Original Article

Graphene oxide aggravated dextran sulfate sodium-induced colitis through intestinal epithelial cells autophagy dysfunction

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ABSTRACT — Graphene oxide (GO) is one of the most promising nanomaterials used in biomedicine. However, studies about its adverse effects on the intestine in state of inflammation remain limited. This study aimed to explore the underlying effects of GO on intestinal epithelial cells (IECs) *in vitro* and colitis *in vivo*. We found that GO could exert toxic effects on NCM460 cells in a dose- and time-dependent manner and promote inflammation. Furthermore, GO caused lysosomal dysfunction and then blocked autophagy flux. Moreover, pharmacological autophagy inhibitor 3-Methyladenine could reverse GO-induced LC3B and p62 expression levels, reduce expression levels of IL-6, IL-8, TLR4, and CXCL2, and increase the level of IL-10. *In vivo*, C57BL/6 mice were treated with 2.5% dextran sulfate sodium (DSS) in drinking water for five consecutive days to induce colitis. Then, GO at 60 mg/kg dose was administered through the oral route every two days from day 2 to day 8. These results showed that GO aggravated DSS-induced colitis, characterized by shortening of the colon and severe pathological changes, and induced autophagy. In conclusion, GO caused the abnormal autophagy in IECs and exacerbated DSS-induced colitis in mice. Our research indicated that GO may contribute to the development of intestinal inflammation by inducing IECs autophagy dysfunction.

Key words: Colitis, Intestinal epithelial cells (IECs), Graphene oxide (GO), Lysosomal dysfunction, Autophagy flux, Cytotoxicity

INTRODUCTION

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a complex chronic idiopathic inflammatory disease of the digestive tract. The conventional view of pathogenesis of IBD is likely multifactorial and includes genetic susceptibility, immune dysfunction, alterations in the microbiota and environmental stress (de Souza *et al.*, 2017; Neurath, 2020; Ananthakrishnan *et al.*, 2018). Due to it being characterized by alternating periods of relapse and remission, it greatly affects all aspects of the patients' lives (Torres *et al.*, 2017; Ungaro *et al.*, 2017). Along with the rapid development of the economy, the incidence and prevalence of IBD have an increasing trend worldwide (Ng *et al.*, 2018). To date, there has been no effective cure for IBD, and the aim of therapy only is achieving and maintaining remis-

sion from inflammatory episodes. However, some factors, such as zinc deficiency, obesogenic diet and physiological stress, are proved to aggravate colitis (Matsunaga *et al.*, 2011; Lee *et al.*, 2017; Higashimura *et al.*, 2019). Therefore, we should also pay attention to avoiding potential risk factors for IBD patients when we search for new treatments.

The development of nanomaterials opens new insights for the development of medicines. Studies about therapeutic actions of nanomaterials combined with the use of medication or sometimes without the use of medication have been increasingly performed (Couvreur, 2013; Zhu *et al.*, 2019; Allen and Cullis, 2013). Previous studies have proved that some nanomaterials have therapeutic effects when used alone (Zhu *et al.*, 2019); some nanomaterials can be used to enhance drugs' effects after loading them with the drugs (Lin *et al.*, 2018). Meanwhile, there are some articles warning that the applications of

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nanomaterials may have negative impacts (Ruiz *et al.*, 2017; Mu *et al.*, 2019). Therefore, we must be cautious in choosing suitable and effective drugs or new nanomaterials for clinical application.

