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Inhibition of H_2O_2-induced TM3 cell apoptosis by oxidative stress by lentinan functionalized selenium nanoparticles through JAK2/STAT-3 and P53 pathways

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Key words: Selenium nanoparticles, Lentinan, ROS, Antioxidant

Abstract: Background: Nano-selenium has been widely used in antiviral and anticancer therapy, and has the advantages of good targeting and low toxicity. For the first time, we combined male reproduction with nano-selenium to investigate its antioxidant effect. This study investigated the protective effect of lentinan functionalized selenium nanoparticles on oxidative stress injury of the hydrogen peroxide (H_2O_2)-induced Leydig cell line, TM3. **Methods:** The suitable concentration of nano-selenium treatment to promote cell proliferation was also discussed. The concentration of 4 μM could significantly promote the growth of TM3 cells. Oxidative stress damage was caused using an 800 μM concentration of hydrogen peroxide. The cells were divided into four groups: normal control group, oxidative stress treatment group, H_2O_2+SeNPs@LNT group, and SeNPs@LNT group. The H_2O_2+SeNPs@LNT group was pretreated with 4 μM of SeNPs@LNT for 12 h, followed by 800 μM of H_2O_2 for 8 h. **Results:** Nano-selenium could significantly promote the proliferation and viability of TM3 cells. SeNPs@LNT treatment increased the level of mitochondrial membrane potential in normal cells and slowed down the decline in mitochondrial membrane potential level caused by oxidative stress injury. In addition, the increase in reactive oxygen species caused by oxidative stress was inhibited by SeNPs@LNT treatment. The apoptosis of TM3 cells was detected, and SeNPs@LNT alleviated the necrosis and apoptosis of TM3 cells induced by H_2O_2. Nano-selenium plays a protective role against oxidative H_2O_2-induced stress injury in TM3 cells through the changes in the Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway and P53 pathway, and the expression levels of other related proteins, protein kinase B (AKT) and C3. **Conclusion:** SeNPs@LNT exhibited good biological activity and antioxidant effect and can thus be used to protect the male reproductive system from oxidative stress.

Introduction

Selenium, as an essential trace element, plays an important role in maintaining the normal reproductive structure and function of human beings and animals. It is involved in the synthesis of at least 25 human selenoproteins and essential dietary components containing selenocysteine in the body (Wadhwani *et al.*, 2016). In nature, selenium can be categorized as organic selenium, inorganic selenium, and nano-selenium (Hosnedlova *et al.*, 2018). Three forms of selenium (inorganic selenium, such as sodium selenite and sodium selenate; organic selenium, such as selenocysteine and selenium-enriched yeast; functionalized and non-functionalized nano-selenium) are used in anti-cancer therapy, dietary supplements, as antibacterial and antiviral agents, clinically or in combination with other antioxidants (Labunskyy *et al.*, 2014; Qazi *et al.*, 2019). Inorganic selenium is strictly controlled due to its high biological toxicity. Biological organic selenium has high safety and high selenium supplementation efficiency and is involved in cell repair, free radical scavenging, reducing the physiological toxicity of chemotherapy drugs (such as cisplatin, etc.), and resisting the accumulation of heavy metals, etc. (Maiorino *et al.*, 2018).

As a new type of nanotechnology product, nano-selenium has improved the effectiveness and safety of traditional selenium products and has been used in targeted drug carriers, diabetes treatment, and the treatment of

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Alzheimer's disease (Michaelis *et al*., 2014; Rayman, 2020). Nano-selenium is synthesized in three ways: physical synthesis, chemical synthesis, and biosynthesis. The physical synthesis method cannot be widely used in the production of nano-selenium due to the limitation of synthesis conditions (high temperature and high pressure, catalyst) and low efficiency, and the use of plant extracts, fungi, and bacteria to synthesize bio-derived nano-selenium overcomes the shortcomings (easy to agglomerate and unstable) of particles made by the chemical synthesis, and their special functionalization can further reduce the cytotoxicity or improve the application functionality (Andersen *et al*., 2003; Boitani and Puglisi, 2008; Hariharan and Dharmaraj, 2020). Throughout the research history of nano-selenium, selenium has been regarded as a toxic substance and even a carcinogen due to its narrow safety range. Until the 20th century, people gradually found that selenium is beneficial to humans and animals; it has antioxidant, anti-cancer, immunity enhancing, and other functions, can treat Keshan disease, cardiovascular diseases, diabetes, cancer, etc., and is an indispensable essential trace element for physiological activities.

