Nitrogen-Doped Multiwalled Carbon Nanotubes Inhibit Fat Deposition via Immunomodulatory Actions

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Abstract

Multiwalled carbon nanotubes (MWCNTs) offer immense opportunities to deliver drug and biomolecules to targeted tissues. However, it's unclear for us about their effects on fat metabolism. Here, we demonstrate that nitrogen-doped carboxylate-functionalized MWCNTs (N-MWCNTs) inhibit fat deposition both in vivo and in vitro primarily by suppressing adipogenesis. N-MWCNTs show good biocompatibility in HEK293 mammalian cells. Intramuscular administration of N-MWCNTs does not affect the body weight gain and feed intake of mice, but reduces the fat mass. In in vitro-cultured adipocytes, N-MWCNTs suppress fat accumulation, accompanying with decreased and increased expression of adipogenic and lipolysis genes, respectively. Transcriptome analysis further certified the N-MWCNT alteration of fat metabolism-related genes. Interestingly, we observed the phagocytosis of N-MWCNTs by macrophage-like cells via TEM imaging. The mRNA sequencing data also showed remarkable variation of the genes involved in TLRs pathway, ultimately leading to down- or up-regulation of inflammatory factors, of which *Tnf α* , *Il1*, *Il7*, *Il10*, and *Il12* are decreased, whereas *Il6* and *Il11* are increased. In conclusion, N-MWCNTs induce the production of inflammatory cytokines through immune responses, which trigger the reduction of fat deposition. These findings support the usage of N-MWCNTs as a promising delivery for anti-obesity agents.

1. Introduction

Nanotechnology is now revolutionizing many fields from industrial manufacturing to human health [1]. Carbon nanotubes (CNTs), as a kind of tube like-structured nanomaterials, have received great interest in the field of pharmaceutical sciences due to their high penetration power and surface area [2–5]. CNTs can be divided into two categories: single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) [5]. Compared with SWCNTs, MWCNTs are composed of multi-layer graphene and have a much wider diameter adjustment range, so they provide a variety of research methods for different biological purposes, such as delivering some large biomolecules and drugs to the cells [6–9]. Doping and functionalization could efficiently improve their solubility, compatability, and safety [10–12]. Scientists have shown in mouse models that nitrogen-doped MWCNTs are better tolerated than the pristine MWCNTs, and induce a weaker inflammatory response [11]. Our investigation has demonstrated the enhancement of nitrogen-doped carboxylate-functionalized MWCNTs (N-MWCNTs) on bone growth with good biocompatibility [12]. Therefore, N-MWCNTs offer immense opportunities as drug or biomolecule carriers and have great application potential in the treatment of diseases.

Obesity is a multifactorial metabolic disease mainly resulting from the imbalance of energy intake and consumption. The global prevalence of obesity and its affiliated comorbidities, such as diabetes, fatty liver, hypertension and high cholesterol, pose a severe threat to human health [13, 14]. Various approaches, including modified nutrition, additional physical activity, pharmaceutical agents, and bariatric surgery, have been applied in clinic to combat obesity [15]. However, the curative effects of these conventional options seem to vary largely among individuals [16]. Therefore, treatments with higher efficacy and specificity are urgently required. The emerging drug delivery systems using nanomaterials,

for example N-MWCNTs, promise new possibilities to improve therapeutic efficacy and specificity through stabilization and spatiotemporally controlled release of anti-obesity agents [17–19]. However, whether N-MWCNT itself affects fat metabolism remains largely unknown.

In the present study, we found that N-MWCNT administration inhibits fat deposition in both mice and *in vitro*-cultured adipocytes mainly by suppressing adipogenesis. Further investigation indicated that N-MWCNTs induce immune responses through phagocytosis and alter the production of inflammatory cytokines, which in turn inhibit fat accumulation. This study reveals that N-MWCNTs could regulate fat metabolism through immunomodulatory actions, which supports the usage of N-MWCNTs as a promising delivery material for anti-obesity agents.

2. Experimental

2.1. Materials

The N-MWCNTs were obtained from Chengdu Organic Chemicals Co. Ltd., Chinese Academy of Sciences. PI/RNase Staining Buffer and FITC Annexin V Apoptosis Detection Kit were from BD Pharmingen (New Jersey, USA). Mitochondrial membrane potential (MMP) assay Kit with JC-1 was purchased from Beyotime (Jiangsu, China). MiniBEST Universal RNA Extraction Kit and One Step TB Green® PrimeScript™ RT-PCR Kit were obtained from Takara Biotechnology (Dalian, China).

