Mitigative Effect of Graphene Oxide Nanoparticles in Maintaining Gut–Liver Homeostasis against Alcohol Injury

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Abstract: Alcoholic liver disease (ALD) develops when the immunotolerant environment of the liver is compromised due to excessive alcohol consumption. ALD progression involves variations in the expressions of multiple genes, resulting in liver inflammation and the development of a leaky gut. It is still unclear which molecular mechanism is involved in ALD progression, and due to that, there are currently no FDA-approved drugs available for its treatment. In this study, the protective effects of graphene oxide (GO) nanoparticles were investigated against ethanol-induced damage in the gut–liver axis in vitro. GO was synthesized using a modified Hummer's method, and characterization was performed. Given the general concerns regarding nanoparticle toxicity, assessments of cell viability, lipid accumulation, DNA damage, cell death, and the generation of reactive oxygen species (ROS) were conducted using various techniques. Furthermore, the gene expressions of pro- and anti-inflammatory cytokines were determined using RT-qPCR. The findings reveal that GO promoted cell viability even against ethanol treatment. Additionally, lipid accumulation significantly decreased when cells were treated with GO alongside ethanol compared to ethanol treatment alone, with similar trends observed for other assays. A gene expression analysis indicated that GO treatment reduced the expression of proinflammatory cytokines while enhancing the expression of antioxidant genes. Moreover, GO treatment led to improvements in gut integrity and a reduction in proinflammatory cytokines in colon cells damaged by ethanol. These findings suggest that GO holds promise as a drug carrier, exhibiting no observed toxic effects. By shedding light on the protective effects of GO against ethanol-induced damage, this study contributes to the burgeoning field of nanoparticle-mediated therapy for ALD.

Keywords: graphene oxide nanoparticles; gut–liver axis; alcoholic liver disease; inflammation; lipolysis

1. Introduction

ALD is a major lifestyle-related disorder and accounts for approximately 4% of liver-related deaths worldwide [1]. When the blood alcohol concentration is below 10 M/L, the alcohol dehydrogenase (ADH) pathway is activated to metabolize ethanol. Beyond this threshold, the Microsomal Ethanol Oxidizing System (MEOS) pathway is triggered, involving the activation of cytochrome P450E1 (CYP2E1) and catalase pathways. These pathways generate elevated levels of reactive oxygen/nitrogen species, leading to the activation of stress response mechanisms [2]. Furthermore, liver damage is not only exacerbated by ethanol but also by alterations in gut homeostasis. Increased ethanol consumption is associated with gut dysbiosis, which disrupts gut homeostasis and consequently impacts liver homeostasis by producing elevated levels of exotoxins [3]. Persistent and excessive alcohol consumption over an extended period may lead to the development of fatty liver disease. As severity progresses, inflammation and fibrosis ensue, eventually advancing to the early stage of liver disease known as liver cirrhosis. Hepatocellular carcinoma (HCC) is the advanced stage of ALD [4]. It is essential to recognize that prolonged and high-volume alcohol intake can have profound implications on liver health, progressing through various stages of pathology.
There are various treatments available, but none have received FDA approval. Most clinicians advise people to make lifestyle modifications by including balance and nutrition in their diet and participating in moderate exercise [5]. These would help with an early disease condition, but for later stages, steroids like corticosteroids and prednisolone are prescribed, though they are discontinued if their side effects are significant. Furthermore, doctors may recommend participating in rehabilitation programs or taking medication to aid with alcohol withdrawal. In the final stages of the disease, there is only one feasible alternative to survival: liver transplantation [6]. Post transplantation, maintaining abstinence for an extended period is crucial. ALD and its treatment has been a research focus for several years, yet the precise mechanism of ALD is inadequately understood, limiting the development of effective drugs. Some herbal compounds have shown hepatoprotective effects; however, their reduced water solubility leads to reduced efficacy. Several investigations are underway on the use of nanoparticles as protective agents, and among these, PEG nanoparticles have received FDA approval [7]. Few studies have even demonstrated the preventive role of nanoformulation, such as Rapamycin-mPEG-PLGA nanoformulation, in metabolism-associated liver disease (MALD). These studies have indicated that the incorporation of a nanoparticle-mediated therapy may result in a better rate of amelioration [8]. Recently, graphene-based materials, especially graphene oxide and reduced graphene oxide nanoparticles, have widely been studied for their higher bioavailability, and they are highly being tested for drug delivery, as anti-cancer agents, for biofunctionalization with proteins, and for bioimaging [9,10]. Among all graphene-based materials, graphene oxide (GO) nanoparticles have been extensively studied by many researchers. They are majorly studied for their antimicrobial activity and biomedical application. It is said that due to the high surface area of GO nanoparticles, they can be functionalized with another compound and can further be used as therapeutic agents [11]. Although GO has been researched for many years for various activities, its protective activity remains potentially unexplored.

