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REVIEW PAPER

Systematic review on toxicity outcomes of graphene oxide coated materials

Pushpa Yadav [1]*, Upasana Yadav [2]

[1]Department of General Pathology and Microbiology, Chandra Dental College and Hospital, Faizabad road, Dharsania, India
[2]Department of Environment, Amity School of Applied Sciences, Amity University, Lucknow, India

ABSTRACT

Graphene and its derived forms have surfaced as promising substances for a wide range of technological and biomedical purposes. However, it is crucial to evaluate their safety and potential risks to ensure their safe use. This systematic review examines the present state of knowledge regarding the toxicity of graphene oxide (GO) through *in-vivo*, *in-vitro*, and other species studies. The aim of this present research was to study toxicity outcomes of GO-coated materials. The literature search was conducted and most important electronic databases (20 studies) were checked and selected for the present study. The findings underscore the need for cautious consideration of GO's potential risks, especially at high concentrations and prolonged exposures. Continued research efforts are essential to gain a deeper understanding of the underlying mechanisms and to develop appropriate safety guidelines for the utilization of GO in various applications.

Keywords: *Graphene oxide, In vitro, In vivo, Toxicity outcomes, Toxicity*

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INTRODUCTION

Nanomaterials based on graphene have attracted a lot of attention due to its wide range of possible uses in a variety of industries, including biomedicine, biotechnology, and environmental technologies. Nonetheless, various researches have indicated their potential to exhibit toxicity in biological systems, which can be modulated by various parameters, including lateral dimension, surface structure, functional groups, purity, dose and duration of exposure [1].

Graphene is a two-dimensional structure composed of carbon atoms that are sp2 hybridized and arranged in a hexagonal pattern. This unique arrangement results in a significant surface area on both sides of graphene sheet [2]. Due to its distinctive two-dimensional structure, graphene exhibits a remarkable array of fascinating and unparalleled properties (Fig. 2). It is renowned for being the strongest material known, possessing exceptional lightness, unrivaled conductivity, and remarkable transparency [3]. Graphene is widely recognized as the most basic form of carbon and holds the distinction of being the thinnest material [4]. The utilization of graphene in biological uses is constrained because of its low solubility in aqueous environments, primarily attributed to its high hydrophobic nature [5].

Reduced graphene oxide, graphene nanosheets, few-layer graphene and graphene oxide (GO) are materials that belong to the graphene family (Fig. 2) [6,7]. GO is a nanomaterial that has been recognized for over 150 years and finds applications in various fields [8]. It acts as the precursor to graphene, an extraordinary two-dimensional material that falls within the family of carbon allotropes. The breakthrough discovery of graphene occurred in 2004, credited to the research team led by Andre Geim at the University of Manchester in England [6]. GO is a chemically modified form of graphene [9] which consists of a monolayer [10] and is thus categorized as a two-dimensional material [5].

GO is mainly composed of oxidation of graphene along with other oxygen-based functional groups like hydroxyl, alkoxyl, carbonyl, carboxylic

* *Corresponding author: Email: ydrpushpa@gmail.com*
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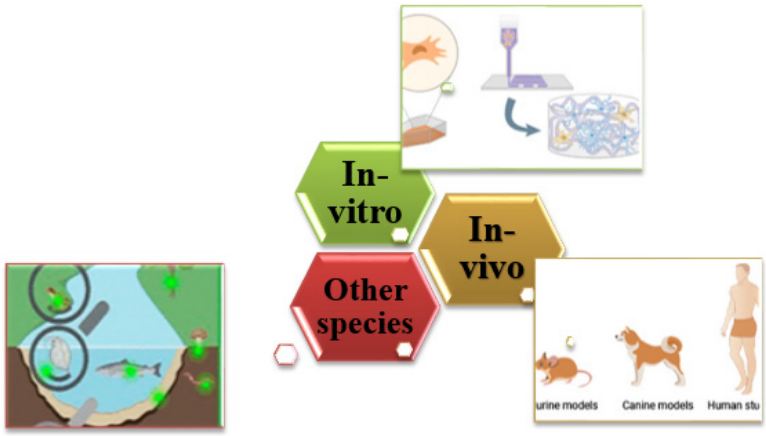

Fig. 1. Toxicity of Graphene Oxide (GO)

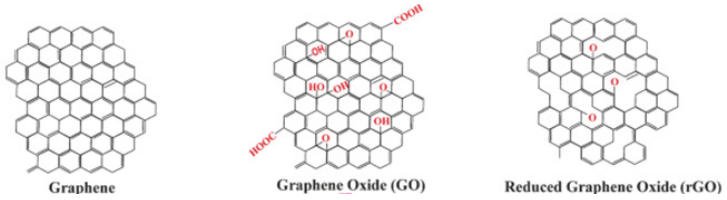

Fig. 2. Structure of Graphene oxide and reduced graphene oxide [3]

acid, and others. These functional groups are located on the sp2-carbon basal plane of GO, resulting in its increased amphiphilicity [1]. GO holds significant value for biological applications due to its intriguing properties of hydrophilicity, high dispersibility in aqueous media, and synthesis methods (Fig. 3). GO can interact with different biomolecules including proteins and nucleic acids and is also employed in nanocomposite materials further expanding its potential applications in the various fields [5]. It is important to note that the synthesis methods employed for GO, results in superior water dispersion stability, and unique