Graphene oxide (GO), as one of the most promising nanomaterials, is extensively used in many domains due to its beneficial properties, which include large surface area, high light transmittance, superlative mechanical strength, unparalleled thermal stability, excellent electrical conductivity, and antibacterial activity (Gholampour *et al.*, 2017; Liao *et al.*, 2018; Syama and Mohanan, 2016; Muthoosamy *et al.*, 2014). Nowadays, its applications are gradually extended from industrial production to the biomedicine fields, including drug delivery, magnetic resonance imaging (MRI), fluorescence imaging, antibacterial activity, biosensors and hyperthermia (Muazim and Hussain, 2017; Plachá and Jampilek, 2019; Muthoosamy *et al.*, 2014). For example, GO combined with the chemotherapy drug cisplatin can potentiate anti-tumor effects in mice which have the CT26 colon tumor (Lin *et al.*, 2018). However, it is worth noting that nanomaterials may produce specific physical or chemical interactions with their environment (Nel *et al.*, 2006; Medina *et al.*, 2007). Possible undesirable results are potential to generate toxicity. Indeed, some studies have demonstrated that GO produces cytotoxic effects during usage (Liao *et al.*, 2018; Yin *et al.*, 2020). Therefore, we should be aware of the cytotoxic effects of GO when we use it, while it is impossible to ignore the benefits of the use of GO. Moreover, the effects of GO on IBD have not been proved and the evaluation of biosafety of the nanomaterial is urgently required.

Autophagy, hereafter referred as macroautophagy, is a fundamental catabolic process to maintain cellular homeostasis and promotes cell survival by clearing damaged organelles, proteins and foreign substances (Galluzzi and Green, 2019). Autophagy is a dynamic, multi-step process and lysosome is the most central organelle in the final step of autophagy processes, hence autophagy is also regarded as the lysosomal degradation pathway (Savini *et al.*, 2019). Abundant evidence implicated that the autophagy-lysosome pathway is involved in regulation of inflammatory response and many inflammatory diseases are associated with autophagy-lysosome pathway dysfunction (Ballabio and Bonifacino, 2020). Autophagy initiation and fusion of lysosomes with autophagosomes are critically involved in the proliferation of inflammation. Especially, fusion of lysosomes with autophagosomes timely could result in efficient diminishment of inflammation (Lapaquette *et al.*, 2015). However, disruption of the process and blockade of autophagy flux would

cause elevated activation of inflammation. Furthermore, recently, emerging research has elucidated that most nanoparticles induce autophagy and autophagy is one of the most important mechanisms involved in nanoparticles-induced toxicity (Mao *et al.*, 2016).

In this study, we investigated the effects of GO on intestinal epithelial cells (IECs) *in vitro* and on colitis mouse *in vivo*. We observed that dysfunctional autophagy and impairment of lysosome were triggered after treatment with GO. Furthermore, the levels of inflammatory related cytokines were reversed when pharmacological inhibitors 3-methyladenine (3-MA) was used in advance. These results revealed that GO causes impairment of lysosome and further results in accumulation of autophagosomes, which is the main mechanism of how GO promotes inflammation of IECs and aggravates colitis.

MATERIALS AND METHODS

Preparation and characterization of GO nanoparticles

The GO sheet was purchased from Sigma-Aldrich. To prepare for suspension of GO platy, it was dispersed in pure water with a concentration of 1 g/L as a stock solution and sonicated for 1 hr by a high powered sonicator before use. The morphology of GO was examined by atomic force microscopy (AFM, Bruker, Santa Barbara, CA, USA) and transmission electron microscope (TEM, Hitachi, Tokyo, Japan). The microstructure was detected by Raman spectroscopy (Renishaw, Gloucestershire, UK). The chemical element composition and chemical bonds were evaluated by X-ray photoelectron spectroscopy (XPS, Axis Ultra DLD, Manchester, UK). The hydrodynamic sizes and zeta potential were detected in pure water and in complete culture medium by dynamic light scanning (DLS, Malvern Instruments, Malvern, UK).

Cell culture

NCM460 cells were obtained from AtaGenix (Wuhan, China) and were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco).

Cellular uptake of GO through TEM, confocal microscopy and flow cytometry

In order to investigate the effects of GO on IECs *in vitro*, the uptake of GO was observed firstly. At the end of the time, cells were collected and washed with PBS 3 times, then fixed in 3% glutaraldehyde at 4°C, followed by fixing in osmium tetroxide, dehydration with ethanol, then polymerization using epoxy resin. Ultrathin sections

of cells were observed with the TEM.