In this study, GO nanoparticles were chosen due to their higher bioavailability. Furthermore, GO’s antioxidant and anti-inflammatory activities have also been reported recently, so in this study, we hypothesized that GO has protective activity against ALD in an ethanol-induced in vitro model. In certain instances, GO did not exhibit toxic effects and even contributed to cell proliferation, suggesting that it has potential benefits in this context. For further investigation, a series of experiments were conducted. Due to ethanol metabolism, oxidative stress and lipid accumulation occur, which are further targeted by GO as it shows its antioxidant and free radical scavenging activities, further targeting lipolysis [12,13]. To our knowledge, this is the first study that has investigated GO nanoparticle-induced protective activity against ALD in an in vitro model.

2. Materials and Methods

2.1. Materials

Graphite powder and H₂SO₄ were obtained from LOBA chemicals, H₃PO₄ was obtained from Himedia, and KMnO₄ was obtained from Sisco research laboratories (SRL, Mumbai, India). DAPI was purchased from Sigma Aldrich (St. Louis, MO, USA), H₂DCFDA was purchased from Merck (Darmstadt, Germany), acridine orange (AO) and ethidium bromide (EtBr) were purchased from SRL, and Oil Red O powder was purchased from Himedia. Materials used for gene expression were as follows: RNAisoplus (cat no. 9109) was from TAKARA (Kusatsu, Japan), cDNA synthesis kit was from Thermo Scientific-AB1453, and Powerup SYBR green master mix was from Thermo Scientific (Waltham, MA USA). Cell lines HepG2 and Caco2 were procured from NCCS (Pune, India); MEM media were purchased from Himedia (Mumbai, India) and Gibco (Billings, MT, USA); and Trypsin EDTA, antibiotic and antmycotic mixture, and fetal bovine serum were purchased from Gibco.
2.2. Methods

2.2.1. Synthesis and Characterization of Graphene Oxide (GO) Nanoparticle

The mixture of $H_2SO_4$ and $H_3PO_4$ was prepared in a 9:1 ratio (90 mL:10 mL). In cooling conditions, this mixture was added to 1 g of graphite powder and 9 g of KMnO$_4$. This reaction is exothermic. After the mixture was ready, it was kept under a stirring condition for 12 h, and a 60 °C temperature was maintained. After 12 h of stirring, the oxidation reaction was ceased using ice-cold distilled water. To see the oxidation, 10 mL of 30% $H_2O_2$ was added to the mixture. As $H_2O_2$ was added, it created bubbles in the solution. Then, the mixture was filtered through Whatman filter paper 1. The collected filtrate was washed with distilled water and 30% HCl solution until the sulphate and manganese impurities were removed. After washing with HCl again, a water wash was carried out. To obtain the raw material, the collected pellet was air-dried for 12 h at 50 °C, and then the material was exfoliated using the sonication method [14,15].

After exfoliation, the sample was again air-dried for 12 h at 50 °C, and then the characterization of the material was carried out using a UV–visible spectrophotometer, Scanning Electron Microscopy (SEM), and Fourier Transform Infrared Resonance (FTIR). For FTIR, KBr pellets were prepared, and the transmission was analyzed. An SEM analysis was conducted after platinum sputtering. UV visible spectroscopy was carried out using a sample diluted with water at a 1:9 ratio.