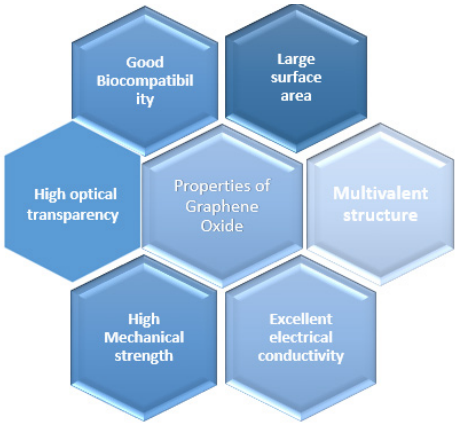

Fig. 3. Properties of GO

mechanical, colloidal, and optical characteristics which typically involve the use of strong oxidizing agents like potassium permanganate. This can introduce a considerable number of defects into the crystalline network of GO. These defects significantly impact the conductive properties of GO, leading to lower conductivity compared to graphene. However, the optical and mechanical properties of GO are relatively less affected by these defects [10].

Toxicity mechanism of GO

GO exhibits a myriad of toxicological effects, across various mechanisms, including cytotoxicity, genotoxicity, inflammatory responses, platelet activation, apoptosis, necrosis, autophagy dysregulation, and epigenetic modifications. Each of these toxicities contributes to the intricate landscape of GO-induced biological perturbations, showing the multifaceted nature of its toxicity. (Fig. 3) [1].

GO elicits a plethora of toxicological effects through diverse mechanisms. Primarily, its physical interaction with cell membranes instigates cytotoxicity, manifesting as aberrant stretching of cell membranes and disruption of cytoskeletal integrity. Furthermore, it induces oxidative stress, a pivotal mediator in the onset of carcinogenesis,

aging, and mutagenesis, leading to DNA damage and apoptosis. This oxidative stress-induced DNA damage can initiate cancer development which poses risks to future generations by affecting reproductive cells, potentially impacting fertility and offspring health. Additionally, GO triggers inflammatory responses, evidenced by inflammatory cell infiltration, pulmonary edema, and granuloma formation, along with platelet activation and subsequent thrombi formation. The release of cytokines and chemokines further exacerbates tissue injury by recruiting circulating monocytes and stimulating cytokine secretion. Moreover, GO induces apoptosis through various pathways, including mitochondrial dysfunction, activation of apoptotic signaling cascades, and autophagy dysregulation (Fig. 4) [5, 11, 12].

Autophagosome accumulation, associated with GO exposure, contributes to cellular dysfunction, while epigenetic modifications such as DNA methylation alterations have been observed, implicating chromatin remodeling and gene expression changes. Notably, GO combined with cisplatin triggers necrosis, exemplified by dysregulation of RIP proteins and HMGB1 release [13, 14].

Fig. 3. Schematic diagram representing the toxicity mechanisms of GO family [1].

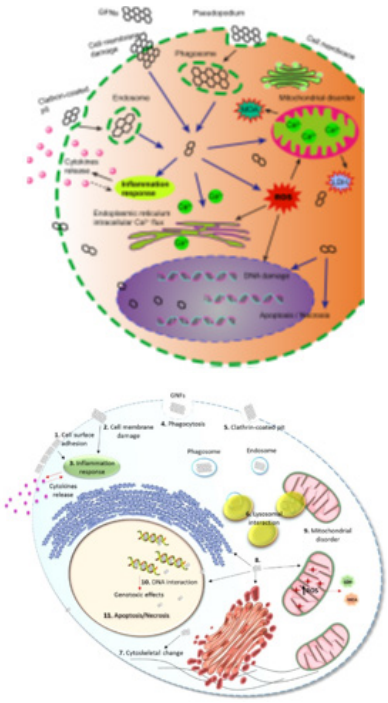

Fig. 4. Possible interactions of graphene family with cell membrane [13]

Over the last few years, graphene has recognized as a valuable tool in the medical field with specific applications including DNA sequencing [8], biosensor development, and promoting cell differentiation and growth [15]. Due to its insolubility in water, the applications of graphene are restricted to passive platforms for detection and cellular work. However, its functional derivative, GO exhibits distinct characteristics which enhance its effectiveness in biomedical applications. Notably, it possesses the unique capability to disperse in various solvents, greatly facilitating handling and utilization diverse biomedical contexts [16]. Additionally, it has been used to transport anticancer medications into cells [17], as well as to attach aptamers for ATP probing and as a carrier for gene delivery [18].

