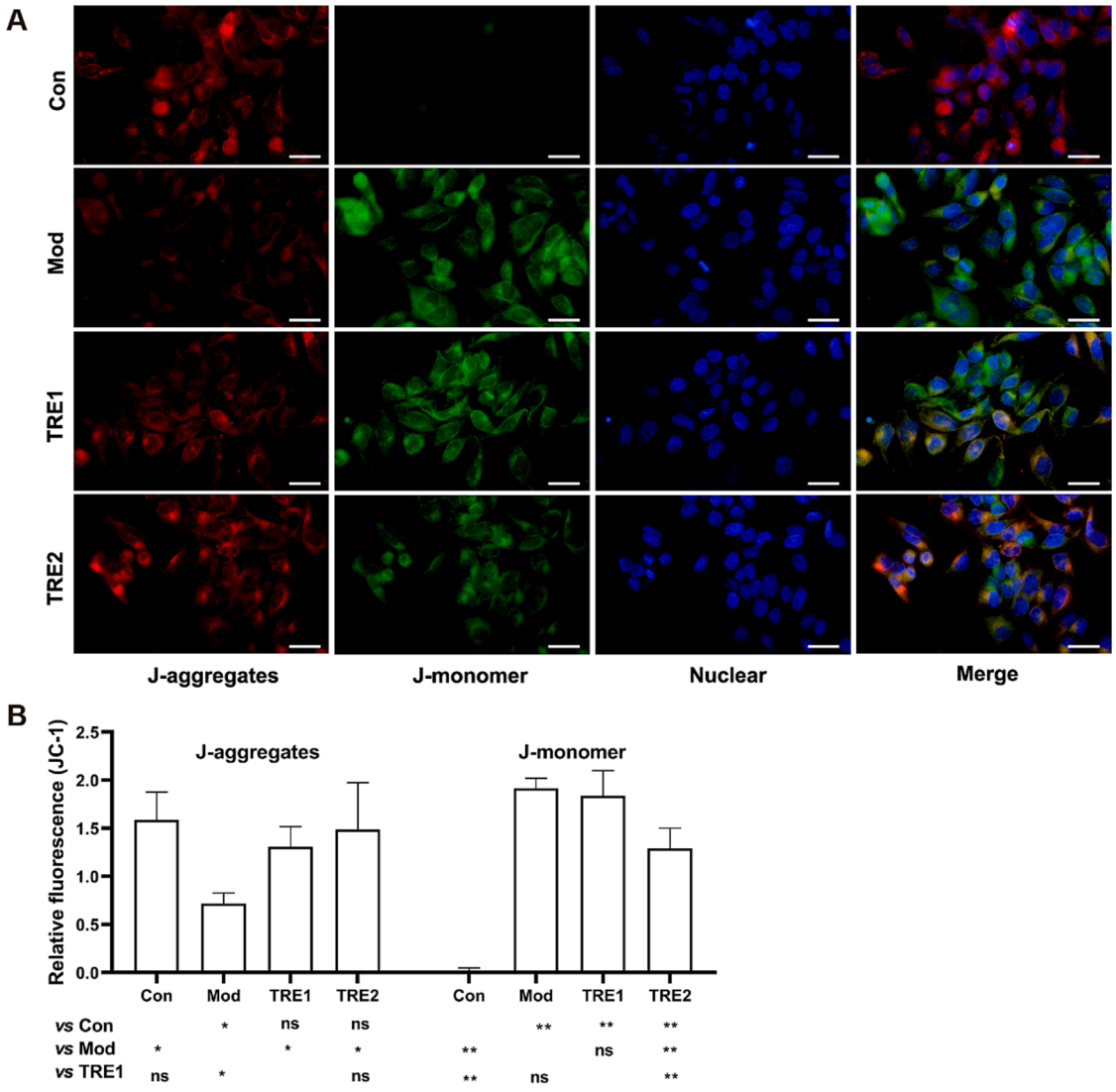

Fig. 6. Effect of Na_2SeO_3 and SeNPs on the $\triangle\Psi m$. Con is normal WRL68 cells; Mod is WRL68 fatty liver cells; TRE 1 and TRE 2 are WRL68 adipose fatty liver cells treated with 1 μM Na_2SeO_3 and 1 μM SeNPs, respectively. JC-1 is a commonly used fluorescent probe for detecting $\triangle\Psi m$. When it is high, JC-1 forms J aggregates in the mitochondrial matrix and produces red fluorescence. In contrast, when $\triangle\Psi m$ is low, JC-1 cannot accumulate in the mitochondrial matrix, resulting in JC-1 mainly existing as a monomer and emitting green fluorescence. A. Immunofluorescence image (400 X). B. Relative fluorescence intensity, n = 3. The white scale is 50 μm. Values are expressed as mean ± standard deviation, using one-way analysis of variance. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. SeNPs effectively reduced intracellular ROS

The fluorescent probe DCHF-DA was used to detect the ROS content in WRL68 cells treated by different groups. Experimental results (Fig. 7) show that FFA treatment significantly increase the ROS content in Mod cells ($p < 0.01$), but 1 μM Na_2SeO_3 and 1 μM SeNPs can significantly reduce the ROS content in WRL68 fatty liver cells, and relatively speaking, the effect of 1 μM SeNPs is more significant than that of Na_2SeO_3 ($p < 0.05$).

3.8. SeNPs effectively improved the antioxidant capacity of WRL68 cells