2.2.2. Cell Culture Studies

HepG2 and Caco2 cell lines were procured from NCCS, Pune. HepG2 cell line was maintained using 10% FBS and MEM media with $1 \times$ non-essential amino acid; for Caco2, 20% FBS and DMEM low-glucose media were used along with $1 \times$ antibiotic and antifungal mixture containing penicillin, streptomycin, and amphotericin. For the purpose of experimentation, cells were treated with low-serum media.

For GO nanoparticle treatment, 1 mg/mL of GO stock solution was prepared in water and sonicated for 15–20 min. After that, the solution was sterilized by autoclaving at 121 °C at 15 psi for 15 min. For ethanol treatment, 1 M of ethanol stock solution was prepared and sterilized using a syringe filter. At the time of treatment, the GO solution was sonicated using a bath sonicator for 5 min, and after that, it was used for the treatment.

(A) Toxicity study:

In brief, an adequate amount of cells were seeded in a 96-well plate. As the confluency reached 60–70%, they were treated with different amounts of GO (10 to 100 µg/mL) in increasing concentration. The next day, GO-containing media were removed, and cells were thoroughly washed with $1 \times$ PBS as any remaining GO can interact with MTT and might give a false positive result [16]. As the cells were washed, they were treated with a final concentration of 0.5 mg/mL of MTT. After 60–90 min MTT-containing media were removed, and formazan crystals were dissolved in 100% DMSO. Then, the results were observed in a multimode reader at 570 nm.

(B) Cell damage analysis:

DAPI Staining

For DAPI, cells were seeded in a 96-well plate (HepG2 25,000 cell/well and Caco2 10,000 cells/well), and the next day, cells were treated with 10 and 50 µg/mL of GO and 0.1 M of ethanol. After treatment, cells were incubated, and their growth characteristics were observed after 24 h. The next day, cells were washed with $1 \times$ PBS and fixed with 4% neutral buffered saline (NBS). After fixation, cells were washed with distilled water until traces of the fixative agent were removed. Furthermore, they were incubated for 15 min with 100 µM of DAPI stain in a dark condition. After incubation, excess DAPI solution was removed, and cells were further washed with PBS. After being washed, cells were observed under a fluorescent microscope in a DAPI filter. Their excitation and emission were also noted via a multimode reader [17].
AO-EtBr Staining

Acridine orange and ethidium bromide were used to check cell death. Acridine orange and ethidium bromide staining was used to detect live and dead cells. Dead cells were stained due to EtBr because of ruptured membranes [18]. The cells were counted in the merged channel, where the green fluorescence was first measured, and then the red fluorescence was measured. Live cells were observed under a fluorescent microscope. Initially, in a 96-well plate, cells were seeded (HepG2 25,000 cell/well), and the following day, they were treated with 10 and 50 µg/mL of GO and 0.1 M of ethanol. On the next day, cells were washed with PBS, and treated with 100 µg/mL of AO/EtBr solution, and immediately observed under FITC (485 excitation/595 nm emission) and TRITC (540 excitation/590 nm emission) filter. In the merged channel, images of cells were observed, and up to 300 cells were counted to screen live and dead cells [19,20].

Lipid Accumulation Study

Cells were seeded in a 96-well plate and treated with 0.1 M of ethanol and 10 and 50 µg/mL of GO the next day. After 24 h of treatment, they were washed with PBS and incubated for fixation in NBS for 15–30 min. Then, cells were washed with distilled water and incubated for 15 min with working Oil Red O (ORO) solution. After incubation, extra ORO was removed by washing cells with Phosphate Buffered Saline (PBS) and observed under a light microscope. Furthermore, to measure absorbance, PBS was removed, and 100% IPA was added in each well and shaken for a few minutes to extract the stain from cells, and the solution was placed in an empty well. Absorbance was measured at 570 nm, and relative fold change over basal was measured [21,22].