2.2. Preparation and characterization of N-MWCNT dispersions in aqueous media

For the purpose of preparing a suspension of MWCNTs, we dissolve 1 mg of N-MWCNTs in 5 mL of phosphate buffer solution (PBS). Subsequently, the suspension was dispersed with sonication for 50 min/40 kHz. Next, we use a scanning electron microscope (JEOL, Japan) to prepare images under an acceleration voltage of 15kV. Additionally, transmission electron microscopy (TEM) imaging was prepared to observe the N-MWCNTS dispersion at an accelerating voltage of 120 kV (Hitachi TCM system, Japan). The axial dimensions and radial dimensions of more than 100 N-MWCNTs were analyzed by ImageJ software.

2.3. X-ray photoelectron spectroscopy (XPS) and UV-visible spectroscopy of N-MWCNTs

In order to analyze the elemental composition of N-MWCNTs, the N-MWCNTs were examined using XPS. An Al-K α (1253.6 eV) X-ray monochromator with the 150 W frequency and the 500 μ m light spot is used for detection. Furthermore, UV-visible absorption spectrum techniques were used to characterize N-MWCNTs. A spectrometer (Lambda 35, Perkin Elmer) was used to record the UV-visible spectrum, with its operating wavelength being between 190 nm and 240 nm.

2.4. Culture of HEK293 cells

The human embryonic kidney 293 (HEK293) cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM/F-12 1:1) (BI, Kibbutz, Israel), 10% fetal bovine serum (FBS) (BI, Kibbutz, Israel), 1% penicillin and streptomycin (Solarbio Technology Co., Beijing, China) and incubated under standard cell culture conditions (37°C with 5% CO₂ humidified atmosphere).

2.5. Annexin V-FITC/PI double staining assay

The FITC Annexin V apoptosis detection kit was used to quantitatively analyze the cell necrosis and apoptosis induced by N-MWCNTs. HEK293 cells were treated with N-MWCNTs ranging from 0 to 1.0 µg/mL for 24 hours, which were washed twice with cold PBS, and then were re-suspended in 1× binding buffer to a concentration of 1×10⁶ cells/mL. We transferred 100 µL of cell suspension (1×10⁵ cells) to a 5 mL tube, added 5 µL each of FITC Annexin V and PI, and gently mixed for 15 minutes at room temperature in the dark. Subsequently, we added 400 µL of 1× binding buffer to each tube, used flow cytometry (BD Bioscience, San Jose, CA) to analyze the cells. Finally, we collected and tested at least 10,000 cells per sample and used FlowJo VX software to analyze the data.

2.6. Flow cytometric analysis of the cell cycle

PI/RNase staining buffer was used to determine the cell cycle. Briefly, HEK293 cells were exposed to different concentrations of N-MWCNTs (0, 0.05, 0.2 or 1.0 µg/mL) at 37°C for 24 hours. Subsequently, the cells were collected, centrifuged and washed twice with cold PBS. Cells were then re-suspended in 70% ice-cold ethanol, incubated at -20°C for 1 hour, and washed with PBS. After fixing and permeabilizing, the cells were incubated with PI/RNase staining buffer for 15 minutes, and immediately measured with a flow cytometer (BD Bioscience, San Jose, CA). Eventually, ModFit LTTM software (Becton Dickinson, CA, USA) was used to calculate the cell distribution of 20,000 cells in different phases of the cell cycle.

2.7. Detection of MMPs

MMP is an important sign of early apoptosis through the endogenous mitochondrial pathway. Thus, we use JC-1 MMP detection kit to detect MMP, to further determine whether N-MWCNTs have any toxicity to cells. First, 2×10⁶ HEK293 cells were treated with various concentrations (0, 0.05, 0.2, and 1.0 µg/mL) of N-MWCNTs for 24 hours. Subsequently, the treated cells were collected and re-suspended in 0.5 mL of medium. We then added 0.5 mL of JC-1 staining solution to the culture medium, mixed it well with the cells, and incubated the mixture at 37°C in the dark for 20 minutes. After that, analysis was performed using a flow cytometer (BD Bioscience, San Jose, CA).

2.8. Animal experiment, sample collection, and determination

3 week-old Kunming male mice with an average body weight of about 20 grams were used to investigate the bioactivity of N-MWCNTs. The experimental protocol was approved by the ethical committee for animals in research of Shandong Agricultural University, China. Mice were randomly separated into 2 groups, which were intramuscularly injected with PBS (Vehicle) or N-MWCNTs at a dosage of 1.0 mg/kg of body weight (bw). The mice were given free access to feed and water. The rearing temperature was

fixed at 25~27°C. Body weight and feed intake of the mice were monitored at 2, 7, and 14 days post injection, respectively.