To examine the uptake of GO visually, GO was co-incubated with FITC-BSA (Bioss, Beijing, China) solution as a mass ratio of 1:1 stored overnight in a dark place. FITC-BSA-GO was centrifuged at 16,000 *g* for 30 min in 4°C, and then the supernatant was removed, the pellet was suspended by complete culture medium to treatment cells, FITC-BSA was used as a negative control. At the end time of treatment, the cells were fixed with 4% paraformaldehyde (PFA) for 30 min and then were washed three times using PBS, and next were permeabilized with 0.1% Triton for 15 min and incubated with 5% bovine serum albumin (BSA). Then, the cells were incubated with rhodamine-phalloidin (100 nM) for 30 min, thereafter cell nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 20 min and then were washed 3 times with PBS. The represented images were captured by confocal microscopy (Zeiss LSM780, Jena, Germany).

To further quantitatively analyze the cellular uptake of GO, cells were treated with FITC-BSA-GO or FITC-BSA for 24 hr, and then were collected and washed in PBS 2 times. The uptake of GO was examined using flow cytometry.

Cell viability assay

Cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assay was used for testing cell viability according to the manufacturer's instructions.

Autophagy flux analysis through fluorescence microscopy

mRFP-GFP-LC3 adenovirus (Hanbio, Shanghai, China) is a common tool to detect autophagy flux. mRFP was used to track the location and expression of LC3. GFP fluorescent protein is sensitive to acidity and would be quenched in an acidic environment, such as lysosomes, so the decrease of GFP indicated the fusion of autophagosomes and lysosomes to form autolysosomes. In the current study, mRFP-GFP-LC3 adenovirus were transfected into cells, and then GO was added to cells for 24 hr. At the ending time, LC3 puncta were examined with a fluorescence microscope. The yellow spots were autophagosomes, and the red spots indicated autolysosomes.

Lysosomal function evaluation

Lysosomal acidification assay through fluorescence microscopy and flow cytometry

LysoSensor Green DND-189 (Yeasen, Shanghai, China) is a kind of fluorescent probe that can accumulate in acidic organelles to study the function of acidic organelles, such

as lysosomes. The fluorescence intensity of the probe is PH- dependent with the degree of acidification of the organelles. In this study, after NCM460 cells were exposed to various concentrations of GO for 24 hr, LysoSensor Green DND-189 was added into the cells for 30 min at 37°C, cells were washed in PBS 2 times, and then visualized observed under a fluorescence microscopy or analyzed by flow cytometry.

Acid phosphatase (ACP) assay kit

Cells were exposed to GO at 25, 50 µg/mL for 24 hr and lysed using cell lysis buffer without inhibitors (Beyotime, China). Then, the supernatant fluid was collected for ACP activity assay according to the manufacturer's instructions (Beyotime, Nanjing, China).

Western blot analysis

Cells or tissues were homogenized in RIPA lysate buffer containing protease inhibitor and phosphatase inhibitor. Lysates were centrifuged and the protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal masses of protein were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) members. The members were blocked with 5% nonfat milk for 1 hr at room temperature. Then, the members were incubated with corresponding primary antibody of microtubule-associated protein 1 light chain 3B (MAP1LC3B/LC3B) and p62 (Proteintech, Wuhan, China) at 4°C overnight. Chemiluminescence was developed using the enhanced chemiluminescence detection system.

Quantative real-time PCR (qRT-PCR)

Total RNAs were isolated in Trizol reagent. 1 mg of RNAs was used as a template for cDNA synthesis by reverse transcription. qRT-PCR was performed with one primer pair amplifying the gene of interest and the other an internal reference (GAPDH). Expression levels of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method.

Mouse experiments

Colitis model

Female C57BL/6 mice between 6 and 8 weeks old were purchased from the Animal Research Center of Southern Medical University (Guangzhou, China) and were maintained in a pathogen-free laboratory environment. The mice were randomly divided into four groups (*n* = 5 per group): control group, GO group, DSS group and DSS-GO group, and allowed access to a rodent diet *ad libitum*. The acute colitis model was induced by oral administration of 2.5% DSS (MP,

Santa Ana, CA, USA) in drinking water for 5 consecutive days, then followed by regular drinking of fresh water for 3 days. A suspension of GO was administered daily by oral gavage at doses of 60 mg/kg every two days from day 2 to day 8 to investigate the effects of GO on colitis. On day 9, the mice were sacrificed and the length of colons was measured.