To better evaluate the degree of cellular oxidative damage, we examined the contents of GSH, GSH-Px and *T*-AOC in WRL68 cells in each group. Compared with Con, we found that the GSH, GSH-Px, and *T*-AOC contents of Mod cells were significantly reduced. Treatment with 1 μM Na_2SeO_3 and 1 μM SeNPs significantly inhibited this effect caused by FFA, and the antioxidant activity level of 1 μM SeNPs was higher than that of 1 μM Na_2SeO_3 ($p < 0.05$) (Fig. 8).

4. Discussion

4.1. Characteristics of SeNPs produced by bacteria

There have been many discussions on the preparation methods of SeNPs, including the use of physical, chemical or biological techniques to reduce the tetra-valent SeO_3^{2-} or *hexa*-valent SeO_4^{2-} to zero-valent Se^0 in recent years. Compared with physical and chemical preparation techniques, we found that the synthesis of SeNPs using bacteria, fungi and plants appear to have greater flexibility and higher safety [26–28]. Indeed, many studies have shown that microorganisms such as *Bacillus*, *Lactobacillus* and *Bifidobacterium* have the ability to reduce SeO_3^{2-} and SeO_4^{2-} to Se^0 [29–33]. Under aerobic and anaerobic conditions, bacteria can form SeNPs through the dissimilatory reduction pathways, including mainly: (i) Painter-type reaction involving thiol groups; (ii) Thioredoxin-thioredoxin reductase system–mediated reduction; (iii) Siderophore-mediated reduction; (iv) Sulfide-mediated reduction; and (v) Dissimilatory reduction [34]. Since the *L. acidophilus* used in this article is a facultative anaerobic bacterium, its reduction of Se oxygen