In another experiment, the mice were injected with 1.0 mg/kg bw N-MWCNTs for 2 days. DXA (MEDIKORS, Korea) was used to determine the whole body fat content. Subsequently, the mice were sacrificed and dissected, inguinal WAT (iWAT) of the mice was obtained and weighed, and its proportion relative to total bw was calculated.

2.9. Isolation, cultivation, and treatment of mouse adipose-derived stem cells (ASCs)

ASCs were isolated from iWAT of 3-week-old female Kunming mice, as previously described [20]. In brief, the iWAT was collected under sterile conditions, which was digested with 1.5 mg/mL collagenase at 37°C for 45 minutes. Subsequently, the digested tissue was filtered through a membrane of 100 µm, and the filtered cells were centrifuged at 1,000 ×g for 5 minutes. The sedimentary cells were re-suspended with Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% FBS, plated to cell culture plates, and cultured under standard cell culture conditions (37°C, humidified, 5% CO₂/95% air). After 4 days, the dis-adherent cells were removed and the adherent cells, defined as ASCs, were cultured to sub-confluence.

The sub-confluent cells were treated with adipogenic stimuli (AS), which consists of 0.5 mM IBMX+5 µg/mL Insulin+0.1 µM Dexamethasone for the first two days, and 5 µg/mL Insulin+0.1 µM Dexamethasone for further 8 days, in the absence or presence of N-MWCNTs ranging from 0.05 to 1 µg/mL.

2.10. Observation of lipid drops in adipocytes via Oil red O (ORO) staining

The treated ASC-differentiated adipocytes were washed with PBS, and fixed with 4% paraformaldehyde for 1 hour at room temperature. Then the cells were stained with a 0.6% ORO solution for 30 minutes. After washing thoroughly with PBS for 3 times, the cells were re-stained with hematoxylin for 5 minutes to show the nuclei. Pictures were taken under an inverted microscope (Nikon, Japan). For quantification, the stained ORO was dissolved with isopropanol, and the absorbance at 510 nm was determined, which was shown as mmol ORO/mg total protein.

2.11 Real time quantitative polymerase chain reaction (RT-qPCR)

N-MWCNT-treated adipocytes and iWAT of mice were collected to determine the mRNA expression of fat deposition-associated genes. MiniBEST universal RNA extraction kit was used to extract the total RNA from cells or tissues and one-step SYBR RT-PCR kit (TaKaRa, Japan) was employed to reverse transcribe the extracted RNA into cDNA. RT-qPCR was performed on ABI Prism 7500 PCR System using UltraSYBR

mix (low ROX) reagent (CWBIO, China). The average Ct value in triplicate is normalized from the average Ct value of GAPDH and the relative expression levels of genes between N-MWCNTs and vehicle groups were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in the Supplementary table 1.

2.12 mRNA sequencing of Vehicle and N-MWCNT-treated adipocytes

ASCs were cultured in 100 mm² dishes to sub-confluence, which were exposed to N-MWCNTs (0.2 µg/mL) or Vehicle (PBS) in the presence of AS for 10 days. The treated cells were collected, and total RNA was extracted using Trizol reagent (Takara, Japan). According to recommendations in the Illumina TruSeq RNA Sample Preparation V2 Guide, the biologically triplicate sequencing libraries were constructed by 4 µg of total RNA. The amplified libraries were sequenced on an Illumina HiSeq 2000 platform. For RNA-sequencing, 150 base pair, single-end reads were binned according to a six base pair barcode. TopHat was used to map the barcoded reads to the mouse genome (GRCm38), and then DESeq was run to assemble and quantify the transcriptome. Transcript abundances were presented as FPKM (Fragments Per Kilobase of transcript per Million mapped reads).

2.13 TEM of N-MWCNTs in macrophage-like cells

ASCs were cultured to sub-confluence in 100 mm² dishes, which were exposed to N-MWCNTs (0.2 µg/mL) and incubated for 48 hours. After that, the cells were washed 3 times with PBS and then digested with trypsin (0.25%) at 37°C for 10 minutes. The collected cells were centrifuged and fixed with 2.5% glutaraldehyde for 24 hours at 4°C. Then the cell particles were fixed with 4% OsO₄, dehydrated in ethanol series (25, 40, 60, 80, 90, 100%), which were eventually embedded in propylene oxide and cut into thin slices. A transmission electron microscope (Hitachi TCM system, Japan) was used to observe and picture the obtained ultrathin section (5 µm).