ROS Study

H2-DCFDA was used to check the ROS level. Cells were seeded in a 96-well plate, as mentioned previously, and after 24 h, they were treated with ethanol and 10 and 50 µg/mL of GO. After 24 h of treatment, cells were washed with PBS and fixed. After that, cells were incubated with 20 µM of H2-DCFDA for 1 h. Furthermore, cells were washed to remove excessive dye. Fluorescence was observed using microplate reader, (Perkin Almer, Waltham, MA, USA), with fluorescent excitation at 484/30 nm and emission at 535/30 nm. Fold change over basal graph was prepared for both cell lines [23].

Gene Expression

For gene expression analysis, cells were first seeded in 6-well plates, and then in their exponential phase, they were treated with 0.1 M of ethanol and 10 and 50 µg/mL of GO. After 24 h of treatment, RNA isolation was manually carried out by using RNAisoplus solution (TAKARA). In brief, cells were washed with RNAisoplus solution and collected in a 2 mL microcentrifuge tube. The collected solution was incubated with chloroform following centrifugation at 12,000× g for 15 min. Further, the pellet was washed with 100% IPA and incubated at room temperature for 15 min. After that, the pellet was centrifuged at 12,000× g for 10 min, and then the collected pellet was again washed with 70% ethanol. The clear pellet was then resuspended in nuclease-free water and stored at −80 °C for future use. Purity of RNA was checked using Nanodrop. The 260/280 and 260/230 ratios should be 2, which indicates pure RNA. Furthermore, purified RNA was used for cDNA synthesis. cDNA was synthesized using a kit, and after that, RT-qPCR was performed for gene expression study.

For gene expression, 2× SYBR green enzyme mix, 500 nM of primer, and 5 ng of cDNA were used. To activate SYBR green enzyme, an initial 95 °C temp was used. Further PCR was carried out at 95 °C denaturation for 30 s, at 60 °C for 45 s of annealing, and at 72 °C for 1 min of extension.

Fold change over basal was calculated by the 2−ΔΔCt method, and mRNA expression was evaluated for HepG2 and Caco2 cells; 18S rRNA was used as a housekeeping gene. Table 1. Represents the list of primers used for gene expression analysis.
Table 1. List of human primers for RT-qPCR.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
</tr>
</thead>
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<tr>
<td>18S</td>
<td>GATGTAGTGGCGCCGCTG</td>
<td>GCCGTGCTCTCCCTTGG</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>AAATCTCCCGGGAACCTC</td>
<td>GGGCTCTGACATGTGCTTT</td>
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<td>EGR1</td>
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<td>GGCCAGTGCTGTTTGTG</td>
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<td>SREBP2</td>
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<td>GAAATCGTGAGCAGTTCACTCAT</td>
</tr>
<tr>
<td>Il6</td>
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<td>GAAGGTACGACCTGACTCT</td>
</tr>
<tr>
<td>TNF</td>
<td>CTGCTGAGGCTGCTGACTTG</td>
<td>ATGGGCTACAGTTTGCATC</td>
</tr>
<tr>
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<td>TGGGCAAAGACACAGAGC</td>
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<tr>
<td>PPAR γ</td>
<td>GCGCTATGACAAGGGAGTTTC</td>
<td>AACTCAACTTGAGGCTCCATAAAG</td>
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2.3. Statistical Analysis

All experiments were performed at least thrice. Data were analyzed by using GraphPad prism software version 9.0. Data were analyzed using two-way ANOVA method with multiple comparison method followed by different test method. Values represent the mean ± SD of six or three individual experiments.

3. Results

3.1. Characterization of Nanoparticles

Graphene oxide is a material which is mainly made up of carbon and oxygen. It has a hexagonal carbon structure with stacks created through π–π interaction, which is attributed to Van der Waals interaction. C=C interactions were observed near 224 nm. There is a bond between carbon and oxygen as well, where oxygen tends to donate electrons to form a stable structure, which is possible through an n–π* interaction. This interaction can be observed near 300 nm in the UV–visible analysis (Figure 1A). It shows a weak interaction that is attributed to C=O [24].