The mouse experiments were approved by the Southern Medical University Ethics and Experimentation of Committee (approval number: L2016189) prior to initiation of the research.

Hematoxylin and eosin (HE)

Freshly colon specimens were fixed in 4% PFA and then embedded in paraffin. Paraffin-embedded blocks were sectioned and stained with hematoxylin and eosin. Finally, the sections were observed by microscope.

Statistical analysis

All experiments were repeated at least three times. All data are represented as the means $\pm$ standard error of the mean (S.E.M.). The differences between groups were analyzed using Graphpad Prism 8.0 and one-way ANOVA.

RESULTS

Characterizations of GO

Physicochemical characterizations, including morphology, thickness, atomic contents, surface structure, charge and size, are shown in Fig. 1 and Table 1. Firstly, TEM and AFM were used to detect the morphology of GO, as shown in Fig. 1A-B, GO was a kind of flat structural nanoparticle with the thickness of approximate 1.5 nm. According to Fig. 1C, two typical characteristic peaks (G peak and D peak) were observed by Raman spectroscopy, the G peak was located in 1596 cm^{-1} and the D peak was located in 1342 cm^{-1} . Furthermore, we also observed a common 2D peak in GO, indicating that GO was single or few layers. Next, XPS was further used to determine the atomic content of GO. Carbon (C) and oxide (O) were the main elements of GO, making up about 95.4% of the total atomic content. In addition, the spectrum of GO showed that the presence of basic chemical bonds: C=C (284.3 eV), C-O (286.4 eV), C=O (288.0 eV) and O-C=O (288.7 eV) (Fig. 1D-E). Finally, the distribution and zeta potential of GO were tested using DLS, and we found that the diameter of GO in pure water was about 251 nm, larger than in complete culture media. The zeta potential of GO was about -25.2 mV and -9.53 mV in pure water and complete culture media, respectively (Table 1).

Table 1. Hydrodynamic sizes and zeta potential of GO in pure water and complete culture medium.

GO	In pure water	In complete culture medium
Zeta potential (mV)	-25.2 ± 0.66	-9.53 ± 0.16
Hydrodynamic sizes (nm)	251.13	201.23

GO is internalized and further exerts cytotoxicity and inflammation in human IECs

The interactions of GO with biological cells are crucial for biosafety evaluation to assure their safe use in biomedical applications. In this study, NCM460 cell line, as a typical human IEC, was used to determine the effects of GO *in vitro*. As shown in Fig. 2A-B, TEM and confocal microscope images vividly showed that GO was internalized by cells and accumulated in cytoplasm. These findings were further confirmed through flow cytometry (Fig. 2C). Subsequently, the GO-induced cytotoxicity was measured by CCK-8 assay. Our results showed that GO could exert cytotoxicity in a dose- and time-dependent manner (Fig. 2D). Concretely, the cell viability started to observably decrease when NCM460 cells were exposed to a low concentration of GO for 24 hr, and cell viability was even under 50% when the concentration was up to $50\text{ }\mu\text{g/mL}$. We chose the concentrations of 25 and $50\text{ }\mu\text{g/mL}$ for further research. To investigate whether GO could trigger inflammatory response in NCM460 cells, we detected the levels of pro-inflammatory cytokines and anti-inflammatory cytokines with qRT-PCR. We found GO could promote the expression of IL-6, IL-8, TLR4 and CXCL2, but reduced the level of IL-10 (Fig. 2E). To conclude, we affirmed that GO could exert cytotoxicity and inflammatory response in NCM460 cells.