2.14 Statistical analysis

Each experiment was repeated at least three times. Data were expressed as mean ± standard error of the mean (SEM) and analyzed by the GraphPad Prism version 5.0 software (San Diego, CA) to determine differences among the groups. Difference between treatments was tested by one-way ANOVA or two-sample student *t*-test. Statistical significance was considered at *p*<0.05.

3. Results

3.1. Physicochemical characterization of N-MWCNTs

The scanning electron microscopy (SEM) pictures exhibited a well-separated suspension of N-MWCNTs after sonication for 50 minutes (Fig. 1A). And the TEM imaging further confirmed that the morphology of N-MWCNTs was generally uniform, showing corrugated or segmented tubes (Fig. 1B). Meanwhile, statistical analysis indicated that the diameter and the width of N-MWCNTs were on the order of micrometers and nanometers, respectively (Fig. 1C, D). The XPS spectrum of N-MWCNTs showed the presence of carbon, oxygen and nitrogen, and the nuclear-level peaks of C, O and N 1s were at about 285 eV, 530 eV and 399 eV, respectively (Fig. 1E). Chemical analysis revealed that the carbon content is the most (77.42%), followed by oxygen (19.02%), and the nitrogen content is the least (3.56%), with C/O ratio is 4.07 and C/(C+N) ratio is 0.96 (Fig. 1F). The UV-Visible spectrum of N-MWCNTs suggested a maximum absorbance at 200 nm and gradually decreased thereafter (Fig. 1G). These results suggest that the N-MWCNTs used here have sufficient purity and uniformity, their structure is relatively stable, and their element ratio is appropriate, which support subsequent biological activity studies.

3.2. N-MWCNTs treatment does not affect the biological activity of HEK293 cells

Next, we tested the biocompatibility of N-MWCNTs in mammalian cells via apoptosis, necrosis, cell cycle, and MMP evaluations. Annexin V/PI staining showed that N-MWCNTs do not lead to enhanced apoptosis or necrosis in HEK293 cells (Fig. 2A-D). Representative cell cycle phase distribution was done by PI/RNase staining followed by flow cytometric analysis, which suggested that the G0/G1, S and G2/M cell population were not noticeably changed ($p>0.05$) (Fig. 2E, F). In addition, the MMP of HEK293 cells was only decreased by 1 $\mu\text{g/mL}$ N-MWCNTs but rather by other treatments (Fig. 2G, H). These data provide strong evidence that N-MWCNTs exhibit good biocompatibility at most of the tested dosages and would not affect the normal life activities of the cells.

3.3. N-MWCNTs administration does not affect the growth and feed intake of mice

To further detect the biocompatibility of N-MWCNTs *in vivo*, mice were intramuscularly injected with N-MWCNTs at a dosage of 1.0 mg/kg bw every 2 days, respectively for 2, 7 and 14 days. The result showed that there is no significant change in total body weight upon N-MWCNT injection at any indicated days (Fig. 3A-C). During the experiment period, no abnormal symptoms or stress occurred in any of the treated mice. In addition, the feed/gain ratio in different groups following 2, 7 and 14 days of injection was not varied as well (Fig. 3D-F). These findings indicate that N-MWCNTs also exhibit good biocompatibility in mice.

3.4. N-MWCNTs administration decreases fat deposition in mice

We next investigated the effect of intramuscular injection of N-MWCNTs at a dose of 1.0 mg/kg bw on fat deposition of mice. Two days after injection, DXA imaging showed that the adipose tissue (red) of the mice in the treatment group was less than that of the mice in the Control group (Fig. 4A), with a modest

reduced fat mass (Fig. 4B). Consistently, the iWAT index in the N-MWCNTs-treated mice was significantly lower than that of the Control (Fig. 4C). In addition, the transcript levels of adipogenesis-related genes including *Cebpa*, *Ap2*, and *Lpl* in N-MWCNTs-treated mice were significantly decreased, while *Cpt1*, a gene involved in fatty acid oxidation, was also down-regulated compared with those in the Control (Fig. 4D). Taken together, these results indicate that the intramuscular injection of N-MWCNTs reduces fat deposition in mice primarily via decreasing the adipogenic program.

