Peer Review File

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Reviewer A

Comment: *The article has potential, but requires a few corrections. There is no summary of the effect of graphene/GO on ferroptosis induction as a potential way to trigger this pathway in CRC. The article lacks a good summary that holds the whole thing together. Has graphene or GO been used to induce smelting via ferroptosis? Without a summary, the article is only a good overview of the methods but lacks any conclusions derived from these methods.*

Reply:

First and foremost, I would like to express my sincere gratitude for your insightful comments and the time you dedicated to reviewing our manuscript. Your feedback is invaluable to us in enhancing the quality and clarity of our work. Regarding your comment about the lack of discussion on the role of graphene/GO as a potential trigger factor in CRC through ferroptosis induction, we appreciate your keen observation. However, currently, there is no literature on the application of graphene/GO through the ferroptosis mechanism in the diagnosis and treatment of CRC. In response to your comment, we have made some revisions and additions to the manuscript, summarizing the prospects and challenges of GO and the ferroptosis mechanism in cancer treatment, and proposing potential directions for GO in cancer treatment

through the ferroptosis mechanism. A part of the revised and added content is as follows:

In summary, the application prospects of GO and ferroptosis in cancer therapy are vast. Despite the need to overcome numerous challenges, the rapid development of nanomaterial science, particularly the advancements in graphene oxide and its substrates, has turned many impossibilities into possibilities, especially in cancer diagnosis and treatment. Furthermore, it's worth mentioning that combining GO with ferroptosis-inducing therapies presents an intriguing path that remains largely unexplored. In the future, GO could be further modified to directly influence the proliferation, migration, and invasion of tumor cells, such as those in CRC, through mechanisms like ferroptosis. Additionally, research into GO's drug-carrying and releasing capabilities could be expanded to include ferroptosis inducers, utilizing GO's delivery potential to enhance ferroptosis induction in cancer cells. Moreover, addressing the toxicity of GO and improving its biocompatibility is crucial for its effective integration into cancer therapy. In summary, while GO and the ferroptosis mechanism offer significant opportunities to advance cancer treatment, their integration necessitates a thorough understanding of their interactions, meticulous management of potential toxicities, and innovative strategies to leverage their combined potential to improve cancer treatment outcomes. This area warrants further research and exploration by the scientific community.

Additionally, we have added a table to summarize the main applications of oxidized graphene and ferroptosis in cancer diagnosis and treatment, categorized by type of study, type of sample used, and the main findings of the referenced literature.

Table. Graphene Oxide and Ferroptosis in CRC Diagnosis and Treatment

Type of Study	Types of Samples Used	Main Findings	Reference(s)
GO and Early Diagnosis	LoVo, HCT116 cell and LoVo cells in human; human blood; human fecal	Chen H et al. developed a GO-based fluorescent aptasensor that provides a simple, one-step, and highly sensitive approach for the detection of mCRC cells; developed a new biosensor based on a GO nanocomposite for the precise measurement of CEA in CRC biomarker detection; Alustiza M et al. developed a novel non-invasive diagnostic method for CRC using magnetic GO to extract volatile organic compounds from feces, serving as a potential screening technique for CRC and precancerous lesions	[1-3]
GO and Drug Delivery Systems	HUVEC, HT-29 cell; CT-26 cell and BALB/c mice	Bardania H et al. constructed a folic acid-modified co-delivery carrier based on graphene nanoparticles (GO-Alb-Cur-FA-5FU) to enhance the effects of 5FU and curcumin (Cur) on colon cancer; Lu YJ et al. developed the dual-targeting MGO-PEG-CET/DOX, which could be suggested as an effective drug delivery system for anticancer therapy. It showed a 29-fold increase in therapeutic efficacy compared to the control by combining chemotherapy with photothermal therapy.	[4, 5]
GO and Photothermal therapy	KM12C cell	He S et al. developed a two-dimensional graphene oxide-template gold nanosheet (GO@SiO_2@AuNS) hybrid, which effectively killed colorectal cancer cells (KM12C) under NIR irradiation.	[6]
GO promotes cancer cell death	HCT116 cell and BALB/c mice; Colon 26 cell	Shen et al. discovered that GO exerts anticancer effects against CRC through ROS-dependent AMPK/mTOR/ULK-1 pathway-related autophagy and apoptosis; Krasteva N et al. found that exposing cancer cells to aminated graphene oxide significantly enhances cytotoxicity by inducing ROS, subsequent DNA damage, and apoptosis, thereby significantly promoting the killing of cancer cells.	[7, 8]
Ferroptosis Triggers Tumor Cell Death	colorectal cancer stem cell; HCT116 cell and BALB/c mice; HCT116,	LV X and others found that IFNγ is a key cytokine capable of blocking intestinal stem cells, and they verified its role in killing colorectal cancer stem cells through triggering GPX4-dependent ferroptosis; ZHAO Y et al. discovered that both in vitro and in vivo, downregulation of CAPG can significantly inhibit CRC cells. Interfering with CAPG can block the cell cycle at	[9-11]

