ANNALS OF APPLIED SCIENCES & TECHNOLOGY

Type of the Paper (Research Article)

Graphite Oxide Nanosheets Induce Dose-Dependent Mortality in Rats: LC_{50} Determination and Toxicity Analysis

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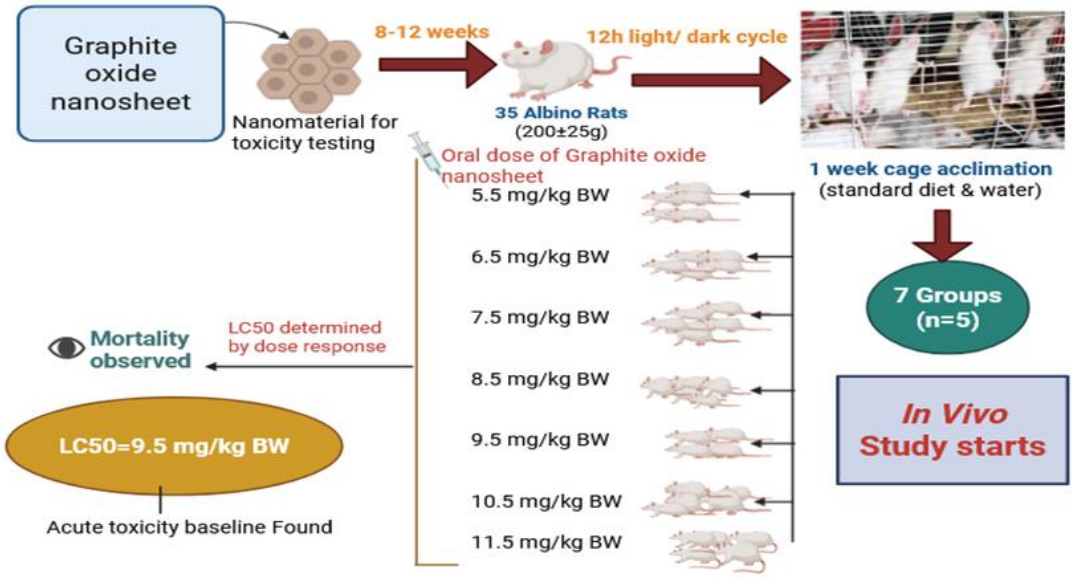
Academic Editor:
Dr. Naseeb Ahmad

Citation: Zahid, A., Khalid, A., Khan, R., Asif, S., Ijaz, M. Z., Ba-tool, F., & Ijaz, A. (2025). Graphite oxide nanosheets induce dose-dependent mortality in rats: LC_{50} determination and toxicity analysis. *Annals of Applied Sciences & Technology, 1*(1), 1–7

Abstract: Graphite oxide nanosheets have emerged as promising candidates for a range of biomedical applications. In this preliminary toxicological study, forty healthy albino rats (8 to 12 weeks old, 200 ± 25 g) were used to determine the median lethal dose (LD_{50}) of graphite oxide nanosheets (GON). The animals were obtained from Government College University, Faisalabad, and acclimated for one week under controlled laboratory conditions housed in stainless steel cages with a 12-hour light/dark cycle, and given unrestricted access to filtered tap water and a standard rodent diet. To estimate the LD_{50}, the rats were randomly divided into seven groups (n = 5 per group). Following a six-hour fasting period, each group received a single intraperitoneal dose of GON at concentrations of 5.5, 6.5, 7.5, 8.5, 9.5, 10.5, and 11.5 mg/kg body weight. This stepwise dosing approach enabled the identification of the dose causing 50% mortality, which was determined to be 9.5 mg/kg body weight. These findings provide important baseline data on the acute toxicity of graphite oxide nanosheets, offering valuable insights for future safety evaluations and risk assessments in biomedical nanotechnology.