3.5. N-MWCNTs treatment inhibits fat accumulation of *in vitro*-cultured adipocytes

ASCs could differentiate into adipocytes by hormone inductions [21]. Considering the negative regulation of N-MWCNTs on fat deposition *in vivo*, we used ASCs to investigate the effect of N-MWCNTs exposure on adipogenesis. ORO staining and quantification showed that the accumulated lipid droplets in N-MWCNTs-treated groups (0.05, 0.2, and 1 $\mu\text{g/mL}$) were significantly decreased compared to the Vehicle (Fig. 5A, B). In addition, the mRNA levels of *Ppar γ* , *Cebpa* and *Lpl* were all reduced significantly by different concentrations of N-MWCNTs (Fig. 5C, D, G). Meantime, 0.05 $\mu\text{g/mL}$ N-MWCNTs markedly decreased the expression of *Fas* (Fig. 5F). Additionally, while *Hsl* was inhibited by 0.05 / 0.2 $\mu\text{g/mL}$ N-MWCNTs, *Cpt1* was significantly increased in N-MWCNTs-treated cells (Fig. 5I). These data collectively evidence that the tested dosages of N-MWCNTs significantly suppress the adipogenic differentiation of ASCs and fat accumulation *in vitro*.

3.6. N-MWCNTs trigger transcriptomic alterations in fat metabolism associated genes *in vitro*

To systematically explore which genes and signaling pathways were transcriptionally affected by N-MWCNTs, we conducted mRNA sequencing on ASCs treated with 0.2 $\mu\text{g/mL}$ N-MWCNTs or Vehicle for 10 days. Hierarchical cluster analysis showed significant transcriptomic differences between treated and Control group cells (Fig. 6A), with 2,415 genes being up-regulated and 2,812 genes being down-regulated by N-MWCNTs, respectively (Fig. 6B). KEGG analysis showed the top 20 most altered signaling pathways, with MAPK and PI3K-Akt signaling pathways being involved (Fig. 6C, Supplementary table 2). Further analysis showed that genes in the PPAR signaling pathway (Fig. 6D, Fig. S1), lipolysis pathway (Fig. 6E, Fig. S2), and adipocytokine signaling pathway (Fig. 6F, Fig. S3), which are closely correlated with fat metabolism, have undergone major changes.

By categorizing the key changed genes in these three fat metabolism related pathways, we divided them into two groups: genes linked to lipogenesis and genes linked to lipolysis. In the first group, fatty acid uptake and utilization associated genes, including *FATCD36*, *Fatp*, and *Fabp5*, were significantly reduced. Besides, the transcription levels of the gene *Gyk* that promotes the accumulation of glycerol, as well as *Adipor1*, *Lepr*, *Acs*, and *Acs15* that are positively related to lipid accumulation, were all significantly decreased (Fig. 7A, B). In the second group, the gene *Ppara* that promotes the oxidation of fatty acids and the metabolism of high-density lipoproteins, the gene *Plin1* that controls triacylglycerol (TAG) hydrolysis and lipolysis in adipose tissue, the gene *Cpt1b* which encodes the key enzyme of fatty acid β -oxidation,

and the gene *Cpt1c* that activates leptin, were all increased in response to N-MWCNTs treatment. Additionally, the transcription levels of fat degradation closely related genes *Rxrg* and *PGC-1 α* , were both increased significantly (Fig. 7C, D). Thus, these data demonstrate that N-MWCNTs lead to vast transcript alterations of fat metabolism associated genes.

3.7. N-MWCNTs-induced alteration of fat metabolism is associated with immune responses

TEM imaging showed the phagosome localization of N-MWCNTs in macrophage-like cells (Fig. 8A), indicating N-MWCNTs might be taken up by macrophages and transported to lysosomes. This supported the RNASEQ data and KEGG analysis, which showed significant activation of several inflammation related pathways (Fig. 6C). In fact, N-MWCNTs treatment significantly impacted the transcription of a number of inflammatory factors, such as *TNF α* , *I κ B*, and *Il1a/6/7/10/11/15/16/17c/18* (Fig. 8B, D), suggesting that N-MWCNTs might trigger an immune response. In addition, we noticed a significant change in the TLRs signaling pathway (Fig. 8C, D, Fig. S4). The phagocytosis of macrophage-like cells would initiate the immune response [22], which led to the decreased transcription of *TLR1/2/6/7/8/9*, *PI3k*, *Casp8*, *CD14*, *ERK*, and *AP1* (Fig. 8C). Meantime, the transcription levels of *TLR5* and *p38* were increased. It was worth noting that the reduced *I κ B* and *AP1* could inhibit the production of several pro-inflammatory factors, such as *TNF α* , *Il1a*, and *Il12*. Moreover, *Il6* and *Il11*, which are associated with metabolic inflammation and insulin resistance, were up-regulated. These findings suggest that N-MWCNTs trigger the immune response via macrophage-like cells, alter the transcription of several inflammatory factors by affecting the expression of *TLRs*.