FTIR is used to identify the chemical bond that is present in the molecule. GO is mainly made up of carbon, oxygen, and hydrogen, and together, all three form various bonds, which can be detected using FTIR. In the FTIR analysis, peaks at 3334, 1734, 1627, 1230, and 1040 cm⁻¹ were observed. All are attributed to the stretching of different chemical groups. Stretching near 3334 cm⁻¹ is attributed to carbonyl/O-H stretching. The peaks near 1734 and 1627 cm⁻¹ are attributed to the carboxyl and carbonyl group, respectively. The band near to 1230 cm⁻¹ indicates the presence of a C-O-C functional group. Vibration at 1040 cm⁻¹ indicates the activity of the C-O group (Figure 1B) [25–28].

Furthermore, SEM and FESEM analyses of GO were conducted to observe its structural orientation. In the SEM images, a sheet-like structure of GO was observed, which was further analyzed in FESEM. In FESEM, a layered structure was properly visualized. The thickness was analyzed using Imagej software version 1.54f, which showed that the thickness of the sheet was near 0.8 to 1 nm. It also showed a flaky morphology (Figure 1C,D) [29].

3.2. Activity in In Vitro Model

3.2.1. Cell Viability Study

The cells were treated with different concentrations of GO, and 100 mM of ethanol was used as a negative control. In the ethanol-treated cells, 30 to 40% cell death was observed for both cell lines, while 100% viability was observed even at the highest concentration of GO (Figure 2). To obtain proper results, the cells were washed with 1× PBS before being treated with MTT.
Figure 1. Characterization of GO. (A) UV–visible spectroscopy visualization of GO showing maximum absorbance at 224 nm; (B) FTIR spectrum of GO; (C) SEM and (D) FE-SEM images of GO showing formation of multiple layers.

Figure 2. (A) Percentage of cell viability for HepG2 cells. (B) Percentage of cell viability for Caco2 cells. Values represent mean ± SD of six individual experiments.

3.2.2. Cell Damage Study
(A) AO-EtBr and DAPI staining:
Cell damage was analyzed using AO-EtBr staining and DAPI staining. An AO EtBr analysis was carried out for HepG2 cells. In the merged image, approximately 300 cells were counted. From those 300 cells, the normal control group showed 10 ± 5 dead cells, but in the ethanol control group, 80 ± 5 cells were observed and were red or orange in color. It was seen that in the treatment, the cells which were treated with both ethanol and nanoparticles showed decreased cell death (Figure 3A).

**Figure 3.** (A) AO EtBr staining in HepG2 cell line. (B) DAPI staining in HepG2 and Caco2 cell lines. (C, D) Relative fold change of both the cell line were analyzed for DAPI via measuring fluorescence for both cell lines. Statistical significance was carried out by two-way ANOVA followed by multiple group comparison with Sidak’s method, where, **p < 0.01, and ***p < 0.001. Values represent mean ± SD of three individual experiments. Above figures are illustrative 10 × objective images of three individual experiments. (Here, E represents the Ethanol treatment).

DAPI staining is used to assess cell activity. Cells were treated for 24 h and further stained with DAPI. Fluorescence images and fluorescence spectra were obtained. For both cell lines, the ethanol-treated cells were more damaged than the control cells. In the ethanol-treated cells, an increase in chromatin condensation was observed. Of 100 cells, 15 ± 2 cells
were damaged and showed higher fluorescence. The fluorescence of the ethanol- and GO-treated cells was decreased and was almost near that of the untreated cells. Furthermore, of approximately 100 cells, very few cells were found damaged (Figure 3B).

(B) Lipid accumulation study:

Due to ethanol, cells produce a higher amount of lipids, which are transferred near the cell membrane [30] and start accumulating. The cells were analyzed microscopically using a Nikon Eclipse Ti2 microscope and were further quantified using a spectrophotometer. Due to ethanol treatment in both cell types, lipid accumulation was increased. Increased lipogenesis was further confirmed by measuring absorbance at 570 nm. In the GO-treated cells, lipid accumulation was decreased, and it showed similar absorbance to the untreated cells. The relative fold change was determined for absorbance (Figure 4A,B), and a comparison was conducted with the ethanol-treated cells.