Graphical Abstract



Keywords: Nanosheets, LC50, Graphite Oxide, Graphite oxide nanosheets, Acute toxicity, Albino rats, Nanotechnology

1. Introduction

Graphite oxide (GO) retains a layered structure similar to graphite, but its carbon planes are richly decorated with oxygen-

containing functional groups (e.g., hydroxyl, carboxyl, epoxy). These modifications increase the interlayer spacing and render the atomic-thin layers highly hydrophilic. Upon ultrasonication, these oxidized layers can be exfoliated in aqueous media, forming graphene oxide nanosheets (GON), which may consist of one or more layers of carbon atoms. Due to its surface chemistry, GO demonstrates excellent dispersion in water and other organic solvents, making it an attractive material for integration into polymer and ceramic composites to enhance mechanical strength, thermal stability, and functional versatility (Krishnan et al., 2023).

However, the oxidation process disrupts the π-conjugated sp^2 network of graphene, which significantly reduces its electrical conductivity. As a result, pristine GO is typically classified as an electrical insulator. Nevertheless, upon chemical or thermal reduction, GO can be partially restored to reduced graphene oxide (rGO), which shows improved conductivity and has been used in flexible electronics, supercapacitors, and sensor technologies (Kavitha, 2022; Kanti et al., 2023). In biomedical applications, graphene-based materials have enabled the fabrication of field-effect transistors (FETs), with functionalized rGO acting as a semiconducting layer in biosensors. These devices have shown sensitivity in detecting biomolecules such as catecholamines, avidin, DNA, and glucose (Cao et al., 2022; Iordache et al., 2023). GO functionalized with glucose oxidase has also been utilized in electrochemical glucose sensors, highlighting its potential in non-invasive diagnostics (Razaq et al., 2022). GO's unique optical and chemical properties allow it to form transparent conductive films, making it suitable for use in solar cells (LEDs), flexible electronics, and touchscreen displays (Zhang et al., 2022; Khine et al., 2024). In particular, GO and rGO have been applied as transparent electrodes and hole transport layers in optoelectronic devices, improving charge mobility and device stability.

Recent advances also include the use of PEG- and hyaluronic acid-functionalized GO in photothermal therapy, where near-infrared (NIR) irradiation of GO-loaded platforms effectively ablates melanoma tumors in murine models (Yadav et al., 2022). The global health crisis caused by COVID-19 has further driven interest in nanomaterials with antiviral properties. Carbon-based nanostructures, including GO and rGO, have demonstrated broad-spectrum antiviral potential, interfering with viral entry, replication, and enzymatic activity in pathogens such as SARS-CoV-2, HSV, HIV, and influenza (Ghulam et al., 2022; Nagarajan et al., 2023).

Moreover, in vitro studies have reported anticancer, antiproliferative, and pro-apoptotic effects of GO and rGO against various tumor cell lines, although cytotoxicity to normal cells has also been observed (Oliveira et al., 2022). These biological responses are strongly influenced by the material's surface chemistry, purity, and functionalization state. Despite its potential, toxicological concerns remain. GO can penetrate biological barriers such as the skin, lungs, gastrointestinal tract, and placenta, raising concerns about developmental and reproductive toxicity (Vedakumari et al., 2022). Studies indicate that GO administered via inhalation or injection can accumulate in vital organs—including the liver, lungs, spleen, and brain—leading to dose-dependent inflammation, oxidative stress, genotoxicity, and mitochondrial damage (Ghulam, et al., 2022). For instance, a mouse study revealed that intratracheally administered GO induced pulmonary edema and acute lung injury, with 47% retention in lung tissue (Kan et al., 2022).

Furthermore, oxidized graphene forms were shown to exert greater cytotoxicity than pristine graphene in human skin HaCaT keratinocyte models, causing mitochondrial disruption and membrane damage (Ghulam et al., 2022). Several recent reports suggest that rGO may pose higher biological risks than GO, especially when used without appropriate surface modification or purification (Zhang et al., 2022). In terms of genotoxicity, fibrous carbon nanomaterials, including graphite nanoparticles, may disrupt mitotic mechanisms either through direct DNA interaction or indirect oxidative damage, depending on surface charge, functional groups, and particle dimensions (Razaq et al., 2022). While some findings suggest GO can induce apoptosis or inhibit angiogenesis in vivo, the biological role of graphite-based nanostructures remains incompletely understood, underscoring the need for rigorous safety evaluations prior to biomedical use (Krishnan et al., 2023).