4. Discussion

This study revealed several major findings about the important biological properties of N-MWCNTs and their effects on fat metabolism. Firstly, N-MWCNTs are stable and show high biocompatibility both *in vivo* and *in vitro*, supporting their safety as a drug delivery nanomaterial. Secondly, N-MWCNT administration inhibits adipogenesis, and ultimately reduces the overall fat content of the organism, indicating their strong potential applicability in the treatment of obesity. Thirdly, N-MWCNTs regulate fat metabolism through immune regulation, and thus have application potentials in immune-related metabolic diseases.

CNTs exhibit amazing functions in immunomodulation, cancer therapy, brain disorder treatment, tissue engineering, and drug delivery [9, 10, 12, 23, 24]. With the development of nanotechnology, the evaluation in drug delivery is more than ever. When using as drug carriers, the first and most important consideration is the safety of the nanomaterial. Here, we analyze the physicochemical properties of N-MWCNTs and determine their effects on cell cycle, apoptosis, and necrosis. Results indicate that N-MWCNTs show good biocompatibility in both human cell lines and mouse models. This apparent low toxicity may be due to nitrogen doping and the addition of carboxylic acid functional groups, which have previously been reported to be more soluble and less toxic in biocompatible media than unmodified MWCNTs [25, 26].

Through *in vivo* and *in vitro* investigations, we confirmed that N-MWCNTs have the capability to inhibit fat deposition. It is well known that decreased adipogenesis and/or increased lipolysis would lead to reduced fat deposition [27, 28]. Low dose administration of N-MWCNTs in the present study led to both decreased adipogenic- and increased lipolysis-associated gene expression in *in vitro*-cultured adipocytes, while only suppressed the adipogenic gene expression in animals. The transcriptome sequencing data indicated that the significant varied signaling pathways include PPAR signaling, regulation of lipolysis in adipocytes, and the adipocytokine signaling. In the PPAR signaling pathway, the increased expression of *Ppara* promotes fatty acid oxidation [29, 30], as evidenced by the up-regulation of *Cpt1*, a gene that translates to a key enzyme in the fatty acid oxidation process (Fig. S1). For the regulation of lipolysis in adipocytes, we noticed a significant increase in the expression of *Plin1*, which plays a major role in TAG hydrolysis and lipolysis [28, 31, 32] (Fig. S2). Additionally, we observed that most of the genes in the adipocytokine signaling pathway have changed to varying degrees, and these genes are closely related to the NF- κ B and MAPK signaling pathways. The NF- κ B signaling pathway related genes *TNF- α* and *FATCD36* are both decreased [33, 34]. In addition, the transcription levels of *STAT3*, *AMPK*, *Pgc-1 α* and *Cpt1* are all increased (Fig. S3). It is notable that *Pgc-1 α* and *Cpt1* induces the expression of genes involved in fatty acid oxidation [35, 36]. In addition, *Acs* and *Acs15*, which activate the synthesis of long-chain fatty acids [37, 38], and *Lepr* that is positively correlated with obesity [39], are decreased by N-MWCNT treatment.

Through electron microscopy observation, we have further obtained evidence that N-MWCNTs are endocytosed by macrophage-like cells through phagocytosis. Meanwhile, the expression of many key factors in TLRs signaling pathway has changed, including the decreased expression of TLR1/2/6/7/8/9 (Fig. S4). Decreased TLR1/2 affect the PI3K-Akt signaling pathway by reducing the expression of PI3K, and PI3K can affect fat synthesis by participating in the insulin signaling pathway [40]. At the same time, the decreased PI3K also down-regulates the expression of I κ B α in the NF- κ B signaling pathway. The reduced ERK and phosphorylated P38 in the MAPK signaling pathway act together on AP1. In the end, the transcript levels of inflammatory factors *Tnfa*, *Il1*, *Il7*, *Il10*, and *Il12* are decreased, whereas *Il6* and *Il11* is increased (Fig. S4). The adipose tissue is not only a passive storage of fat, it is also an important producer of inflammatory cytokines through macrophages and other immune cells during obesity. The inflammatory factors link the immune response of macrophages to fat metabolism [41, 42]. The inhibited TNF α promotes fat degradation by participating in the adipocytokine signaling pathway (Fig S3). IL-1 β hinders insulin signal transduction and lipogenesis in adipocytes [43, 44]. IL-6 acts as a potent stimulator in increasing fatty acid oxidation [45], and acts in autocrine/paracrine fashions to promote beiging of white adipocytes [20]. There is also evidence that IL6 reduces fat formation in mammals [46]. Based on these findings, we can conclude that N-MWCNT treatment boosts complex immune responses, which is actually a low-grade inflammation. Through the link between innate immunity and metabolism, they induce changes in the expression of fat metabolism-related genes. Therefore, the reduction of fat deposition is at least partially dependent on the N-MWCNT-initiated immune responses.