![Figure 4](image-url)

**Figure 4.** Lipid accumulation. (A) Relative fold change in absorbance was calculated for HepG2 cells, and images were captured using phase contrast microscope. (B) Relative fold change in absorbance was calculated for Caco2 cells, and images were captured in phase contrast microscope. (C) Images of HepG2 cell line at 10×. (D) Images of Caco2 cell line at 40×. For both cell lines, ethanol-treated cells showed higher lipid accumulation. Statistical significance was carried out by two-way ANOVA followed by multiple group comparison with Sidak’s method, where *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Above figures are illustrative 10× objective images of three individual experiments. (Here, E represents the Ethanol treatment).

(C) Reactive oxygen species analysis:

Cells exposed to ethanol produce an increased amount of reactive oxygen species (ROS) through the activation of cytochrome p450E1. H2DCFDA is a non-fluorescent compound that easily enters cells. In the presence of esterase, it is converted into a non-fluorescent compound called 2′,7′-dichlorofluorescein. Due to the presence of ROS, these non-fluorescent compounds quickly transform into a highly fluorescent compound known as 2′,7′-dichlorodihydrofluorescein. The ethanol control group exhibited higher fluorescence compared to the control/untreated group for both cell lines, but this trend was reversed in the GO treatment group. The relative fold change was measured for both cell lines, demonstrating that the ethanol-treated cells exhibited greater fluorescence compared to the treatment group (Figure 5A,B).
Additionally, ethanol downregulates the lipolytic genes of the PPAR family, which were upregulated in the treatment group. Furthermore, GO increases the antioxidant activity of cells by upregulating the expressions of genes responsible for ROS production, fat accumulation, and inflammation, where, *** $p < 0.001$, and **** $p < 0.0001$. Values represent mean ± SD of three individual experiments. (Here, E represents the Ethanol treatment).

(D) Gene expression analysis:

Excess ethanol in the body causes various changes in gene expression, leading to increased inflammation. According to Figure 6, treatment with ethanol in cells increases the expressions of genes responsible for ROS production, fat accumulation, and inflammation, while treatment with GO decreases their expressions. Additionally, ethanol downregulates the lipolytic genes of the PPAR family, which were upregulated in the treatment group. Furthermore, GO increases the antioxidant activity of cells by upregulating the expressions of AMPK, NrF2, and HO-1, which were downregulated due to ethanol exposure. It also increases IL10 anti-inflammatory regulation to reduce inflammation. Ethanol-induced damage to gut permeability results in secondary damage to the liver [28]. GO helps to restore gut permeability by upregulating the expression of tight junction proteins, which are downregulated due to ethanol exposure. Moreover, it decreases inflammation by reducing the activity of TNFα.

![Figure 5](image-url)  
**Figure 5.** ROS estimation. (A) Relative fold change in fluorescence of H2DCFDA stain for HepG2. (B) Relative fold change in fluorescence of H2DCFDA stain for Caco2. Statistical significance was determined by two way ANOVA followed by multiple group comparison with Sidak’s method, where, *** $p < 0.001$, and **** $p < 0.0001$. Values represent mean ± SD of three individual experiments. (Here, E represents the Ethanol treatment).

![Figure 6](image-url)  
**Figure 6.** Gene expression study was carried out for both cell lines. (A) Proinflammatory and lipolytic gene expression fold change for HepG2 cells. (B) Antioxidant and anti-inflammatory gene expression...
fold change for HepG2 cells. (C) Tight junction activity of Caco2 cells. Statistical significance was measured by two-way ANOVA, followed by multiple group comparison and Tukey’s method. (D) Proinflammatory TNFα activity in Caco2 cells. (EGO1-ethanol + 10 µg/mL GO, EGO2-ethanol + 50 µg/mL GO.) Statistical significance was measured by one-way ANOVA followed by multiple group comparison followed by Dunnett’s method, where *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Values represent mean ± SD of three individual experiments.