In male reproduction, the key role is mediated by two selenoproteins, phospholipid hydrogen peroxide (H_2O_2) glutathione peroxidase (PHGPx/GPX4) and selenoprotein P (Liu *et al*., 2019). GPX4, as an oxidation-inactivated protein, constitutes more than half of the mitochondrial envelope in the middle of mature spermatozoa, ensuring the stability and viability of spermatozoa (Zi *et al*., 2018). GPX4 is thought to protect developing spermatozoa from oxidative stress-induced DNA damage early in the spermatogenesis process (Liu *et al*., 2020). Strong expression of GPX4 protein in seminiferous tubules and immune signals were also observed in the epididymis and ejaculated sperm (Yan *et al*., 2021). Selenophosphorin P, as a selenium transporter, is expressed in vesicle-like structures in the basal region of Sertoli cells for selenium uptake (Novak and Mollen, 2015). Its function is closely related to the expression of ApoE receptor 2 (ApoER2). ApoER2 is a receptor highly expressed on the membrane surface of neurons and the initial segment of the epididymis. Its role in the brain is to affect the migration of neurons to the appropriate location in the developing brain, and its role in male reproduction is to transport selenoprotein P to the Sertoli cells of the testis for uptake and utilization. It affects the function and expression of aggretin protein and GPX4 (Liu *et al*., 2017). Other proteins, GPX1 and GPX3, are also expressed in male reproductive tissues and secretions located in the epididymal epithelium to protect epididymis and mature spermatozoa from oxidative stress (Banerjee *et al*., 2020). Selenium is the oxidation–reduction (Redox) center of these enzymes and is essential for their biochemical activities (Zorov *et al*., 2014).

Lentinan is a polysaccharide with a β-1,3-linked glucose backbone and a β-1,6-linked glucose branch chain (Zorov *et al*., 2014). Lentinan possesses a large number of bioactive groups. As an antioxidant component, lentinan can significantly reduce the malondialdehyde content of neurons and prevent lipid peroxidation in neurons. At the same time, Lentinus edodes polysaccharide can increase SOD activity in animal blood and promote lymphocyte proliferation. The antioxidant mechanism may involve a hydrogen migration scheme, which transfers hydrogen atoms to other molecules, terminates the chain reaction of free radicals, and avoids cell membrane damage and oxidative degradation of nucleic acids by removing superoxide anions (Xu *et al*., 2019). Past studies have shown that in lentinan-functionalized nano-selenium particles (SeNPs-LNT), the three spiral single-stranded glucan is dispersed in the water medium, proving the interaction between the hydroxyl and nano-selenium, improving the dispersion stability in water and adjust the redox reaction rate or the content of SeNPs-LNT, synthesis of selenium nanoparticles of different size. Compared with simple nano-selenium, Lentinus edodes functionalized nano-selenium has greater stability, stronger biological targeting effect, and better biocompatibility and effectiveness than inorganic and organic compounds (Xu *et al*., 2019; Tavoosi *et al*., 2020).

Aiming at the physiological and biochemical effects of selenium in male reproduction and the application of nanomaterials in antioxidants, this study evaluated the cytoprotective effects and the mechanism involved in SeNPs-LNT on H_2O_2-induced oxidative stress injury in mouse mesenchymal cells, TM3. The study showed that it can significantly reduce the ROS level, reduce the apoptosis of cells, act on mitochondria, change the mitochondrial membrane potential, and regulate the physiological function of cells through Janus kinase 2/signal transducer and activator of transcription 3 (JAk2/STAT-3) pathway and P53 pathway, and C3 and AKT-related proteins are involved.

Materials and Methods

Materials

Cell Counting Kit-8 (CCK8) (C0038) and BCA protein detection kit (P0009), Reactive Oxygen Species (ROS) Assay Kit (S0033), and Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Assay Kit (C1062) were purchased from Beyotime Biotechnology (Shanghai, China). Anti-STAT-3, anti-JAK2, anti-P-P53, anti-AKT, anti-C3, anti-β-actin, anti-rabbit lgG secondary antibody, and anti-mouse IgG secondary antibodies for western blotting were from Cell Signaling Technology (Boston, USA). Fetal bovine serum (FBS) (10099) and Dulbecco's modified Eagle's medium (DMEM) (11965092) were from Gibco (New York, USA). Mitochondrial membrane potential Assay Kit (JC-1) (MAK160) was purchased from Sigma (MO, USA).

Preparation and characterization of SeNPs

SeNPs@LNT was prepared by Professor Chen Tianfeng team at Jinan University. Data for the synthesis and characterization of nano-selenium can be obtained from an earlier publication (Zorov *et al*., 2014).

Cell culture and treatment

The Leydig cell line TM3 was isolated from mouse stromal cells and obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% FBS, streptomycin (100 μg/mL), and penicillin (100 U/mL). The cells were treated with 0, 125, 250, 500, 600, 700, 800, and 1000 μM H_2O_2 for 8 h to observe the cell viability and

screen out the appropriate concentration of oxidative stress. The concentration of 800 μM of H_2O_2 was selected for the treatment according to the standard of IC50, which caused a decline in cell viability but did not cause complete necrosis. The Leydig cell line TM3 was treated with SeNPs@LNT at 1, 2, 4, 8, 16 and 32 μM for 24 h to observe the cytotoxicity of selenium. The cell viability was the strongest at 4 μM of SeNPs@LNT, and the cells were treated with 4 μM intervention concentration. TM3 cells in the logarithmic growth phase were cultured for 12 h, and the solution was changed after observing adherence. TM3 cells were randomly divided into the control group, H_2O_2 group, SeNPs group, and H_2O_2+SeNPs group. For the H_2O_2+SeNPs group, TM3 cells were pretreated with SeNPs@LNT 4 μM for 12 h, followed by H_2O_2 800 μM for 8 h (Pi *et al.*, 2013).