Despite its wide range of applications, the use of GO is limited due to concerns about its toxicity. Researchers often face the challenge of finding a balance between the positive therapeutic effects of GO and possible toxicity-related adverse effects. Therefore, selecting an appropriate experimental model, whether *in-vivo* or *in-vitro*, becomes crucial for assessing the nanoparticle's toxicity. The toxic impacts of GO are influenced by various factors, including the route of administration, dosage, synthesis method, and physicochemical properties of GO. These factors contribute to the complexity of comparing different studies on GO toxicity [12].

Graphene-based systems, although relatively newer in development compared to other carbon materials, exhibit significant potential for various biomedical applications [19]. However, before incorporating graphene-based materials, it is crucial to adopt a proactive approach by thoroughly determining any potential toxicity, which is relatively less understood compared to other carbon nanostructures like carbon nanotubes [6]. While the utilization of GO holds great promise in advancements and potential breakthroughs the biomedical field, it is essential to recognize the associated risks to human health. Therefore, conducting toxicological studies and evaluating human safety is imperative. It is crucial to explore the extent of GO potential toxicity and determine its safety threshold for use in order to ensure responsible and informed utilization [20]. So, the purpose of the research was to study the toxicity outcomes of GO coated materials *in-vitro*, *in-vivo*, and other species.

MATERIALS AND METHODS

The protocol of the study was built on approved reporting articles for systematic review (PRISMA-P) declaration and all changes were properly reported. The Cochrane handbook and the PRISMA statement were followed in conducting and reporting this systematic review, respectively. Our literature search included MEDLINE, Google Scholar, PubMed, Cochrane Library, and Web of Science and Scopus, among other computerized databases. The related keywords in the following search terms such as GO, toxicity, and *in-vitro* and *in-vivo* studies were used with limitation to publications in English. Several eligibility factors, including inclusion and exclusion criteria, were taken into consideration when doing the study selection.

Inclusion criteria

- Articles which are published between 2012 to 2023
- Human clinical trials
- *In-vitro* studies
- *In-vivo* studies
- Full text articles
- Articles which are published in English language

Exclusion criteria

- Non-human studies
- Incomplete or irrelevant studies
- Articles which are not written in English language
- Duplicate articles

RESULTS

The present systematic review initially yielded 546 articles upon conducting the search. Among them, 10 articles were identified as duplicates and subsequently removed. An additional 68 articles were excluded from the review for various reasons. For the inclusion in the study total 468 articles were screened.

During the detailed screening process, 214 articles were further eliminated. Subsequently, the full-text articles were assessed and removed based on specific criteria, including studies with small sample sizes, incomplete or irrelevant data, as well as case reports, case studies, and letters to the editor.

Following the completion of the screening and analysis, a total of 20 studies were included in the present systematic review (Fig. 5).

These 20 studies encompassed various categories, including 7 *in-vitro* studies [21-27], 7 *in-vivo* studies [28-34], and 6 studies involving other species [35-40] (Tables 1, 2, and 3).