4. Discussion

The liver is an immunotolerant organ [31], and due to an unhealthy lifestyle, this immunotolerant environment becomes disrupted, further causing metabolism-related disorders like ALD or MALD. Prolonged and excessive alcohol consumption can have detrimental effects on the body’s metabolism and immune system [32,33]. The liver is particularly susceptible to the negative impacts of alcohol, as it is responsible for metabolizing approximately 90% of ethanol [32]. In the presence of an excessive amount of ethanol, metabolism occurs via the MEOS pathway, which includes the activation of CYP2E1 and catalase [34]. Due to the upregulation of CYP2E1, the elevation of ROS also takes place, resulting in increased inflammation and oxidative stress [35]. Furthermore, increased CYP2E1 activity is responsible for the expression of the sterol regulatory binding protein gene (SREBP1C), which, in turn, activates the fatty acid synthase (FAS) and acetyl co-A carboxylase (ACC) [36]. The sterol regulatory gene is also accountable for inflammation as it enhances the activity of tumor necrosis factor (TNFα) [6].

The liver is not only damaged due to ethanol exposure, but also due to gut dysbiosis. Heavy alcohol consumption alters the gut microbiota, leading to gut dysbiosis [37]. This serves as a secondary hit model. As a result of gut dysbiosis, toxins produced by the altered microbiota enter the portal vein and reach the liver, where they activate TLR4-mediated inflammation by activating liver resident Kupffer cells [38]. Heavy alcohol exposure disrupts the gut permeability by affecting the tight junction activity of gut epithelial cells. Because of the decreased gut permeability, toxic material or microbes can travel to other organs and induce inflammation [39]. There are various mechanisms which are known for alcohol-related liver disease but are less explored for their therapeutic activity. A few medications are available for liver disease, but none are FDA-approved.

Here, we investigated the activity of graphene oxide (GO) nanosheets, which were synthesized using a modified Hummer’s method involving various chemical reactions, which do not produce any toxic gases that could be harmful to individuals [14]. GO is well known for its biocompatibility and can be applied in drug delivery, biosensors, and cancer treatment [9]. Additionally, it is recognized for its antioxidant and anti-inflammatory properties [40].

Since GO primarily consists of carbon, oxygen, and hydrogen, various bonds are formed between these elements, such as carboxyl, carbonyl, and hydroxyl groups, which can be detected through FTIR peaks. Additionally, electron microscope images (Figure 1C,D) reveal that GO nanosheets have a flaky nature and exhibit a sheet-like structure. The presence of acids during the oxidation process leads to the formation of these sheet-like structures [41]. These sheets possess a higher surface-to-volume ratio, making them particularly interesting for studying their biological activity [42].

Further in vitro studies were conducted to analyze the effects of GO on cell viability. The results showed that at a concentration of 100 µg/mL, GO exhibited 100% cell viability for both cell types. Previous research [43] has also reported that concentrations up to 40 µg/mL do not induce any toxic activity in cells. Furthermore, GO has been studied for Caco2, HT29, and 3T3 cell lines, and they showed cell proliferative activity [43–46]. Interestingly, in our study, it was observed that, though excess GO was present in the media, the cells remained unharmed even at higher concentrations. Additionally, the interaction of GO with cell lines was evaluated, and its anti-inflammatory and antioxidant activities were assessed through a gene expression analysis. GO is known for its diverse range of activities,
one of which is its radical scavenging ability. Studies [47] have demonstrated that GO, along with other graphene-based materials, exhibit significant radical scavenging activity. In our study, we observed that due to exposure to ethanol, cells produced higher amounts of ROS, as shown in Figure 5. However, GO’s structure and scavenging activity was effectively reduced ROS production. Furthermore, GO was found to increase superoxide dismutase activity [48], resulting in decreased ROS production. This led to an increase in the antioxidant mechanisms involving AMPK, Nrf2, and HO-1 in the treated cells. The further activation of AMPK would also help to activate autophagy in cells. ROS-induced damage contributes to cellular dysfunction through various mechanisms, including apoptosis. Ethanol exposure is known to induce both apoptosis and necrosis, as depicted in Figure 3. Due to apoptosis and necrosis, cell inflammation takes place, which is attenuated with the help of GO. It is shown that GO also timely regulates and helps in macrophage polarization. It helps to activate the M2 type of macrophage polarization and reduces inflammation by scavenging ROS [49]. As GO demonstrated its scavenging activity against ROS, it also mitigated cell damage. These findings suggest that GO may possess anti-apoptotic activity [50], which contributes to its ability to reduce oxidative stress.