Cell viability assay

Untreated TM3 cells were plated in 96-well plates at a concentration of 5×10^4/mL. TM3 cells in 96-well plates were incubated overnight at 37°C. TM3 cells in each group, as described above, were treated with 100 μL CCK-8 solution in a 96-well plate and incubated for 1 h. The absorbance at 470 nm was read with a microplate reader (Varioskan Flash, Thermo, Waltham, USA). Cells from four different experiments were analyzed for each treatment.

Detection of mitochondrial membrane potential (ΔΨm)

TM3 cells were cultured in a 6-well microtiter plate with a cell density of 5×10^4/mL for 24 h according to different treatment groups. After adding 1 mL of cell culture medium, 1 mL of JC-1 staining working solution was added, and the mixture was fully mixed. The cells were incubated at 37°C for 20 min; then, the supernatant was removed by aspiration and washed twice with JC-1 staining buffer. Cell culture medium, which may contain serum and phenol red, was added, and the mitochondrial membrane potential was examined under a fluorescence microscope (Leica, Wetzlar, German).

Intracellular active oxygen levels

TM3 cells were cultured on a 96-well droplet plate at a density of 5×10^4/mL for 24 h. For the nano-selenium and H_2O_2 treatment group: TM3 cells were pretreated with 4 μM nano-selenium for 12 h and treated with 800 μM H_2O_2 for 8 h. Then, the cells were stained with 10 μM DCFH-DA for 0.5 h. Two techniques, including microscopy and fluorometry, were used to measure intracellular ROS levels. Images were collected by fluorescence microscope (Leica, Wetzlar, German) in each group at the same exposure time. Fluorescence intensity of 488 nm excitation and 525 nm emission was measured with a fluorescence plate reader (Thermo Fisher Scientific, Waltham, USA).

Apoptosis detection by annexin V-fluorescein isothiocyanate

TM3 cells were cultured in 6-well plates at a concentration of 5×10^4/mL. After oxidative stress induction and drug treatment, the culture medium was aspirated and washed once with PBS. For *in situ* fluorescence detection of adherent cells, 195 μL Annexin V-FITC binding solution was added and mixed gently. Then, 10 μL propidium iodide staining solution was added, mixed well, and incubated in the dark for 10–20 min at room temperature (20°C–25°C) in an ice bath. Aluminum foil can be used to protect from light. Cell assays were performed as soon as possible after staining, usually within 1 h.

Western blotting

TM3 cells were cultured on a 12 cm cell culture dish at a density of 8×10^4/mL according to different treatments for

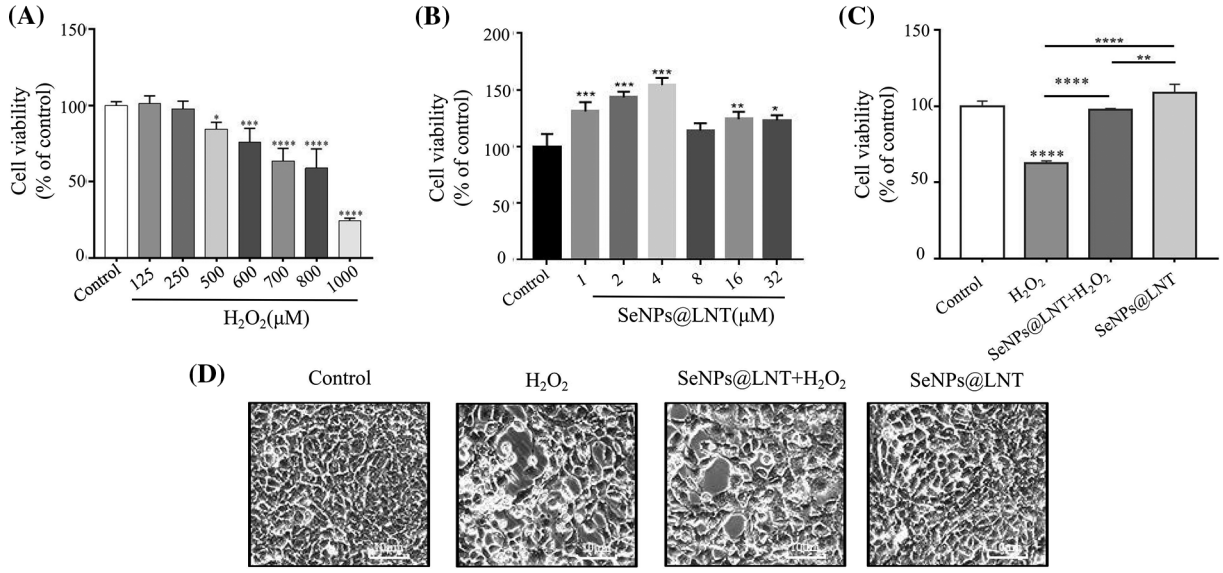

FIGURE 1. Effect of lentinan-functionalized nano-selenium particles (SeNPs@LNT) on H_2O_2-induced TM3 cells. (A) TM3 cells were exposed to increased concentrations of H_2O_2 (from 0 to 1000 μM) for 8 h. (B) TM3 cells were exposed to increased concentrations of SeNPs@LNT (from 0 to 32 μM) for 12 h. (C) TM3 cells were treated with 4 μM SeNPs for 12 h before being challenged by 800 μM H_2O_2 for 8 h. Cell viability was determined by CCK-8. (D) The TM3 cell was detected under a fluorescent microscope as a bright field. All data were presented as mean ± SD of three separate experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. The results were obtained from three repeated experiments.