GO induces autophagy response in NCM460 cells

Autophagy was regarded as an important method of cell death in various nanoparticles-triggered cytotoxicity. To investigate whether autophagy is the possible mechanism of GO-induced cytotoxicity and inflammatory response in NCM460 cells, we evaluated autophagy in NCM460 cells after exposure to GO. We first observed the ultrastructure of NCM460 cells after treatment with GO using TEM. As shown in Fig. 3A, characteristic morphologic structure of autophagosomes could be found in NCM460 cells after treatment with GO for 24 hr, suggesting the activation of autophagy response in NCM460 cells. Furthermore, double fluorescent adenovirus mRFP-GFP-LC3, as an autophagy indicator, was used to visu-

Fig. 1. Physicochemical characterization of GO. (A) Representative TEM image of GO in agglomeration state. (B) Representative AFM image (upper figure) and the thickness analysis (lower figure) of GO. (C) Raman spectroscopy of GO. (D and E) Basic chemical bonds and atomic contents were detected by XPS.

ally observe the formation of autophagosomes and autolysosomes. As shown in Fig. 3B, the fluorescent images showed autophagosomes accumulated in cells but autolysosomes could not be observed, which implied GO triggered an accumulation of autophagosomes in NCM460

cells. In addition, the protein LC3B played a crucial role in the formation of autophagosomes, and the conversion of LC3B-I to LC3B-II is a hallmark of mammalian autophagy. Given the fact that autophagy is a highly dynamic process, we detected autophagy response at

Fig. 2. GO induced NCM460 cells cytotoxicity and inflammation. (A) NCM460 cells were exposed to GO at the concentration of 50 μ g/mL for 24 hr or not. Ultrastructural images of cells were captured by TEM. Red arrow indicates the localization of GO. (B and C) Uptake of FITC-BSA-GO was detected with confocal microscope and flow cytometry after exposure to GO for 24 hr. (D) The cytotoxicity was examined with CCK-8 following exposure to GO at 0, 10, 25, 50, 100, 200 μ g/mL for 24 hr or at 25 and 50 μ g/mL for 0, 6, 12 and 24 hr. (E) The relative mRNA levels of cytokines were detected by qRT-PCR following treatment with 25 and 50 μ g/mL GO for 24 hr. (* $p < 0.05$, ** $p < 0.01$ vs. ctrl or 0).

Fig. 3. GO induced autophagy in NCM460 cells. (A) NCM460 cells were treated with 50 µg/mL GO for 24 hr, and then the ultra-structures were observed with TEM. (B) Fluorescence images of NCM460 cells after transfection with mRFP-GFP-LC3 adenovirus followed by treatment with GO. The yellow dots represent autophagosomes. (C) Western blot analysis and bar graph of LC3B-II/LC3B-I and p62 in NCM460 cells after treatment with 50 µg/mL of GO for 1 hr, 6 hr, 12 hr and 24 hr. (* $p < 0.05$, ** $p < 0.01$ vs. 0 hr).

several different time points. As shown in Fig. 3C, we found that LC3B-II/LC3B-I protein ratio was markedly elevated over time, which further confirmed the activation of autophagy response in NCM460 cells. Surprisingly, western blot analysis showed the level of p62 protein was decreased at 1 hr, and subsequently even enhanced to over the control group after exposure to GO for 24 hr. Given that p62 is the substrate for autophagy and would be degraded by autolysosome, the increased expression of p62 suggested the dysfunctional autophagy. Based on these results, we speculated that excessive autophagy occurred in cells with the continuous accumulation of GO or that autophagy flux was blocked due to dysfunctional lysosome.

GO exerts lysosomal dysfunction

In the complete autophagy process, autophagosomes fused with lysosomes would form autolysosomes to degrade cellular contents. Therefore, lysosomal functions are vital for the autophagy response. Lysosomal impairment is considered to be the primary reason of autophagy flux blockage, which results in autophagy dysfunction. The optimal lysosomal pH is a crucial factor allowing lysosomal proteases to regulate degradation. ACP is a kind of main component of acid hydrolase in lysosomes, which is considered to be the characteristic enzyme of lysosomes. It is responsible for lysosomal degradation and characterizes the lysosomal degradation capability. In order to clarify whether GO triggered NCM460 cells to have an abnormal autophagy response though inducing lysosomal dysfunctions, lysosomal acidity and activ-

ity of ACP were detected, respectively. As shown in Fig. 4A-B, green fluorescence of LysoSensor Green DND-189 was decreased after exposure to GO, indicating that acidity of lysosomes was weakened in a dose-dependent manner, which was consistent with the results of flow cytometry. Similarly, the activity of ACP was significantly decreased by treatment with GO (Fig. 4C). Taken together, these results indicated that GO affected lysosomal activity of proteases by changing lysosomal acidification and further resulted in impairment of capacity of lysosomal degradation. In this study, GO not only induced activation of autophagy but also affected the function of lysosomes. This in turn caused a large accumulation of autophagosomes.