As GO has shown to decrease ROS activity, it also has a positive effect on lipolysis. Several carbon-based materials with graphene as their core structure have been found to exhibit lipolytic activity. In a recent study [51], researchers observed lipolysis activity in a graphene-based material and concluded that the presence of graphene as the core structure contributes to significant lipolytic activity by binding with adipocyte integrin β1. Similarly, in the current study, GO demonstrated the downregulation of SREBP1C, a key regulator of lipid accumulation. Through a gene expression analysis and Oil Red O staining, it can be inferred that the ethanol-treated cells experienced increased lipid accumulation due to heightened SREBP1C activity. However, when the cells were treated with a graphene-based material, the lipid accumulation was reduced (Figure 4), which was also seen by Liu J.’s research group [52]. Moreover, the activity of SREBP1C was found to be influenced by CYP2E1, and it displayed a negative correlation with genes from the PPAR family [53]. In ethanol-treated HepG2 cells, CYP2E1 expression was found to be increased, which subsequently led to an increase in SREBP1C activity. Nevertheless, in the treated cells, the expression of CYP2E1 was decreased, contributing to enhanced ROS scavenging activity.

SREBP1C is also responsible for inflammation through the activation of TNFα [54]. During lipolysis, potential anti-inflammatory mechanisms are also activated. In terms of gene expression, the activities of IL6 and TNFα decreased when the cells were treated with GO along with ethanol. This decrease in TNFα activity also contributes to barrier function. It has been reported that graphene-based materials are safe and aid in maintaining blood barrier integrity [55]. Similarly, in the Caco2 cell line, GO demonstrated an increased expression of tight junction (TJ) proteins, such as ZO-1, and adherent junction proteins like claudin and occludin. Together, these proteins contribute to gut permeability and protect against ethanol-induced damage.

In summary, it can be stated that the structural composition of GO grants the potential to serve as a scavenging material, aiding in the reduction in ROS accumulation. This, in turn, contributes to the downregulation of proinflammatory cytokines and the upregulation of anti-inflammatory cytokines such as IL10. Additionally, GO has displayed lipolytic activity, which facilitates the decrease in SREBP1C expression. Moreover, it plays a role in the maintenance of TJ and provides protection against secondary damage. The planar structure of GO helps it to scavenge free radicals and also helps in lipolysis. Additionally, its increased anti-inflammatory activity helps in maintaining the integrity of the tight junction barrier.

5. Conclusions

Alcohol-related liver disease is a major contributor to chronic liver diseases globally and is associated with a higher mortality rate. In this study, we aimed to investigate the potential of GO in ameliorating the gut–liver axis. GO is widely recognized for its biocompatibility and has been extensively studied in various fields. In this research, we
assessed its effectiveness against an in vitro model of alcohol-related liver disease. The results demonstrate that GO exhibited a protective effect by effectively scavenging radicals and promoting lipolysis, both of which are crucial for mitigating the disease (Figure 7). Additionally, a gene expression analysis validated the protective properties of GO. Further, in vivo studies need to be carried out to gain a proper understanding of the mechanism targeted by GO for ALD. These findings indicate that GO holds promise as a drug delivery agent or for nanoformulation by binding with other therapeutic agents, which may enhance the activity of GO and the therapeutic agent as well.

Figure 7. Possible protective mechanism of GO for ALD. Due to excessive alcohol administration, mechanisms like autophagy and antioxidant mechanisms are highly impacted and cause oxidative stress, lipogenesis, and cell death. Furthermore, gut dysbiosis also occurs and produces lipopolysaccharide, which travels to liver, causing secondary damage by activating inflammation. Due to GO treatment, lipolysis takes place and also decreases inflammation and oxidative stress by upregulating antioxidant mechanisms. Additionally, GO helps in maintaining tight junction barrier integrity and protects liver against secondary hit.

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