24 h. TM3 cells were treated in different ways, as mentioned above, and placed in a lysis buffer to obtain cellular proteins. Protein concentration was determined by the BCA method. For loading, 40 μg of the sample was resolved by SDS-PAGE and transferred to a nitrocellulose membrane under the appropriate voltage. After that, TBSE buffer containing Tween-20, TrIS-Base, and 10× TBS (pH 7.4) was sealed with skim milk for 1 h. The primary antibody was diluted at 1:1000, and an appropriate amount of primary antibody was added to drown the PVDF membrane, and the reaction was conducted overnight at 4°C. After that, it was incubated with a secondary antibody for 1–2 h. Enhanced chemiluminescence reagents were used for X-ray development (Clinx, Shanghai, China).

Results and Discussion

Construction of oxidative stress model

TM3 cells were treated with concentration gradients of 0, 125, 250, 500, 600, 700, 800, and 1000 μM H_2O_2 for 8 h. After the induction of oxidative stress, cell viability after treatment with each concentration was detected by CCK-8, and the results are shown in Fig. 1A. With the increase in the concentration of H_2O_2, the cell viability gradually decreased. When the concentration was 800 μM, the cell viability was 58.81% ($p < 0.0001$), and the difference was statistically significant. The concentration of 800 μM was selected as the concentration of the H_2O_2 treatment according to the standard of IC50, which caused cell viability decline but not complete necrosis.

Characteristics and cytotoxicity of SeNPs@LNT

The synthesis and characterization data of nano-selenium can be referred to in this article (Zorov *et al.*, 2014). The results of treating cells with different concentration gradients of nano-selenium for 12 h are shown in Fig. 1B. In the set concentration gradient range, the cell viability gained a certain degree of peak after nano-selenium treatment. When the concentration was 4 μM, the cell viability was 154.00% ($p < 0.001$), and the difference was statistically significant. This concentration was chosen as the concentration of drug treatment. The cell viability did not increase significantly when the concentration was increased after treatment with 4 μM of SeNPs@LNT, and the cell density under a microscope was consistent with Fig. 1B. Compared to the inorganic and organic selenium as controls, nano-selenium had less cytotoxicity.

Antioxidant and cell proliferation effects of nano-selenium in vitro

The cell viability of the normal control group and oxidative stress group after 12 h of nano-selenium pretreatment was shown in Fig. 1C. The cell viability of the oxidative stress group was 62.71%, which was significantly different from