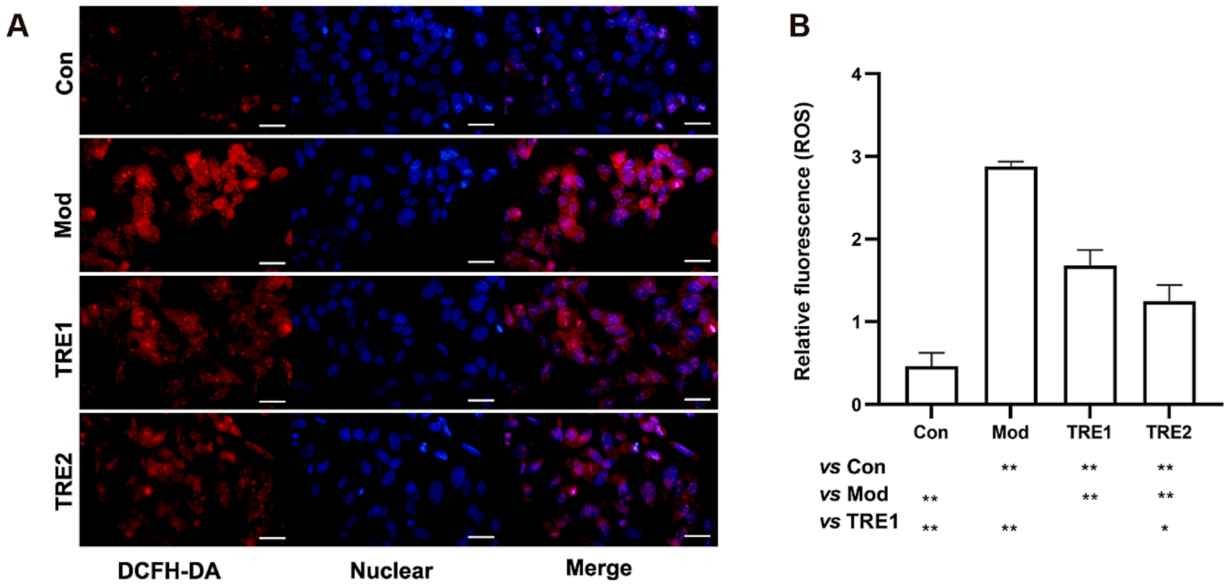

Fig. 7. Effects of Na_2SeO_3 and SeNPs on ROS in WRL68 cells. Con is normal WRL68 cells; Mod is WRL68 fatty liver cells; TRE 1 and TRE 2 are WRL68 adipose fatty liver cells treated with 1 μM Na_2SeO_3 and 1 μM SeNPs, respectively. This figure shows the detection of ROS levels in SeNPs-treated cells using DCFH-DA. A. Immunofluorescence image (400 X). B. Relative fluorescence intensity, n = 3. The white scale is 50 μm. Values are expressed as mean ± standard deviation, using one-way analysis of variance. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

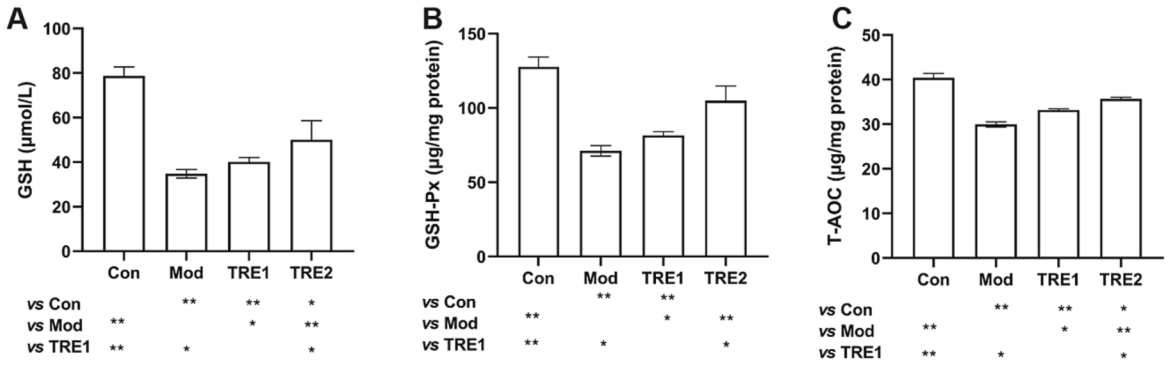

Fig. 8. Intracellular antioxidant indexes (n = 3). Con is normal WRL68 cells; Mod is WRL68 fatty liver cells; TRE 1 and TRE 2 are WRL68 adipose fatty liver cells treated with 1 μM Na_2SeO_3 and 1 μM SeNPs, respectively. A. GSH content in each group of cells; B. GSH-Px content in each group of cells; C. *T*-AOC content in each group of cells. Values are expressed as mean ± standard deviation, using one-way analysis of variance. *: p-value < 0.05, **: p-value < 0.01, ***: p-value < 0.001.