The bulk of GON sheets had a thickness of approximately 1-2 nm, according to atomic force microscopy (AFM) observations, suggesting few-layered graphene oxide structures (Trikkaliotis et al., 2021). The existence of oxygen-containing functional groups, such as hydroxyl, carboxyl, and epoxy groups, which are indicative of oxidized graphene, was verified by FTIR and XPS investigations. The hydrophilic character and dispersibility of GON in aqueous media are facilitated by these functional groups (Qu et al., 2021). The average lateral dimension of the GON

sheets, as determined by dynamic light scattering (DLS) and verified by TEM (Ji et al., 2019).

2. Materials and Methods

2.1. Ethical Approval and Compliance

All experimental procedures were performed following the ethical guidelines of the Institutional Animal Care and Use Committee (IACUC) at the University of Sialkot. The study conformed to the OECD Test Guideline 425 for acute oral toxicity testing (OECD, 2008).

2.2. Experimental Design

Healthy adult albino rats (8–12 weeks old, 200 ± 25 g) were procured from the Government College University animal facility. Animals were acclimatized for seven days under controlled conditions: temperature 22 ± 2 °C, humidity 55 ± 10%, with a 12-hour light/dark cycle. Rats were housed in stainless steel cages and given ad libitum access to filtered tap water and standard rodent feed. To determine the LD_{50} of graphite oxide nanosheets (GON), rats were randomized into seven groups (n = 5). Following a 6-hour fasting period (water allowed), each group received a single intraperitoneal injection of GO nanosheets at doses of 5.5, 6.5, 7.5, 8.5, 9.5, 10.5 and 11.5 mg/kg body weight.

2.3. Preparation of Stock Solution

Graphite oxide nanosheets were dispersed in sterile 0.9% saline solution and sonicated for 30 minutes using a DSA100-SK ultrasonic sonicator (ROSH) to ensure homogeneous suspension.

2.4. Administration of Graphite Oxide

Freshly prepared GO suspensions were administered intraperitoneally via a 1 ml syringe. Injection volume was calculated based on each animal's body weight to maintain dosing accuracy.

3 Results

3.2 LC_{50} Test.

The sublethal doses were determined after the pilot experiment of LC_{50} as shown in the graph 1. Rats were divided into 7 groups and exposed to different concentrations of GO Nanosheets. LC_{50} determined was 9.5 mg/kg BW.

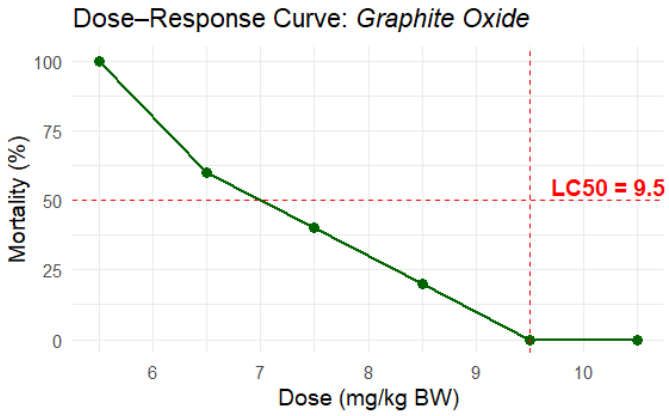


Figure 1: presents the results of the pilot experiment conducted to determine the median lethal dose (LC50) of graphite oxide nanosheets in albino rats.

3.3 Behavior signs during the experiment

Animals given low doses of GO NPs did not exhibit any harmful indications or changes in their physiological state. Animals are affected by GO Nanosheets when their concentration in bodily fluids and tissues beyond the necessary and typical limits and turns hazardous. The 7.5 mg/kg BW dose that was previously administered in the current study did not have any harmful effects on the rats' organs. However, when the rats were given dosages of 8.5 mg/kg BW, aberrant CNS stimulations and aberrant behavior were noted. The animals displayed behaviors such as leaping and jumping within their cages and clawing the area where the test dose was administered. The 9.5 mg/kg BW mice showed bleeding and hemorrhaging in the tail ends at the higher doses. At the end of the four-day trial, the animals' abdomens frequently grew enlarged, causing them to lose their ability to walk normally and to exhibit sluggish, writhing movements of their necks and tails. Ultimately, the animals died mostly from gasping.