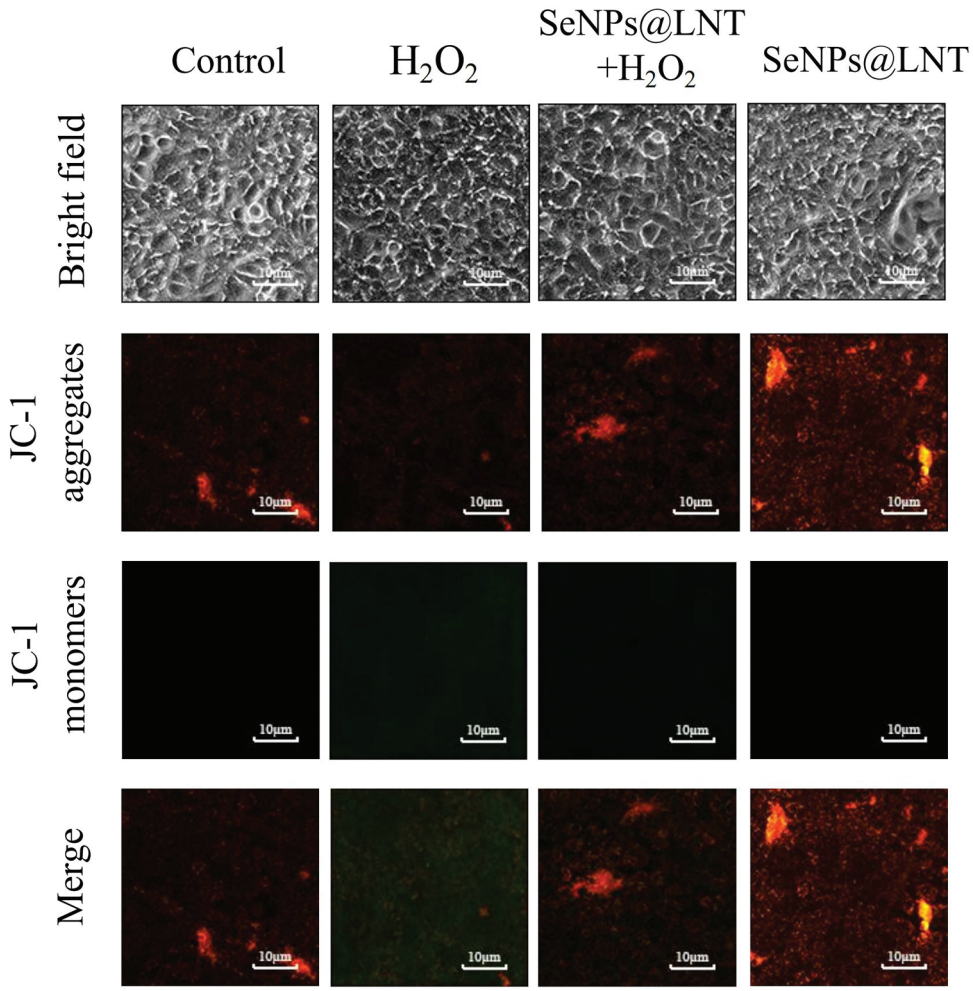

FIGURE 2. The mitochondrial membrane potential of the TM3 cell was detected with JC-1 fluorescent probe under a fluorescent microscope. The ratio of JC-1 monomer and polymer (red and green fluorescence) in different treatment groups was obvious. The results were obtained from three repeated experiments.

the control group ($p < 0.0001$). The cell viability of the H_2O_2+SeNPs group was 97.79%, which was statistically significant compared to the H_2O_2 group ($p < 0.0001$). The cell viability of the SeNPs group was 108.56%, which was statistically significant with the oxidative stress group and the H_2O_2+SeNPs group. The microscopic results of the cells are shown in Fig. 1D. In the oxidative stress group, the refractive ability of the cells increased, the shape of the cells became round, and some cells showed apoptosis and necrosis.

SeNPs@LNT inhibited the decrease of mitochondrial membrane potential induced by hydrogen peroxide

JC-1 kit was used to detect the mitochondrial membrane potential level of each treatment group, and the results are shown in Fig. 2. In the nano-selenium treatment group, the membrane potential was higher, JC-1 aggregated in the mitochondrial matrix, forming polymers, and the red fluorescence level was stronger. However, the level of JC-1 monomer in the oxidative stress treatment group (H_2O_2 group) was higher, and green fluorescence was detected by fluorescence microscopy.

Selenium nanoparticles can be targeted to mitochondria to play their biological role. Mitochondria are key organelles of energy metabolism and ROS production, which trigger the induction of mitochondrial permeability transition pore (mPTP). This triggering phenomenon is the response of mitochondria to the amplification of oxidative stress ROS signal. The plasticity of mitochondrial structure and function is important in maintaining cell homeostasis. Damage to their function causes the accumulation of ROS and triggers inflammation (Liochev, 2013; Zhang *et al.*, 2016). The subsequent detection of ROS revealed that oxidative stress caused the accumulation of ROS. According to relevant reports, selenium nanoparticles (SeNPs) synthesized by *Lactobacillus casei* ATCC 393 can alleviate H_2O_2-induced intestinal epithelial barrier dysfunction, reduce ROS production, and reduce mitochondrial membrane potential through the mitochondrial pathway (Yan *et al.*, 2021). Liu *et al.* (2017) showed that PC-SeNPs could significantly improve mitochondrial fragmentation in their study on the reversal of palmitate-induced apoptotic uptake by phycocyanin functionalized selenium nanoparticles in pancreatic β-cells (Liu *et al.*, 2017).

The production of reactive oxygen species was inhibited by SeNPs@LNT

The quantitative and fluorescence results of detecting intracellular ROS levels are shown in Figs. 3A and 3B. The intracellular ROS level in the oxidative stress group was 162.59% ($p < 0.0001$), and the intracellular ROS level in the H_2O_2+SeNPs group was 65.00% ($p < 0.0001$). There was no significant increase in ROS in the control group and nano-selenium treatment group.

The results of fluorescence data showed that green fluorescence increased in the oxidative stress group, and nano-selenium treatment could reduce the increase of ROS level caused by H_2O_2.

Regulating oxidative stress can initiate different cellular responses, from signaling pathways that trigger cell protection to initiating coordinated activation of mitochondrial fission and autophagy to optimizing the clearance of abnormal mitochondria and cells to protect the spread of damage to neighboring mitochondria and cells. The redox state of the cell environment is mainly determined by the ratio of reduction/oxidation cofactors to proteins that carry a large number of redox-sensitive amino acid residues and functional groups, NAD(P)H/NAD(P), and reduced glutathione and oxidized glutathione (GSH/GSSG), which together form a compartmentalized redox buffer in which all components are in redox equilibrium under cell homeostasis conditions. When stressed, ROS induced by H_2O_2 can affect metabolic pathways through glycolytic pathways, oxidative phosphorylation, and lipid metabolism (lipolysis and lipogenesis). They act on mitochondria, endoplasmic reticulum, and cytoplasmic networks of various second messengers, as well as antioxidases (superoxide dismutase SOD, glutathione peroxidase) to change the intracellular environment (Tsubata, 2020). Tavoosi *et al.* (2020) treated H_2O_2-induced pancreatic beta-cells with cerium and yttrium oxide radionuclides and nano-selenium, and their intracellular ROS levels were significantly reduced. Xu *et al.* (2019) used *Lactococcus lactis* NZ9000 to biosynthesize potan-capped selenium carriers, SeNPs, that can inhibit the increase in ROS, the decrease in adenosine triphosphate, and the decrease in mitochondrial membrane potential in H_2O_2-exposed IPEC-J2 cells (Xu *et al.*, 2019). Studies on the anti-tumor effect of nano-selenium have reported that modified nano-selenium particles can localize in mitochondria and trigger changes in ROS through endocytosis. As a signaling