Autophagy inhibitor 3-MA decreases the expression of pro-inflammatory cytokines and increases the expression of anti-inflammatory cytokine *in vitro*

Both the excessive activation of autophagy and the blockade of autophagy flux can bring about autophagosomes accumulation. In order to reduce the number of autophagosome, we should take steps to inhibit the production of autophagosomes or to restore the function of lysosomes. Obviously, inhibiting autophagy is easier than rescuing the function of lysosomes. 3-MA as a classical autophagy inhibitor can inhibit induction of autophagy at an early stage because it is a selective PI3K inhibitor and also blocks the formation of autophagosomes. We further assessed the levels of LC3B-II/LC3B-I and p62 in the presence of pharmaceutical inhibitor 3-MA. As shown in Fig. 5A, pre-treatment with 3-MA induced a dramatic decrease in the ratio of LC3B-II /LC3B-I and the level of p62 protein when compared with the cells treated with GO alone. To further explore the effects of accumulation of autophagosomes in GO-induced inflammation, we tested the expression of IL-6, IL-8, TLR4, CXCL2 and IL-10 in the absence or presence of 3-MA. These results showed that inhibition of autophagy by 3-MA almost abrogated the high levels of pro-inflammatory factors induced by GO, and rescued the level of anti-inflammatory cytokines (Fig. 5B). In conclusion, these data indicated that accumulation of autophagosomes might involve to inflammation of GO-induced in NCM460 cells and suppression of the number of autophagosomes partially attenuated the GO-induced inflammation response.

GO aggravates DSS-induced colitis and induces autophagy response *in vivo*

We have proved that GO caused negative effects in NCM460 cells, in order to explore the impacts of GO in

colitis *in vivo*, DSS-treated and control mice were administered with GO. DSS-GO group mice presented an even worse inflammatory condition in colon than mice receiving GO or DSS alone in drinking water, as evidenced by a significant shortening of the colon (Fig. 6A-B). Similarly, HE stained sections of colonic tissue presented a significantly higher inflammatory cell infiltration and increased histological scores (Fig. 6C). However, GO group did not show shortening of the colon, suggesting that GO might be deleterious only in pre-existing inflammation. Dysfunction of autophagy is involved in the onset of intestinal inflammation, including IBD. In order to clarify the role of autophagy in the development of GO aggravating DSS-induced colitis, we measured the expression of autophagy-related protein in intestinal tissue. Obviously, the level of LC3BII was higher in DSS-GO group compared with other groups, which indicated that autophagy may play a crucial role in GO exacerbating colitis (Fig. 6D).

DISCUSSION

To date, IBD is still an incurable disease, and more and more dangerous factors that may cause adverse effects on IBD patients have been found. Along with the development of new technology and increased presence of materials, a large number of scientific experiments have been conducted to improve the conditions of IBD patients (Corbo *et al.*, 2017; An *et al.*, 2020). Cure and prevention are two important aspects of the process of disease treatment. Therefore, it is important to take precautions to avoid potential risk factors for IBD patients. A previous article revealed that oral administration of titanium dioxide nanoparticles enhances intestinal inflammation in the DSS mouse model of colitis and suggests a cautionary use of titanium dioxide in pharmaceutical formulations (Ruiz *et al.*, 2017). This forces careful decisions of what medications to use.