Enhanced fat deposition would lead to overweight and even obesity. The inhibitory function of N-MWCNTs on fat deposition poses this nanomaterial as a candidate delivery vehicle for anti-obesity

agents. In fact, nanomaterials have successfully delivered drugs, DNAs, or microRNAs for treatment of cancer, neurological diseases, and metabolic syndrome [19, 24, 47, 48]. Compared to conventional therapy, the targeted-nanosystems have high tolerability, reduced side effects, and enhanced efficacy [18, 47]. However, its application to obesity and metabolic disorders is still largely in the development phase [49]. For example, nanoparticles and nanopatches could induce the browning of adipocytes to increase fat loss [50]. Administration of nanoparticles would target WAT vasculature, stimulate angiogenesis that is required for the transformation of adipose tissue [51]. PLGA/PVA nanospheres were formulated for the systemic delivery of RSG specifically to macrophages to alleviate inflammation in white adipose tissue and liver [52]. Studies on the use of nanosystems to combat obesity gave evidence that the benefits of nanotechnology include enhancing the physiological stability of drugs from the body's defense mechanisms, and controlling both tissue and cell distribution profiles of drugs [49]. This study provides N-MWCNTs as a promising delivery material for anti-obesity agents, including conventional chemical drugs as well as genetic and microRNA materials. Of course, future investigations using obese animal models in the presence or absence of delivering some certain anti-obesity agents are needed to verify the obesity-fighting effect of the N-MWCNTs.

5. Conclusion

We confirmed that N-MWCNTs have good biocompatibility both *in vitro* and *in vivo*. The nanomaterial significantly inhibits fat deposition primarily via suppressing the adipogenic process. The altered expression of fat metabolism-associated genes is attributable to the immune response triggered by phagocytosis of the N-MWCNTs. These findings provide a new idea for N-MWCNTS as a candidate delivery vehicle for anti-obesity agents.

Declarations

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Y.T., H.L. and X.X. designed the research. X.X., G.H. and D.H. performed and analyzed most experiments. W.Z., G.Y., Y.L., Y.L., H.L., X.L. and Y.D. helped with animal experiments and biochemical experiments. H.L. and X.X. wrote the paper. All authors discussed the results and commented on the manuscript.

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Ethics approval and consent to participate

The experimental protocol was approved by the ethical committee for animals in research of Shandong Agricultural University, China.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

All authors gave their consent for publication.

Competing interest

The authors declare no conflict of interest.

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Figures

Figure 1

Physicochemical characterization of N-MWCNTs. A Representative SEM images of N-MWCNTs. Scale bar: left 10 μm , right 1 μm . B Representative TEM images of N-MWCNTs. Scale bar: left 200 nm, right 100 nm. C The diameter distribution of N-MWCNTs. D The width distribution of N-MWCNTs. E XPS spectrum

scanning of N-MWCNTs. F Relative atomic concentrations (%) of elements in N-MWCNTs. G UV-visible spectrum of N-MWCNTs.

Figure 2

N-MWCNTs treatment does not affect the biological activity of HEK293 cells. A Flow cytometry analysis of Annexin V/PI-stained HEK293 cells incubated with N-MWCNTs (0.05, 0.2 and 1.0 µg/mL) or Vehicle for 24 h. The Q1, Q2, Q3, and Q4 quadrants contain necrotic, late apoptotic, early apoptotic, and normal cells, respectively. B-D Statistical comparison of the proportions of (B) necrosis, (C) apoptotic, and (D) normal cells in different treatments upon flow cytometry analysis shown in A (n=6). E Representative cell cycle phase distribution done by PI/RNase staining followed by flow cytometric analysis (n=6). F Statistical analysis of cells in G0/G1, S and G2/M upon flow cytometric analysis shown in (E) (n=6). G Flow cytometric analysis of HEK293 cells exposed to N-MWCNTs or Vehicle via JC-1 MMP analysis (n=6). (Q2:

JC-1 aggregates, Q3: JC-1 monomers). H Ratio of JC-1 aggregates to JC-1 monomers (n=6). Data are expressed as means $\pm$ SEM. *p<0.05 vs. Vehicle.