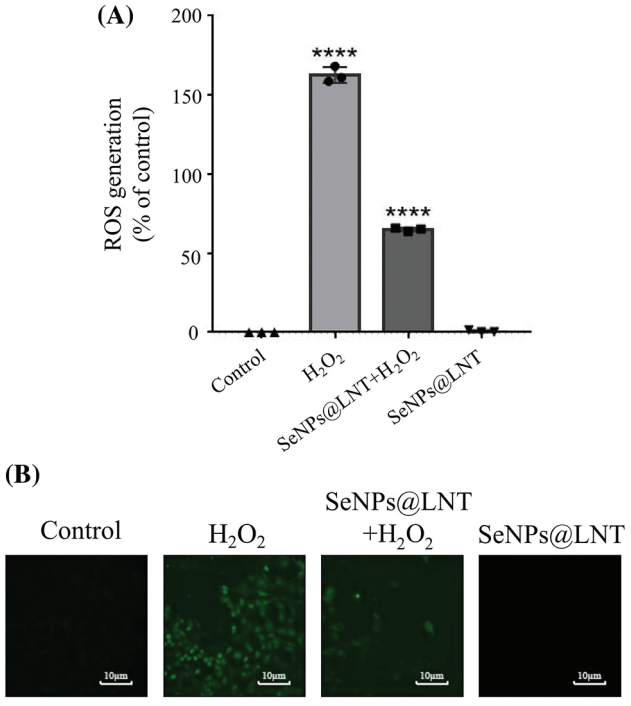

FIGURE 3. Effect of lentinan-functionalized nano-selenium particles (SeNPs@LNT) on H_2O_2-induced TM3 cells on the production of reactive oxygen species (ROS). TM3 cells were treated with 4 μM SeNPs@LNT for 12 h before being challenged by 800 μM H_2O_2 for 8 h. ****$p < 0.0001$. The results were obtained from three repeated experiments.

medium involved in cell growth, progression, and death, ROS has a role in the regulation of cell physiology (Su *et al*., 2019). It contains superoxide radicals, hydroxyl radicals, and singlet oxygen, which can destroy cell proteins, lipids, and nucleic acids. At the same time, the generation of ROS stimulates the activation of various intracellular signaling pathways, such as the P53 pathway, the mitogen-activated kinase pathway, the phosphatidylinositol 3-kinase/protein kinase B pathway, etc.

SeNPs@LNT reduced apoptosis and necrosis induced by oxidative stress

As shown in Fig. 4, apoptotic cells were stained only with green fluorescence, while the necrotic cells were stained with red and green double stains, and normal cells did not stain with any fluorescence. Under a fluorescence microscope, the oxidative stress group showed increased necrosis and apoptotic cells, and the degree of necrosis was less after nano-selenium treatment. There was no obvious apoptosis and necrosis in the control and the nano-selenium groups.

SeNPs@LNT prevented oxidative stress-induced cell damage through the Janus kinase 2/signal transducer and activator of transcription 3 signaling pathway and P53 signaling pathway

Proteins related to cellular pathways activated by SeNPs@LNT are shown in Fig. 5. Oxidative stress induced the decrease in STAT-3 expression and C3 protein level and increased the expression of P-P53 protein. SeNPs@LNT enhanced the expression of JAK2 and decreased the expression of AKT protein. A simulation diagrams for the relevant mechanisms is shown in Fig. 6. Oxidative stress is also an important factor in inducing programmed cell death. We observed the production of ROS, the initiation of apoptosis, and changes in JC-1. The production of mitochondrial ROS causes dysfunction of the electron transport chain and disrupts the regulation of energy production. Most apoptotic signals