Type of Study	Types of Samples Used	Main Findings	Reference(s)
	SW480 cell	the G1 phase and induce apoptosis and ferroptosis in CRC cells by upregulating the P53 pathway; LIU X et al. discovered that TRIM36-mediated FOXA2 promotes the progression of CRC by inhibiting the Nrf2/GPX4 ferroptosis signaling pathway.	
Ferroptosis and the occurrence and development of tumors	HCT8, SW480 cell; MC38 3D tumour spheroidsand HT29 organoids	Lei S et al. discovered that the loss of AMER1 promotes distant metastasis of CRC by inhibiting iron death mediated by SLC7A11 and FTL; Cui W et al. discovered that a gut microbiota metabolite, IDA, promotes the progression of CRC by inhibiting ferroptosis.	[12, 13]
Ferroptosis and drug resistance	HCT116, SW480, Hep3b cell and BALB/c mice ; HCT8 , HT29 cell	He Y et al. discovered that butyrate reverses ferroptosis resistance in CRC by inducing c-Fos-dependent suppression of xCT; Zeng K et al. discovered that CDK1 inhibitors may be an attractive strategy for treating oxaliplatin-resistant CRC patients.	[14, 15]

Abbreviation GO: Graphene Oxide; mCRC: metastatic colorectal cancer ; CEA: carcinoembryonic antigen; FA-: folic acid-; Alb: Albumin; 5FU: 5-fluorouracil; Cur: curcumin; NIR: near-infrared; MGO: magnetic graphene oxide; PEG: polyethylene glycol; CET: cetuximab; DOX: doxorubicin; ROS: reactive oxygen species; CAPG: Capping Actin Protein; FOXA2: forkhead box transcription factor A2; IDA: trans-3-indoleacrylic acid; CDK1: cyclin-dependent kinase 1.

Finally, modifications and improvements have been made to other issues in the manuscript, including language, updating references, and addressing other details.

Reference

[1] CHEN H, ZHANG S, HSIAO Y-C, et al. Graphene Oxide and Fluorescent-Aptamer-Based Novel Aptasensors for Detection of Metastatic Colorectal Cancer Cells [J]. Polymers, 2022, 14(15).

[2] TAO C, ROUHI J. A biosensor based on graphene oxide nanocomposite for determination of carcinoembryonic antigen in colorectal cancer biomarker [J]. Environmental Research, 2023, 238(Pt 1): 117113.

[3] ALUSTIZA M, RIPOLL L, CANALS A, et al. A novel non-invasive colorectal cancer diagnostic method: Volatile organic compounds as biomarkers [J]. Clin Chim Acta, 2023, 542: 117273.

[4] BARDANIA H, JAFARI F, BANESHI M, et al. Folic Acid-Functionalized Albumin/Graphene Oxide Nanocomposite to Simultaneously Deliver Curcumin and 5-Fluorouracil into Human Colorectal Cancer Cells: An In Vitro Study [J]. Biomed Res Int, 2023, 2023: 8334102.

[5] LU Y-J, LIN P-Y, HUANG P-H, et al. Magnetic Graphene Oxide for Dual Targeted Delivery of Doxorubicin and Photothermal Therapy [J]. Nanomaterials (Basel, Switzerland), 2018, 8(4).

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[8] KRASTEVA N, KEREMIDARSKA-MARKOVA M, HRISTOVA-PANUSHEVA K, et al. Aminated Graphene Oxide as a

Potential New Therapy for Colorectal Cancer [J]. Oxidative Medicine and Cellular Longevity, 2019, 2019: 3738980.

[9] LV X, HE F L, DAI Y, et al. IFNγ synergies with cold atmospheric plasma in triggering colorectal cancer cell ferroptosis via the IFNγ/IFNR2/APC/TCF4/GPX4 axis [J]. Aging (Albany NY), 2023, 15(17): 8692-711.

[10] ZHAO Y, MA R, WANG C, et al. CAPG interference induces apoptosis and ferroptosis in colorectal cancer cells through the P53 pathway [J]. Mol Cell Probes, 2023, 71: 101919.

[11] LIU X, YAN C, CHANG C, et al. FOXA2 Suppression by TRIM36 Exerts Anti-Tumor Role in Colorectal Cancer Via Inducing NRF2/GPX4-Regulated Ferroptosis [J]. Adv Sci (Weinh), 2023: e2304521.

[12] LEI S, CHEN C, HAN F, et al. AMER1 deficiency promotes the distant metastasis of colorectal cancer by inhibiting SLC7A11- and FTL-mediated ferroptosis [J]. Cell Rep, 2023, 42(9): 113110.

[13] CUI W, GUO M, LIU D, et al. Gut microbial metabolite facilitates colorectal cancer development via ferroptosis inhibition [J]. Nat Cell Biol, 2024, 26(1): 124-37.

[14] HE Y, LING Y, ZHANG Z, et al. Butyrate reverses ferroptosis resistance in colorectal cancer by inducing c-Fos-dependent xCT suppression [J]. Redox Biol, 2023, 65: 102822.