Figure 3

N-MWCNTs treatment does not affect the growth and feed intake of mice. The mice body weight and feed/gain ratio at day 2 (A and D), day 7 (B and E), and day 14 (C and F) after intramuscular injection of N-MWCNTs. Data are expressed as means $\pm$ SD (n=10). *p< 0.05 vs. Vehicle.

Figure 4

Intramuscular injection of N-MWCNTs decreases fat deposition in mice. A Representative pictures of DXA imaging of the mice treated with Vehicle or N-MWCNTs (n=9). Red represents areas with more than 50% fat. B Fat mass (%) of mice from each treatment (n=9). C The iWAT index (%) of mice from each treatment (n=9). D Relative mRNA levels of fat deposition-associated genes (*Pparγ*, *Cebpa*, *Ap2*, *Fas*, *Lpl*, *Hsl*, and *Cpt1*) in iWAT of mice in different groups, determined by RT-qPCR (n=9). *Gapdh* was used as an internal control. Data are expressed as means ± SEM. *p<0.05 vs. Vehicle.

A

Figure 5

N-MWCNTs treatment inhibits fat accumulation of in vitro-cultured adipocytes. A ORO staining of the intracellular lipid droplets following N-MWCNTs or Vehicle treatments for 10 days. Scale bar: 100 µm. B The bound ORO was dissolved with isopropyl alcohol and absorbance at 510 nm was measured, which was quantified to mmol ORO/mg protein (n = 6). C-I The mRNA levels of *Pparγ*, *Cebpa*, *Ap2*, *Fas*, *Lpl*, *Hsl*, and *Cpt1* in ASCs-differentiated adipocytes treated with N-MWCNTs or Vehicle. *Gapdh* was used as an internal control (n = 6). Data are expressed as means ± SEM. *p<0.05, **p<0.01 vs. Vehicle.

Figure 6

The mRNA sequencing data of ASC-differentiated adipocytes treated with 0.2 $\mu\text{g/mL}$ N-MWCNTs or Vehicle. A Cluster analysis of gene expression in Vehicle and N-MWCNTs treatment groups ($n=3$) (red indicates elevated expression and green shows decreased expression). B The volcano plot showing differentially expressed genes (DEGs) in cells treated with N-MWCNTs. The x axis shows the log2 fold change (magnitude of change), while the y axis shows the -log10-adjusted p value (statistical significance). Red points represent up-regulated genes and blue points represent down-regulated genes. The vertical line in the figure is a two-fold difference threshold. C KEGG enrichment terms with the top 20 lowest p-value upon N-MWCNTs treatment. D Cluster analysis of gene expression involved in PPAR signaling pathway in Vehicle and N-MWCNTs treatment groups. E Cluster analysis of gene expression involved in lipolysis pathway in Vehicle and N-MWCNTs treatment groups. F Cluster analysis of gene expression involved in adipocytokine signaling in Vehicle and N-MWCNTs treatment groups.

Figure 7

Fat metabolism associated genes are significantly altered by 0.2 $\mu\text{g/mL}$ N-MWCNTs. A Heat map on expression of differentially expressed genes linked to lipogenesis following N-MWCNTs treatment. B RT-qPCR validation of gene *FATCD36*, *Acc2*, and *Fabp5* shown in A. *Gapdh* was used as an internal control. C Heat map on expression of differentially expressed genes linked to lipolysis following N-MWCNTs treatment. D RT-qPCR validation of gene *Ppara*, *Cpt1b*, and *PGC-1 α* shown in C. *Gapdh* was used as an internal control. Data are expressed as means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. Vehicle.

Figure 8

N-MWCNTs induce macrophage-like immune response in ASC-differentiated cells. A TEM images of macrophage-like cells in N-MWCNTs- treated cells, internalizing with N-MWCNTs and forming phagosomes. The red arrows mark the N-MWCNTs. Scale bar: left 5 μ m, middle 1 μ m, right 500 nm. B Heat map of changes in expression of inflammatory factors associated with lipid genesis and metabolism upon N-MWCNTs treatment. C Heat map of changes in expression of genes that associated

with TLR signaling pathway upon N-MWCNTs treatment. D RT-qPCR validation of gene TNF α , Il1a, Il6, P38, ERK, and AP1 shown in B and C. Gapdh was used as an internal control. Data are expressed as means $\pm$ SEM. *p<0.05, **p<0.01 vs. Vehicle.

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