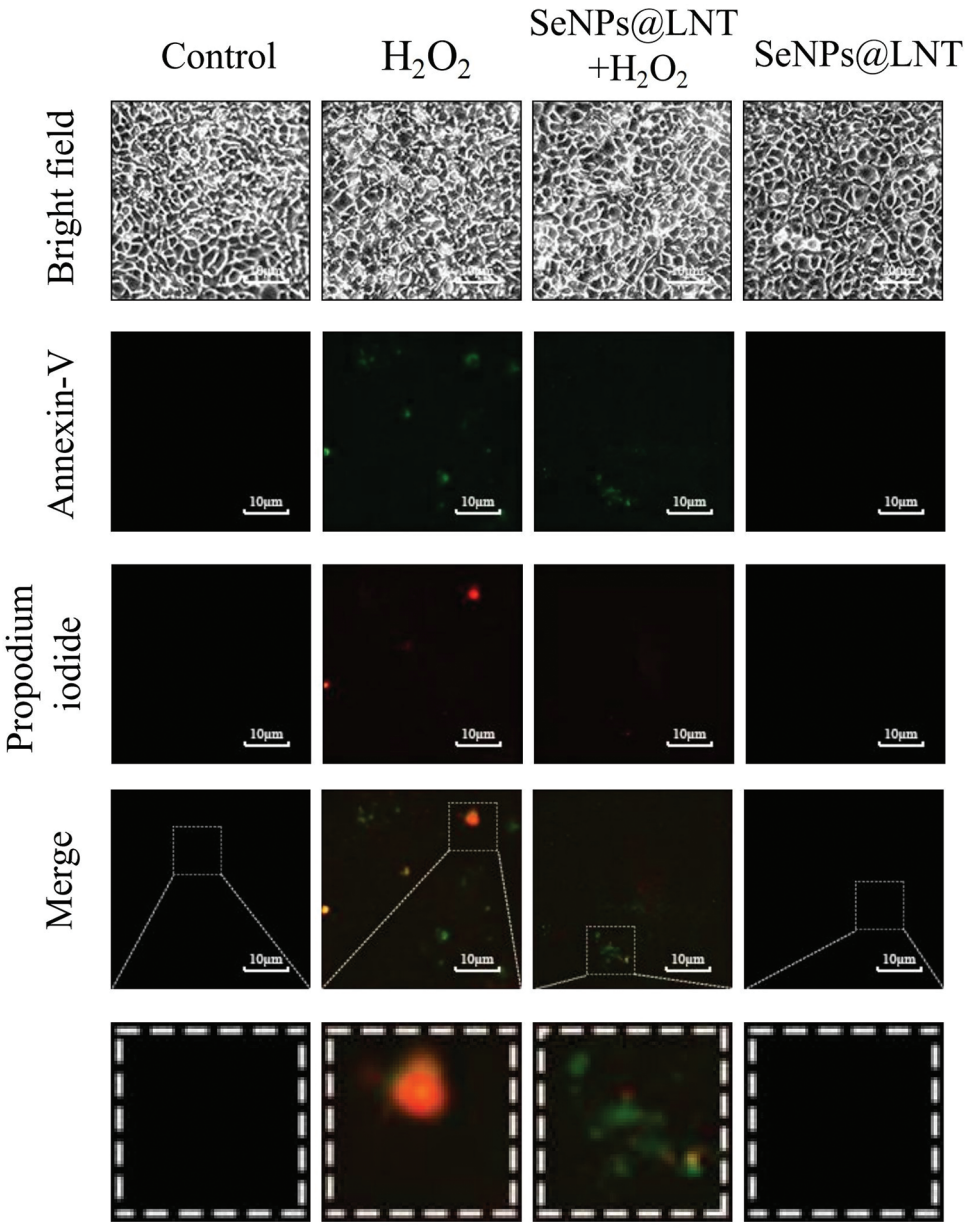

FIGURE 4. Under the fluorescence microscope, Annexin V-fluorescein isothiocyanate staining shows green fluorescence in early apoptotic cells, and propidium iodide staining shows red fluorescence in necrotic cells and late apoptotic cells. Apoptosis and necrosis in different treatment groups could be observed. The results were obtained from three repeated experiments.

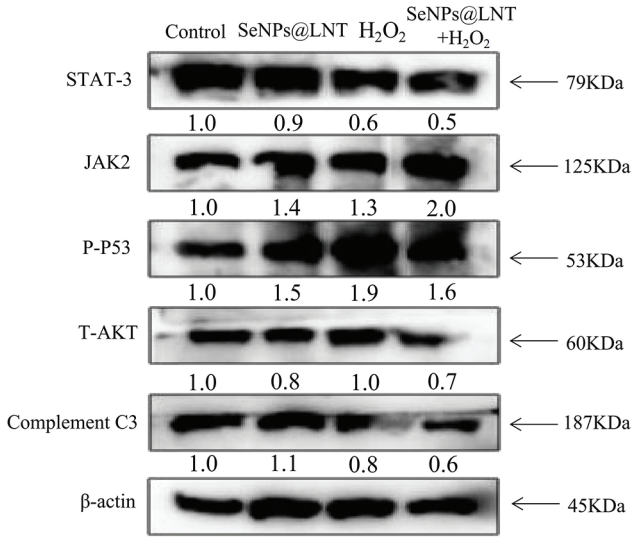

FIGURE 5. The signaling pathways activated by lentinan-functionalized nano-selenium particles (SeNPs@LNT) treatment. The TM3 cells exposed to different treatments expressed the pro-apoptotic and anti-apoptotic protein tracked by western blotting. β-actin was used as the loading control. The results were obtained from three repeated experiments.

originate from mitochondria and are regulated in part by members of the Bcl-2 family of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2, Bcl-xL) proteins, which control mitochondrial transmembrane potential. Various stimuli, including oxidative stress, can activate mitochondrial endogenous apoptotic pathways. Concurrently, we observed changes in STAT-3, JAK2, and P-P53 proteins.