anions may be through the respiratory selenite reductases and nitrite reductase in the sulfide-mediated reduction dissimilation pathway. Under anaerobic environment, electrons are transferred through the electron transport chain (ETC) to reduce tetra-valent SeO_3^{2-} to zero-valent Se^0, hence reducing their cytotoxicity [34].

Due to the instability of Se^0 in nature, it requires the addition of polysaccharides or polypeptides as stabilizers to encapsulate Se^0 and prevent Se^0 from being re-oxidized into other valence states during the process of synthesizing SeNPs using physical and chemical methods [35]. However, when microorganisms synthesize SeNPs, they can use existing organic compounds within the cell such as polypeptides and polysaccharides to stabilize Se^0, thereby facilitating the formation of SeNPs; at the same time, the biological activity of SeNPs may also be related to the organic matter encapsulated during their production process [29,32,36]. For example, surface proteins and polysaccharides in SeNPs prepared by *Bacillus opalba* Y4 participate in the formation of SeNPs and increase their stability; SeNPs prepared using *Lactobacillus casei* 393 contain 14.2 % of polysaccharide molecules [18]. In this study, SeNPs were produced by *L. acidophilus* HN23, and they were found to contain 65.8 % of Se (Supplementary Table S4) and a certain proportion of polypeptides and polysaccharides. The complex structures formed by these biomolecules may exhibit a certain stabilizing effect on Se^0.

4.2. *Effects of Se on cellular lipid deposition*

Through cell biology experiments, we found that FFA could cause metabolism disorders and lipid deposition in WRL68 cells, which might have a negative impact on the function of ROS-producing mitochondria (Figs. 6, 7). In NAFLD, mitochondrial function is regulated by down-regulating the ETC and enhancing fatty acid oxidation capacity, leading to excessive ROS production in various ETC components upstream of cytochrome *c* oxidase. At the same time, β-oxidation of fatty acids can also produce a large amount of ROS in liver metabolism [37,38]. When mitochondria maintain normal physiological status, fatty acids can be decomposed through β-oxidation to reduce intracellular lipid deposition. But when lipids continue to accumulate, it will lead to massive oxidation of fatty acids. As a result, a large amount of ROS will be generated by ETC and fatty acid β-oxidation pathways, causing considerable oxidative stress to mitochondria and even the entire cell, and further inducing cell inflammation and apoptosis [37,39,40].

In addition to using lipid-lowering drugs and insulin sensitizers, the current NAFLD treatment model also requires the help of antioxidants

such as vitamin E present in the phospholipid bilayer of cell membranes to reduce liver damage caused by oxidative stress in patients with NAFLD or non-alcoholic steatohepatitis (NASH) [5], designed to protect cells from oxidative damage caused by ROS [41]. Similarly, acetylcysteine and pentoxifylline are often used as liver antioxidants to reduce liver oxidative damage, thereby alleviating liver lipid deposition caused by high-fat diet [42–44]. In this study, we found that both selenite and SeNPs could reduce FFA-induced mitochondrial oxidative stress damage by lowering ROS levels in WRL68 cells, thereby protecting WRL68 cells (Figs. 6, 7).