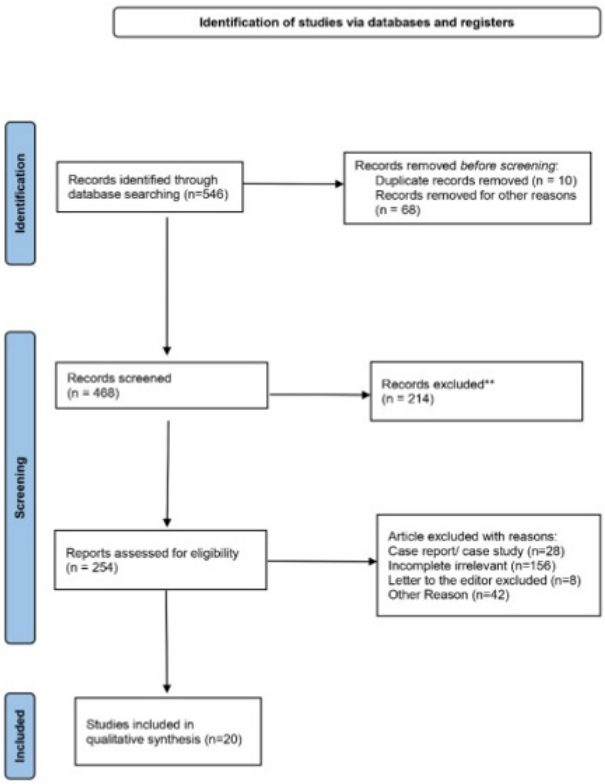

Fig. 5. PRISMA flow diagram and eligible database selected for this study

Table 1. Toxicity outcomes of GO *in-vivo*

In-vitro studies	Author	Dose (ug/mL)	Duration (hr)	Type of toxicity	Toxicity outcomes
CaCo-2 cell line	Dominguez et al. (2023)[21]	0-250	24 and 48	Cytotoxicity	- Reduction in the levels of glutathione (GSH), changes in markers inducing oxidative stress.
MiR-21and miR-29a in human cell lines	Hashemi M et al. (2019)[22]	15	24	Genotoxicity	- Changes observed in expression levels of specific genes such as miR-21, miR-29a, Bax, BcL2 and PTEN. - Mitochondrial activity affected at cellular level, impact basal expression of gene and mitochondrial membrane potential.
Embryonic stem cells (ESC)	L Hu et al. (2019) [23]	50	24	Genotoxicity	- Modifications in mitochondrial activity, alterations in miRNA expression and protein synthesis.
Cardiomyoblast cell line H9c2	Arbo MD et al. (2019)[24]	20-100	24	Cytotoxicity, Genotoxicity	- Disruption in mitochondrial functions, production of reactive oxygen species, interactions with DNA.
Macrophage Cells, THP-1 cells, HUT102 cells, MEL cells, HEK293 cells	Ma et al. (2015)[25]	20	1-24	-	- Polarization of macrophages towards proinflammatory M1 phenotypes.
MDA-MB-231	Liu Y et al. (2013)[26]	100-500	48	Genotoxicity, cytotoxicity	- Disrupted the process of DNA synthesis, altered programmed cell death, impact on the progression and regulation of cell cycle, alterations in metabolic pathways.
HeLa cells	X Zhang et al. (2012)[27]	0-80	24	Cytotoxicity	- Release of lactate dehydrogenase enzyme, production of reactive oxygen species, reduced activity of superoxide dismutase enzyme, and decline in cell viability.

Table 2. Toxicity outcomes of GO *in-vitro*

In-vivo studies	Author	Dose	Duration	Type of toxicity	Toxicity Outcomes
Male mice	Rhazouani A et al. (2021)[28]	2 and 5 mg/kg	5 days	Cytotoxicity	- Elevated levels of peroxidase, augmented levels of malondialdehyde, liver inflammation.
Zebra fish embryos	Cao Z et al. (2021)[29]	10, 50 and 100 mg/mL	-	Cytotoxicity, Neurotoxicity	- Increased activation of oxidative stress, enhanced activity levels of acetylcholinesterase, altered expression pattern of gene involved in neurodevelopment and neurotransmitter.
Male Sprague Dawley rats S	Zhang Let al. (2021)[30]	5-100 mg/kg	7 days	Pulmonary toxicity	- Lung damage influenced by dosage, lung edema, promotes the process of autophagy.
Earthworm (Eisena fetida)	Zhao S et al. (2021)[31]	5, 10, 20 and 30 g/kg	7, 14, 21 and 28 days	Cytotoxicity	- Reactive oxygen species (ROS) imbalance, oxidative degeneration of lipids, impaired stability of lysosomal membrane.
Mice	An W et al. (2018)[32]	50 and 100 ug/mL	24 hrs	Occular inflammation	- Eye inflammation, thickening of corneal stromal layer, cell death in corneal tissue, reduction in cell viability.
Mice	Hashemi E et al. (2016)[33]	100 and 400 g/mL	24 hrs	Cytotoxicity	- Significant amount of cell mortality, marked elevation in reactive oxygen species levels, significant amount of cell mortality.
Sprague- Dawley rats	Han S et al. (2015)[34]	0.5 or 4 mg/mL	6 hrs	Pulmonary toxicity	- Mild toxic reactions observed in lungs of rats.

Table 3. Toxicity outcomes of GO in other species

Other species	Author	Dose	Duration	Type of toxicity	Toxicity Outcomes
Pichia pastori	Foadin T et al. (2022)[35]	0-4000 ppm	24 hrs	Cytotoxicity	- Decrease growth and development of cells, elevated levels of ROS of cells, injury to cell membrane which results cell damage.
Drosophila melanoglaster	Sood K et al. (2019) [36]	300 ug/mL	1-16 days	Neurotoxicity	- Disruption of co-ordination between the nervous system and muscles in larvae.
Pseudomonas putida	Combarros RG et al. (2016) [37]	0.05, 0.5, and 1.0 mg/mL	72 hrs	Cytotoxicity	- Suppression of bacterial growth, detrimental effect of survival of bacteria.
Daphnia magna	Lv X et al. (2018) [38]	44.3 and 45.4 mg/mL	72 hrs	Cytotoxicity	- Increased oxidative stress which was associated with cellular damage, tissue injury or functional impairment.
Ceriodaphnia dubia	Souza JP et al. (2018) [39]	1.25 mg/mL	48 hrs	Cytotoxicity	- Elevation in the level of ROS at sub lethal concentration.
Escherichia coli	Tu Y et al. (2013) [40]	100 ug/mL	2.5 hrs	Cytotoxicity	- Partial disruption of cell membrane, reduction in concentration of phospholipid on cell membrane surface.