JAK2/STAT-3 pathway is a signaling pathway related to immune regulation, cell proliferation, differentiation,

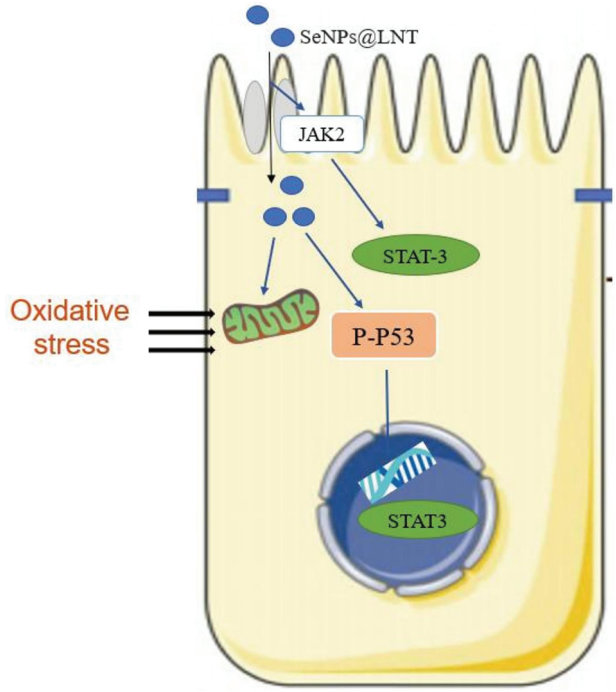

FIGURE 6. Proposed signaling pathway triggered by lentinan-functionalized nano-selenium particles (SeNPs@LNT) and H_2O_2 inTM3 cells.

apoptosis, and hematopoiesis (O'Shea and Plenge, 2012). STAT is phosphorylated by JAK; it is then dimerized, transferred to the nucleus through the nuclear membrane, where it acts on DNA, and regulates the expression of related genes (Yu *et al.*, 2009). A variety of cytokines and growth factors transmit signals through the JAK/STAT signaling pathway, including interleukin 2–7, epidermal growth factor, platelet-derived growth factor, and interferon (Boengler *et al.*, 2008). Tyrosine kinase-associated receptors on cell membranes to receive signals from cytokines and growth factors. To some extent, the activation of this pathway reflects the changes in DNA methylation, the degree of cellular inflammation, and the expression of other cellular mediators (Xin *et al.*, 2020). Its downstream target genes encode transcribed cytokines and growth factors that are further involved in the physiological functions of cells.

Concurrently, we observed that SeNPs@LNT changed the level of P-P53 protein in TM3 cells. P-P53, as a phosphorylated form of the P53 molecule, can be induced to activate by many stress signals, such as DNA damage, oncogene activation, and nutritional deprivation. It is mainly involved in cell metabolism, antioxidant, and DNA damage repair. In the regulation of the cell cycle and cell apoptosis, P53 may induce a large number of genes involved in the execution of the signal, including executing death receptor (Fas, Dr4), apoptosis factor, and BH3 domain to promote apoptosis protein structure to activate the extrinsic apoptotic pathway, and on the outer membrane of mitochondria permeability transition induced membrane hole open, causing changes in the level of membrane potential (Chen, 2016). The serine/threonine kinase AKT, as a proto-oncogene, is activated by extracellular signals (mainly growth factors via P13K signaling) and is involved in cell survival and inhibition of apoptosis, as well as tumor progression (Zhao *et al.*, 2017). AKT is involved in the accumulation of ROS by stimulating oxidative metabolism and promoting mitochondrial oxygen consumption. We found that nano-selenium treatment reduced the level of AKT molecule to a certain extent, but the oxidative stress treatment (H_2O_2 group) did not stimulate its production, and this may be related to the degree of oxidative stress. As a part of innate immunity, C3 is involved in the clearance of pathogens and damaged cells in opsonization, leading to the coating of pathogens with antibodies or complement proteins to promote the phagocytosis of debris with foreign pathogens (Copenhaver *et al.*, 2019). The cascade activation of its products initiates three effector pathways, inflammation, phagocytosis, and membrane attack, which carry out the coordinated defense of the organism (Ricklin and Lambris, 2016). The treatment with nano-selenium up-regulated the expression of C3, and the effect of oxidative stress on the decline of cellular immune function could be seen.

The sperm characteristics, DNA integrity, chromatin quality, antioxidant enzyme levels, testosterone concentrations, and testicular histomorphology were assessed in a study of germ cell damage induced by the anti-cancer drug cisplatin with nano-selenium particles, describing the concomitant antioxidant and reproductive stimulatory effects of selenium-based preparations

(Rezvanfar *et al*., 2013). In fact, the results of several studies are consistent with the conclusions of this study.

Conclusions

In this study, functionalized nano-selenium was used to regulate cell function through the JAK2/STAT-3 pathway, P53 pathway, AKT, C3 molecules, etc., acting on mitochondria, changing their membrane potential level, and alleviating the apoptosis and necrosis of TM3 cells against the elimination of ROS. The detection of cytokines and antioxidant enzymes was not carried out. The specific path of nano-selenium entering the cells could be further determined by fluorescence. The status of other organelles in the cells was not detected. As a new kind of drug, nanomaterials have attracted extensive attention in tumor immunity, antiviral and antioxidant properties, the treatment of nervous system diseases, and so on. Nanomaterials have the characteristics of strong targeting and high bioavailability. How nano-selenium drugs participate in the regulation of signaling pathway molecules and the expression of cytokines is worthy of further investigation.

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Author Contributions: Miaomiao Li and Zilin Zheng designed the study and analyzed the experimental data. Junyi Ke and Jieyi Luo drafted the manuscript. Fan Jiang and Yanxia Qu carried out the experiments. Bing Zhu, Yinghua Li, and Liandong Zuo refined the manuscript and coordinated the study, were responsible for the laboratory management, reviewed and revised articles, and confirmed the final draft. All authors read and approved the final manuscript.

Availability of Data and Materials: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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