Se is also involved in the synthesis of seleno-proteins, such as glutathione peroxidase GPXs and thioredoxin reductase TrxRs, which are the main endogenous antioxidant substances in the liver. They can neutralize hydrogen peroxide and organic peroxides inside and outside cells, and inhibit the production of ROS-activated inflammatory signaling factors, such as nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), p53, glucocorticoid receptor, to reduce oxidative stress damage [45–47]. At the same time, these seleno-proteins are also involved in regulating the proliferation and autophagy of liver cells, promoting liver repair and improving NAFLD treatment [45,48]. Since we also detected changes in the inflammatory factors IL-1 and IL-6, as well as GSH and GSH-Px activities in hepatocytes in this experiment, we suggest that SeNPs may promote the productions of GSH and GSH-Px in WRL68 cells, thereby reducing cellular damage. More importantly, SeNPs are found to have higher activity than Na_2SeSO_3 (Figs. 5, 8).

4.3. SeNPs produced by L. acidophilus have stronger biological activity than selenite

Lactobacilli are considered safe and are the first choice for microbial producers of SeNPs. Compared with Na_2SeO_3, SeNPs are less toxic. For example, the results of acute toxicity tests in mice conducted by Zhang et al. (2001) show that the toxicity of SeNPs is only one-seventh that of Na_2SeO_3. The results of this experiment also show that at the same Se concentration, the cytotoxicity of SeNPs produced by *Lactobacillus* is very low, only 1/4 to 1/6 times that of Na_2SeO_3 (Fig. 3). Obviously, the difference in cytotoxicity between the two is likely related to the valence state of Se. Therefore, it is reasonable to speculate that high-valent Se exerts greater oxidative stress on cells than Se^0.

The organic compounds secreted by *L. acidophilus* can not only serve as stabilizers for SeNPs, but also have certain physiological activities. Research has found that a variety of probiotics, including *Lactobacillus*, can produce a large amount of short-chain fatty acids, polysaccharides, polypeptides and other substances to improve liver metabolism. Furthermore, research on extracellular polymeric substances (EPS) produced by *Lactobacillus* has shown that EPS may have multiple biological activities such as anti-inflammatory, anti-tumor, and cholesterol-lowering [49–51].

EPS from *Lactobacillus* can significantly improve hypertension in mice [52], and upregulate the expression GLUT-4, AKT-2 and AMPK genes to improve glucose metabolism in insulin-resistant WRL68 cells [53]. *L. acidophilus*, as well as *Bacillus subtilis*, *Bifidobacterium*, etc. are common probiotics used clinically to treat NAFLD patients. The active substances produced by these probiotics can improve liver steatosis, liver inflammation, etc. through the entero-hepatic axis, and have the effect of reducing serum TG, cholesterol, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) [54,55]. Therefore, in this study, SeNPs from *L. acidophilus* had a better therapeutic effect on FFA-induced fatty liver model because SeNPs contain both Se and prebiotics.

5. Conclusions

In summary, *L. acidophilus* HN23 could reduce selenite and prepare SeNPs rich in polysaccharides and polypeptides. Under the same Se concentration, the cytotoxicity of the prepared SeNPs was lower than that of selenite; it could also more effectively reduce the lipid deposition and increase the mitochondrial membrane potential and antioxidant capacity of hepatocyte. This effect may be related to the different chemical valence state of Se atoms and the organic components in SeNPs. In general, SeNPs prepared by *L. acidophilus* exhibit certain therapeutic effects on NAFLD. This study thus opened up a new drug treatment approach for the treatment of non-alcoholic fatty liver disease.

CRediT authorship contribution statement

Xianglan Lei: Conceptualization, Data curation, Formal analysis. **Yuxuan Peng:** Methodology, Writing – original draft. **Yan Li:** Data curation, Formal analysis, Methodology. **Qianyuan Chen:** Data curation, Methodology. **Zhenguo Shen:** Writing – review & editing. **Wen Yin:** Writing – review & editing. **Viktar Lemiasheuski:** Writing – review & editing. **Siyang Xu:** Visualization, Writing – original draft. **Jin He:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2024.107165.

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