DISCUSSION

The systematic review of toxicity outcomes associated with GO exposure provides valuable insights into the potential risks and safety considerations. The analysis of the selected studies reveals a diverse range of toxicity endpoints evaluated across different biological systems. One prominent finding from the reviewed studies is the dose-dependent nature of GO toxicity. Several investigations demonstrated that higher concentrations of GO were associated with increased cytotoxicity and adverse effects on cellular viability, suggesting the importance of considering the exposure levels of GO due to its potential risks.

Furthermore, the reviewed studies shed light on the mechanisms underlying GO-induced toxicity. It has been observed that the interaction between GO and cellular components can create oxidative stress and cellular damage by producing ROS. Additionally, physical characteristics of GO, like size and surface charge can influence its toxicity profile. Understanding these mechanisms is crucial for designing strategies to mitigate the potential adverse effects of GO.

The bio-distribution and long-term effects of GO exposure were also investigated in some of the included studies. It was observed that GO could accumulate in various organs, including the liver, lungs, and spleen, highlighting the need to assess the potential systemic effects of GO beyond the site of administration. Moreover, studies exploring the chronic exposure to GO reported persistent inflammation and fibrotic responses, raising concerns regarding its long-term safety. Although the majority of the reviewed studies highlighted the potential toxicity of GO, it is important to note that variations in study design, experimental conditions, and GO characteristics make it challenging to draw definitive conclusions. While a few specific studies have evaluated the effects on organs such as the liver, spleen, and kidney, including assessing injury symptoms, damage indices, and levels of damage, the developmental toxicity of graphene-based nanomaterials (GFNs) remains a critical area of concern. It can potentially induce structural abnormalities, growth retardation, behavioral and functional abnormalities, and even death. Research on the reproductive and developmental toxicity of GFNs is particularly significant and is expected to attract substantial attention in the future. Based on studies of the toxicity of other nanomaterials, long-term exposure may pose significant health risks (Fig. 6) [1].

Additionally, the relevance of the observed toxicity outcomes in experimental models to human exposure levels warrants further investigation. To address these uncertainties and enhance the safety assessment of GO, future research should focus on standardized protocols, well-defined exposure scenarios, and thorough characterization of GO nanoparticles.

Nanomaterials are widely recognized as potent resources for nano-technological applications across industries, cosmetics, and healthcare sectors [41]. GO also have the ability to penetrate physiological barriers and cellular structures through various exposure routes or administration methods, ultimately leading to in-vivo and in-vitro toxicity. The specific routes of administration and entry pathways, as well as the distribution within the different tissues and subsequent excretion, along with the diverse patterns and locations of cellular uptake, can collectively determine the extent of GO toxicity [42-44].

Toxicity of GO in-vitro (Cell lines)

GO has been extensively studied as a representative member of GFNs in *in-vitro* toxicity

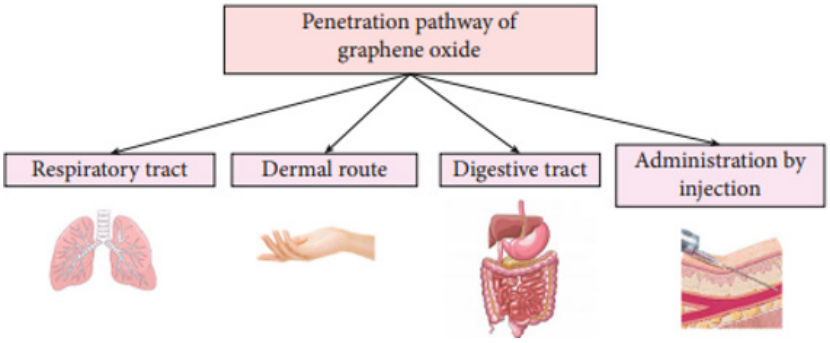

Fig. 6. Penetration pathways of GO [12]

investigations. In-vitro studies have consistently demonstrated the cytotoxicity of GO across a variety of cell types, leading to alterations in cell viability and morphology. Moreover, GO have been shown to disrupt membrane integrity and induce DNA damage, further highlighting their potential adverse effects on cellular health [45-47]. It has been found to decrease cell adhesion, triggering a reduction in the ability of cells to adhere to their surrounding substrates. Additionally, GO have been reported to induce cell apoptosis, a programmed cell death mechanism. Notably, these nanomaterials have the ability to enter cellular organelles such as lysosomes, mitochondria, cell nuclei, and endoplasm, potentially disrupting their normal functions and contributing to cellular toxicity [48].

Research studies have examined the toxicity of GO in cells, particularly on human breast cancer cells (MDA-MB-231). Studies have reported that the incubation of these cells with GO led to a notable decrease in cell viability. This effect was found to be dose-dependent, suggesting that the extent of exposure to GO played a critical role in the observed reduction in cell viability [26].