Recently, with the applications of GO, its benefits as well as adverse effects have been of particular concern. In this study, we evaluated the effects of GO on intestinal inflammation, providing evidence for further pharmaceutical processing. We tested the characteristics of GO before study; as shown in Fig. 1, GO is a lamellar material with characteristic D peak and G peak, and the ratio of carbon to oxygen determines that it is hydrophilic. According to the result of Table 1, GO has a negative charge of -25 mV in pure water, while the GO showed a decreased negative charge of -9.53 mV in complete culture medium. The results were due to DMEM's containing of many positive charge cations (for example Na⁺,

Fig. 4. GO triggered lysosome dysfunction of NCM460 cells. NCM460 cells were incubated with LysoSensor Green DND-189 for 30 min after exposure to GO at the concentrations of 25 and 50 $\mu\text{g/mL}$ for 24 hr. Lysosomal acidification was surveyed through flow cytometry (A) and fluorescence microscopy (B). (C) Activities of acid phosphatase were measured. (** $p < 0.01$ vs. ctrl).

K^+ , Ca^{2+} , Mg^{2+} and so on), which neutralized the negative charge of GO to some degree. Generally, the higher the zeta potential is, the more stable the particle dispersion system will be. To be specific, nanoparticles with zeta potential higher than +25 mV or lower than -25 mV have higher degrees of stability. In contrast, dispersions system with a low zeta potential value will eventually agglomerate due to Van Der Waals interparticle attractions and thus destabilize the whole system (Krasteva *et al.*, 2019). Furthermore, the hydrodynamic sizes of GO in pure water were larger than in complete culture media, which indicated that the large-sized GO may agglomerate and even sink in the complete cultural medium. DLS tested the small-size GO samples staying in the culture medium. This result is consistent with a previous research (Feng *et al.*, 2018). Our results indicated that GO is more stable in pure water than in complete culture medium.

A growing number of studies have investigated the

adverse effects of GO nanoparticles. For example, Wang *et al.* revealed that the effects of GO on human fibroblast cells are dose related. It is definite that GO exhibits no toxicity to human fibroblast cells when the concentration is less than 20 $\mu\text{g/mL}$; nevertheless, GO exhibits obvious toxicity when the concentration is more than 50 $\mu\text{g/mL}$ (Chang *et al.*, 2011). In the current study, GO similarly induced cytotoxicity in a dose-dependent manner in NCM460 cells. However, GO triggered significant toxic levels at the dose of 10 $\mu\text{g/mL}$, further, the cell activity was even below 50% when the concentration was 50 $\mu\text{g/mL}$. It is believed this is a result of NCM460 cells that are more sensitive to GO. Also, it was found that GO promoted up-regulation of IL-6, IL-8, TLR4, and CXLC2 and inhibited the expression of anti-inflammatory cytokine IL-10.

Researchers elucidated a series of mechanisms in nanoparticles-induced toxicity, and abnormal autophagy

Fig. 5. Inhibition of autophagy partially reversed GO-induced inflammation in NCM460 cells. NCM460 cells were treated with 50 $\mu\text{g/mL}$ GO for 24 hr, and 3-MA (5 mM) was added to the cells for 1 hr prior to the GO treatment. CQ (10 μM) was added for the duration as the positive control. (A) Western blot analysis and bar graph of the expression of protein levels of LC3B-II/LC3B-I and p62 in cells. (B) The relative mRNA levels of IL-6, IL-8, TLR4, CXCL2, IL-10 were detected with qRT-PCR. (* $p < 0.05$, ** $p < 0.01$ vs. 0, ## $p < 0.01$ vs. GO50).

and lysosomal dysfunction are recognized as the potential toxic mechanisms of nanomaterials (Li and Ju, 2018). Mounting evidence indicates that the autophagy-lysosome pathway plays a key role in regulating the inflammatory response. Several research results identified the autophagy-lysosome pathway involvement in pro-inflammatory processes (Zhang *et al.*, 2016; Wang *et al.*, 2018; Agrawal *et al.*, 2015). For example, Lu Zhang *et al.*

proved that magnetic ferroferric oxide nanoparticles lead to endothelial dysfunction and inflammation by disturbing the process of autophagy in HUVECs (Zhang *et al.*, 2016). Furthermore, autophagy plays multiple roles in immune homeostasis, and dysfunction of autophagy is associated with the pathogenesis of a variety of diseases (Tan *et al.*, 2019; Haq *et al.*, 2019; Li *et al.*, 2020). Hence, autophagy may have an impact on the onset or