4. DISCUSSION

Extended Discussion on LC50 and Dose-Dependent Toxicity:

The determination of the LC50 is a fundamental step in toxicological studies to understand the acute toxicity of nanomaterials such as graphite oxide nanosheets. LC50 refers to the dose required to cause death in 50% of the test population, providing a quantitative measure of a substance's toxicity (Tariq et al., 2025). In this study, the LC50 of GO nanosheets was estimated at 9.5 mg/kg BW, indicating a relatively high acute toxicity at doses approaching this level. Graphene-based nanomaterials, including graphite oxide, have

attracted significant attention due to their unique physicochemical properties and biomedical applications (Laraba et al., 2022). However, their potential toxicity is still a subject of ongoing investigation. The observed mortality at 9.5 mg/kg BW suggests that GO can induce severe systemic effects, possibly related to oxidative stress, inflammation, and cellular damage (Banoon and Ghasemian, 2022).

The dose-dependent increase in mortality (zero deaths at 7.5 mg/kg BW, partial mortality at 8.5 mg/kg BW, and high mortality at 9.5 mg/kg BW) aligns with classical toxicology principles, where higher doses generally cause more pronounced adverse effects (Sanmugam et al., 2024). This dose-dependent toxicity likely reflects the balance between the body's defense mechanisms and the overwhelming cellular damage induced by the nanoparticles. Mechanistically, GO nanosheets can generate reactive oxygen species (ROS), leading to oxidative damage to lipids, proteins, and DNA in vital organs (Zhang et al., 2022). This oxidative stress can compromise cellular integrity, trigger apoptosis, and provoke inflammatory cascades, contributing to organ dysfunction and mortality (Mohammad and Shaker., 2023). It is also essential to consider that the LC_{50} determined here may vary with different routes of exposure, animal species, and physicochemical characteristics of the nanomaterials (e.g., size, surface charge, functionalization). For example, intravenous administration might result in a different toxicity profile compared to oral or inhalational routes (Li et al., 2022).

Beyond mortality, sub-lethal doses can induce various biochemical and histopathological changes that impact animal health and organ function (Tariq et al., 2025). Hence, while LC_{50} is a critical parameter, comprehensive toxicity assessment should also consider chronic exposure, bioaccumulation, and recovery potential (Jin et al., 2025). In recent studies, the median lethal doses for graphene oxide and related nanomaterials have been reported in similar ranges but can be influenced by surface modifications aimed at reducing toxicity (Zhou et al., 2020). Surface coatings such as polyethylene glycol (PEG) or biocompatible polymers have shown promise in mitigating acute toxicity by decreasing nanoparticle aggregation and immune activation (Zhang et al., 2022).

Dose determination also informs safety margins for biomedical applications, including drug delivery and imaging. Establishing the LC50 enables researchers to select doses that avoid acute toxicity while maintaining therapeutic efficacy (Yadav et al., 2022). It is noteworthy that animal variability and small sample sizes can affect the precision of LC50 estimation. Employing larger cohorts and advanced statistical models such as probit analysis can enhance the accuracy of toxicity thresholds (Razaq et al., 2022). Furthermore, recent advances in nanotoxicology emphasize the role of nano-bio interactions at the molecular level, which dictate toxicity outcomes (Nirmala et al., 2022). Understanding these interactions helps design safer nanomaterials with reduced adverse effects.

In conclusion, the LC50 value of 9.5 mg/kg BW for GO nanosheets highlights the potential acute toxicity of these nanomaterials in vivo. Continued research integrating dose-response relationships, mechanistic pathways, and surface engineering strategies is essential to safely harness the biomedical potential of graphite oxide and related nanomaterials.

Aknowledgment:

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