According to a study by Hu et al. [49], GO exhibited cytotoxicity that varied with its concentration. However, when GO was incubated with 10% fetal bovine serum, the cytotoxic effects were largely reduced. This reduction was attributed to GO's high capacity to adsorb proteins. At low concentrations of fetal bovine serum (FBS) (1%), human cells exhibited sensitivity to the presence of GO and showed concentration-dependent cytotoxicity. Interestingly, this cytotoxicity was significantly reduced at 10% FBS, the concentration typically used in cell culture media. The studies demonstrated that the cytotoxicity of GO nanosheets arises from direct interactions with the cell membrane, leading to physical damage. This harmful effect is largely mitigated when GO is incubated with FBS due to GO's exceptionally high protein adsorption capacity. This observation of FBS-mitigated GO cytotoxicity offers a potential and convenient approach to engineering nanomaterials for safer biomedical and environmental applications. Various cell lines have been used in research investigations to examine toxicity, genotoxicity, and possible mechanisms of GO. These cell lines include a variety of human animal cells, involving stem cells, immune cells, normal cells, immortalized cells, and blood components [49].

After one day of GO incubation, neuronal PC12 cells' metabolic activity decreased in a dose-dependent way. This reduction in metabolic activity affected mitochondrial function and compromised the integrity of the cell membrane. Notably, even at low concentrations, GO still exhibited cytotoxic effects on the cells [50].

The cytotoxicity of GO has been observed to be influenced by its lateral size. Studies have demonstrated that the extent of cytotoxicity is linked to the density of functional groups of GO presented on materials [51].

Three types of GOs with the various lateral dimensions and functional group densities were studied on human lung cells (BEAS-2B) alveolar epithelial (A549). The findings revealed that both GO and thermally reduced GO exhibited greater toxicity when compared to chemically reduced GO [52].

The surface charge of GO plays a crucial role in inducing cytotoxicity. Research studies have indicated that the surface charge of GO influences the internalization and absorption of the material by cells. The amount of electronic charge on the surface of GO and its derivatives is significant; however, this charge is not directly related to the extent of toxicity. Instead, the surface charge influences the particles' aggregation status and their ability to be internalized by cells. Higher surface charge results in greater repulsive force secondary organs among particles, leading to reduced aggregation, which facilitates the entry of GO and its derivatives into cells by maintaining their small size. Conversely, a mild positive charge may help GO stay outside cells if it does not damage the cell membrane. Therefore, the ideal GO derivatives should possess a lower positive electronic charge to minimize their toxic effects on cells [53].

Red blood cells may be hemolyzed as a result of the interaction between GO and the cell membrane, which can also cause morphological alterations and cell lysis. This is due to electrostatic interaction between the positively charged phosphatidylcholine located on the outer membrane on red blood cells and negatively charged oxygen groups that are present on the surface of GO. As a result of these interactions, red blood cells may undergo hemolysis, impacting their appearance and integrity [54]. The toxicity of GO was investigated in a study on the rat

cardiomyoblast H9c2 cell line. The findings revealed that GO exhibited cardiotoxic effects, such as mitochondrial disruption, ROS and interactions with DNA [24].

According to a study conducted by Lammel et al., it was found that GO induces a dose-dependent toxicity by causing damage to the plasma membrane. This damage is characterized by the disruption of the structural integrity of the plasma membrane, which is believed to be a result of the strong physical interaction between GO and the phospholipid bilayer. The study also reported that GO may pass through the plasma membrane, altering the shape of cells and increasing the count of apoptotic cells [55].

Gurunathan et al. examined the effects of bacterially reduced GO on MCF-7 cells. The findings revealed, GO that had been decreased by bacteria both showed toxicity to MCF-7 cells in a dose dependent way [56].

Akhavan et al. conducted a study showing that both GO sheets and nanoplatelets exhibited cytotoxicity and genotoxicity towards human mesenchymal stem cells. The toxicity observed was found to be dependent on the size and concentration of GO materials [57].

Toxicity of GO in-vivo

Understanding the in-vivo toxicity of GO is crucial when considering their application in drug delivery. Chronic toxicity and lung granuloma death were induced by the administration of GO in mice. When GO was administered, lung toxicity developed that was dose-dependent and characterized by granulomatous lesions, pulmonary edema, fibrosis, and inflammatory cell infiltration. In rats, the administration of BSA-capped graphene also resulted in a pulmonary inflammatory response [58].

Zebrafish is widely acknowledged as animal model for investigating the in-vivo toxicity of nanomaterials because of its genomic similarity to humans. Moreover, zebrafish embryos showed higher sensitivity than adult organisms when compared to chemical agents [59].

Gollavelli and Ling conducted a study to assess the in-vivo toxicity of graphene in zebrafish embryos (Danio rerio). The researcher microinjected embryos with multifunctionalized graphene, which had been coated in polylactic acid and fluorescein o-methacrylate. The scientists reported that there were no discernible changes or anomalies in the survival rate of the fish embryos. However, it was noted that the graphenes were extensively distributed within the zebrafish [60].