Fig. 6. Administration of GO exacerbates DSS-induced intestinal inflammation in mice through activation of autophagy response *in vivo*. Mice in DSS-induced model of acute colitis received GO by oral gavage as indicated and were sacrificed at day 9. (A and B) the length of colons were measured. (C) HE staining of colon sections displayed severe barrier breakdown with extensive inflammatory cells infiltration reaching the muscularis mucosae, and colon histological scores were estimated. (D) Western blot analysis and bar graph of autophagy-related proteins LC3B-II in colon of mouse. (* $p < 0.05$, ** $p < 0.01$ vs ctrl, ## $p < 0.01$ GO vs. DSS-GO, && $p < 0.01$ DSS vs. DSS-GO).

progression of various human diseases associated with a chronic inflammatory state, including IBD.

We further investigated the relationship between autophagy and inflammation in NCM460 cells. According to the guidelines for use and interpretation of assays for monitoring autophagy in higher eukaryotes and measuring autophagosome flux (du Toit *et al.*, 2018), we used multiple assays to monitor autophagy in NCM460 cells after exposure to GO. Electron microscope image analysis, fluorescence images of cells after transfected with mRFP-GFP-LC3 adenovirus, and the ratio of LC3-II to LC3-I can be used to monitor autophagosome formation. The present study showed increased conversion of LC3B-I to LC3B-II and decreased levels of p62 after exposure to GO for 1 hr, suggesting induction of autophagy by GO in NCM460 cells. However, the level of p62 increased with the prolongation of exposure time, and was even higher than the control cells. Moreover, we found that autophagosomes accumulated in cells after treatment with GO for an extended period of time. This reflected either increased autophagy activity, or reduced fusion of autophagosomes with lysosomes. The latter could occur when lysosomal functions were impaired, resulting in defect of fusion with autophagosome, or inefficient degradation of autolysosomes. Therefore, the functions of lysosome were evaluated. We found that degradation ability of lysosomes was weakened. These results suggested that the autophagy-lysosome pathway was impaired as time progressed due to dysfunction of lysosomes. This was also the reason of enhanced expression of p62 in cells. Furthermore, the autophagy inhibitor 3-MA effectively inhibited the levels of autophagy-related proteins in cells. Furthermore, 3-MA attenuated the production of IL-6, IL-8, TLR4, and CXCL2 and increased the expression of IL-10. These results suggested that GO triggered activation of autophagy and lysosomal dysfunction, which further led to blockage of the autophagy-lysosome pathway and accumulation of autophagosomes. This was the main mechanism of GO-induced toxicity and inflammation in IECs.

To explore the drug safety, the model of DSS-induced colitis is used to obtain more comprehensive toxicological information (Huang *et al.*, 2020). In the current study, our results showed that oral administration of GO exacerbated DSS-induced colitis *in vivo*, while it had no significant effect on healthy mice. It is believed that healthy mice with complete intestinal mucosa could excrete GO quickly and thus GO would not induce significant toxic levels, while the intestinal mucosa of mice in the state of inflammation was incomplete with increased permeability and led to more residue of GO in the system. In addition, inflammation of the colonic mucosa is also accompa-

nied by an *in situ* secretion and accumulation of positively charged proteins. This character of colitis would cause the attraction of negatively charged materials due to electrostatic interaction (Zhang *et al.*, 2020). Hence, GO with negative charge preferentially adheres to the inflamed intestine when passing through the colon after oral feeding, which is one of the main reasons that GO caused injury in colitis mice but no influence in healthy mice. Further, the levels of LC3B were higher in the DSS-GO group while there were no obvious differences between control mice and DSS mice, indicating residual GO in colon induced autophagy response in DSS-GO group mice. This would be the probable reason of oral administration of GO aggravating DSS-induced colitis.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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