In a mouse study, the intravenous administration of GO at low (0.1 mg) and moderate (0.25 mg) doses did not exhibit any indications of toxicity. However, when a high dose of GO (0.4 mg) was administered, chronic toxicity was observed. Four of the nine mice died from suffocation during 1 to 7 days after injection as a result of the accumulation of GO obstructing their main airways. The liver, spleen, and lungs were where GO deposited predominantly. The mice that survived exhibited the significant chronic poisoning in their liver and lungs. Histopathological analysis revealed a dose-dependent inflammatory response in the lungs, characterized by the accumulation of neutrophils, the presence of foamy alveolar macrophages, and the formation of epithelioid granulomas. Despite a minimal accumulation of GO in the kidneys, the presence of GO in the liver indicated that its primary clearance pathway might involve secretion into the bile tract system. These observations bring into question the suitability of GO for human applications, given that its shape poses challenges for efficient elimination by the kidneys [61].

Another research conducted on Swiss male mice demonstrated the occurrence of extensive pulmonary thromboembolism for 15 minutes only after intravenous administration of 250 mg/kg body weight GO [62].

After intravenous treatment of 1 mg/kg body weight of graphene nanosheets, immunological responses were seen in the lungs of C57BL/6 besides previously reported pulmonary inflammation and thrombosis [63].

The toxicity of GO demonstrated dose-dependent effects on both human and animal cells. In mice, low and medium doses of GO showed minimal to no impact [61]. However, administration of doses equivalent to 10 ng GO per gram of body weight resulted in significant pathological alterations, such as infiltration of inflammatory cells, pulmonary edema, and the formation of granulomas. Conversely, GO exhibited favorable biocompatibility with red blood cells at extremely low doses, but at a concentration of 80 ng/mL, it induced hemolysis [60].

Direct lung injections of a variety of graphene solutions, including aggregated graphene, pluronic-dispersed graphene, and GO were performed on mice. ROS were produced in

mitochondria, inflammatory, and apoptotic pathways, and a severe and long-lasting lung damage was developed as a result of the administration of GO. However, mice exposed to scattered and aggregated graphene did not show any obvious lung damage [65].

To assess the possible negative impacts of GO on the eyes, a research study was carried out on Japanese white rabbits. The rabbits received intravitreal injections of GO at doses of 0.1 mg, 0.2 mg, or 0.3 mg, and were observed for a duration of 49 days. Over the course of the observation period, no clinical indications of ocular changes were detected, and GO demonstrated minimal influence on both intraocular pressure and visual acuity. The evaluation of these factors was conducted using slit lamp biomicroscopy and indirect funduscopic examination [66]. Over time, the GO content in the eyes decreased gradually, and at the end of the experiment, histological examination revealed only a very small amount of the residual GO. No retinal abnormalities were observed in the eyes that received GO injections. These findings demonstrate GO injections into the eyes up to 0.3 mg do not adversely affect them [66].

Oral administration of GO within the concentration range of 0.5 to 100 mg/mL has been found to induce damage to both secondary organs (neurons and reproductive organs) and primary organs, such as the intestine. This effect is attributed to the ability of GO to translocate into intestinal cells, causing the loss of microvilli and distributing itself adjacent to or surrounding mitochondria. Prolonged exposure to GO exacerbates these effects, leading to a hyper-permeable state of the intestinal barrier and an increase in the mean defecation cycle length. Consequently, the observed toxicity of GO is likely due to the combined impact of oxidative stress on the intestinal barrier [67].

ROS generation was closely related to this damage. It was discovered that GO translocated into intestinal cells, causing microvilli to disappear, and that it was distributed within the surrounding mitochondria [68]. Additionally, GO delayed the fecal elimination cycle and influenced the genetic expression which were involved in intestinal growth and defecation behavior. The combined effect of oxidative stress, increased permeability of biological barriers, and changes in fecal cycle suggests that prolonged exposure to GO harmful for environmental species [69].

Toxicity of GO in other species

GO has been shown to have detrimental effects on soil microorganisms, impacting their growth, and overall ecosystem function. Aquatic organisms, such as algae and daphnia, have exhibited sensitivity to GO exposure, leading to changes in their physiological and reproductive parameters. Some studies have also suggested potential toxicity of GO towards bacteria, impacting their viability and microbial communities.

GO was evaluated for its effectiveness against both gram negative Escherichia coli (E. coli) and gram-positive Staphylococcus aureus. The findings revealed that E. coli exhibited resistance to GO [70]. Researches conducted by Hu et al. [71] and Feng and Liu et al. [72] provide evidence of the advantageous properties of GO against E. coli. These investigations revealed that aggregated GO, particularly those with smaller average sizes, demonstrated the increased efficacy in inhibiting bacterial growth. The interaction of E. coli with graphene nanosheets resulted in disruption of both its outer and inner membranes. This phenomenon underscores the significant role of direct contact between E. coli and graphene nanosheets in damaging the bacteria's membranes, ultimately leading to antibacterial effects.

Daphnia magna, belonging to the subclass Phyllopoda, was subjected to toxicity evaluation using GO particles sized between 200-300 nm. After a 72-hr exposure to GO, the EC50 (effective concentration for 50% effect) and LC50 (lethal concentration for 50% mortality) values were determined to be 43.3 mg/L and 45.4 mg/L, respectively. These values indicate the concentration at which GO has a significant impact on Daphnia magna, both in terms of its effects and mortality rate [38].

When GO interacts with E coli, it undergoes reduction, resulting in the formation of reduced GO (rGO).The reduction process hinders bacterial proliferation and causes surface detachment. Consequently, the presence of rGO following bacterial reduction of GO illustrates its inhibitory effects on the growth and attachment of E. coli cells [73].

In the colony-forming assay, when E. coli was incubated with either 85 mg/mL of GO or rGO for 2 hr, more than 90% of the bacteria lost their viability. Transmission electron microscopy analysis (TEM) further revealed significant damage to the cell membranes and leakage of cytoplasm.

These effects could be attributed to either oxidative stress or physical disruption caused by the interaction of GO or rGO with the bacterial cells [74].

In a study conducted by Tu et al. [40], a new mechanism elucidating the cytotoxicity and antibacterial effects of graphene was discovered. The researchers observed that graphene, particularly GO nanosheets, induced the extraction of phospholipids from the membranes of E. coli cells, leading to their destruction. During a 2.5-hr incubation period of E. coli with a concentration of 100 mg/mL of GO, three distinct stage of cell damage were seen under TEM. The first stage, referred to as Stage I, exhibited the initial cell morphology that demonstrated short-term tolerance to GO. In the subsequent stage, Stage II, the cell membranes experienced partial loss of integrity, accompanied by a decrease in surface phospholipid density. Finally, Stage in III, the cell membranes suffered severe damage, with some cells completely lacking their cytoplasm [40].

In contrast, several randomized controlled trials (RCTs) have reported promising findings regarding the use of GO and GO-based nanocomposites in various applications. Soundarajan S. et al. [75] found that an amla seed-mediated GO-Ag nanocomposite mouthwash efficiently reduced plaque, gingival inflammation, and colony-forming units (CFUs) among patients with plaque-induced gingivitis. Sevagaperumal et al. [76] concluded that the GO-AgNp irrigant is an effective, biocompatible antimicrobial agent, comparable to 2.5% sodium hypochlorite and superior to 2% chlorhexidine and normal saline. Eskandari et al. [77] demonstrated that GO-DAP significantly improved root canal disinfection. Additionally, Andrews et al. [78] conducted the first-in-human double-blind randomized controlled trial on the inhalation of thin graphene oxide nanosheets to determine their effects on acute pulmonary and cardiovascular function. They found that acute inhalation of highly purified, thin nanometer-sized GO nanosheets did not cause overt detrimental effects in healthy humans. These findings demonstrate the feasibility of carefully controlled human exposures in a clinical setting for the risk assessment of graphene oxide and lay the foundation for investigating the effects of other two-dimensional nanomaterials in humans.

Additionally, GO has raised considerable interest in tissue engineering and regenerative medicine due to its exceptional mechanical properties, electrical conductivity, and physiochemical, antibacterial, and biological capabilities. GO-based materials in various forms, including 2D and 3D structures, have been shown to stimulate the proliferation and differentiation of cells into specific lineages through interactions with biomolecules. However, the main challenge associated with utilizing GO-based materials is their potential toxicity. Most research has focused on toxicity at the cellular level rather than the genetic level. Since GO-based materials can interact with various biomolecules, especially DNA, further studies are necessary to elucidate the apoptosis pathways. Although ROS production, which leads to cell death, has been extensively studied, investigating the effects of GO on organs such as the lungs, liver, intestines, and kidneys is also critical [79].

CONCLUSION

GO nanomaterials are being developed for a wide range of applications but come with potential safety concerns for human health. This review examines the toxicity outcomes of GO across diverse study groups, including *in-vivo*, *in-vitro*, and other species. However, extensive research on the toxicity and health risks of these nanomaterials is necessary to mitigate the risk of long-term side effects. These findings are crucial for human health risk assessment and for establishing safe usage guidelines for graphene-based materials in the workplace. To understand the toxicological mechanisms of GO, it is essential to identify the molecular targets involved in its toxicity and evaluate both its benefits and risks to health. This comprehensive evaluation will help harness the advantages of nanotechnologies while minimizing the risks to human health. Achieving this goal requires the production of a highly defined graphene family in terms of the number of layers, surface area, and functionality. Despite the increasing research on the bioavailability and toxicity of graphene and its derivatives, much remains to be uncovered to ensure safe and effective formulations. Furthermore, more studies are needed to address the toxicity of GFNs using effective experimental methods and systematic research approaches.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest associated with this publication.

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