[15] ZENG K, LI W, WANG Y, et al. Inhibition of CDK1 Overcomes Oxaliplatin Resistance by Regulating ACSL4-mediated Ferroptosis in Colorectal Cancer [J]. Adv Sci (Weinh), 2023, 10(25): e2301088.

Changes in the text: see Page 12-14,16-17, line 363-372, 437-463.

Reviewer B

Comment 1: *The topic of the review is novel and relevant in the context of colorectal cancer diagnosis and treatment. Interesting ideas are expressed in the length of the manuscript, but more depth into them is necessary.*

Throughout several sections of the review, there was a notable lack of coherence as information from various papers appeared disjointed and insufficiently integrated. Additionally, the summary statements at the end of these sections were overly generalized, failing to provide a focused synthesis of the discussed literature. For example, in the section Applications of Ferroptosis in CRC Research:

Huang Y et al. demonstrated that inhibition of Nrf2 enhances the sensitivity of CRC chemotherapy by promoting ferroptosis and apoptosis [82]. He Y et al. found in a CRC mouse model that butyrate induces xCT inhibition dependent on c-fos, reversing the upper ferroptosis in CRC [83]. Zeng K et al. showed that CDK1 induces resistance to oxaliplatin by inhibiting ferroptosis, suggesting that CDK1 inhibitors might be an attractive strategy for treating patients with oxaliplatin-resistant CRC [84].

1a) How is this all connected? ;1b) Does butyrate come from the diet? How is it related to CRC treatment and ferroptosis? ; 1c) What is CDK1?; 1d) And oxaliplatin, what kind of chemotherapeutic drug is it? Are these studies in mice or human studies?

Reply 1: Firstly, we gratefully thank you for the precious time you spent making constructive remarks.

1a) All of these documents illustrate the application of ferroptosis mechanisms in sensitizing or overcoming resistance to traditional therapies in the treatment of CRC.

1b) Based on the description in the "Material and Methods" section of the paper by He Y et al: butyrate (100 mM in drinking water), or butyrate plus erastin ((30 mg/kg weight, i.p., every three or four days)).

CRC exhibits relative insensitivity to ferroptosis. However, supplementation with butyrate sensitized CRC mice to ferroptosis induction, demonstrating significant translatability in vivo. Therefore, butyrate supplementation represents a promising approach to overcoming ferroptosis resistance in CRC.

1c) Thank you for pointing out that the abbreviation "CDK1" was not accompanied by its full name upon its first appearance in the manuscript (this has been corrected in the manuscript). CDK1, also known as Cyclin-dependent kinase 1, is a highly conserved Ser/Thr protein kinase that plays a vital role in cell cycle progression. In the study by Zeng K et al., CDK1 was identified as a critical contributor to oxaliplatin resistance through in vitro and in vivo CRISPR/Cas9 screening.

Oxaliplatin is a third-generation platinum-based antitumor drug commonly used in oxaliplatin-based chemotherapy, primarily as the first-line treatment for advanced CRC and in adjuvant therapy following complete resection of the primary tumor. However, its therapeutic efficacy is often limited by frequent drug resistance in patients.

1d) Huang Y et al. conducted their study using in vitro CRC cell lines (HCT-116 and LOVO cells) as well as an in vivo HCT116 xenograft mouse model. He Y et al. utilized CRC mouse models (xenograft model and azoxymethane/dextran sodium sulfate (AOM/DSS) model). Zeng K et al. employed in vitro CRC cell lines (HCT8 and HT29 cells). Overall, these studies utilized CRC cell lines and/or CRC mouse models, without further application in human subjects.

Changes in the text: see Page 12, line 340-343, 347.

Comment 2: - *Expand this: "Additionally, GO-based nanocomposites, such as silver-GO and Cu2O-GO nanocomposites, exhibit significant anticancer effects on cancer cells under light irradiation, showcasing the tremendous potential of GO as a phototherapeutic agent in cancer treatment." How? (line 129)*

Reply 2: Thank you for pointing out the issue. We apologize for not mentioning in the manuscript how the GO-based nanocomposite material demonstrated significant anticancer activity against cancer cells under light irradiation. And we have made the following supplement in the manuscript.

For instance, Hou C et al. developed a water-dispersible Cu2O–reduced graphene oxide (CRGO). The study demonstrated CRGO's selective antitumor activity under visible light using an MTT assay on HK-2, MDA-MB-231 (from human breast adenocarcinoma), and A549 cells. Notably, HK-2 cells showed nearly 100% viability even at high CRGO doses (640 ug/mL) during the first hour of irradiation, whereas cancer cells (MDA-MB-231 and A549) experienced significant viability reduction at much lower CRGO concentrations (40 ug/mL). These findings

underscore CRGO's capacity for selective cancer cell destruction under visible light exposure, with control groups maintaining near 100% viability, confirming that the observed anticancer effects were directly attributable to CRGO's light-induced activity.

Changes in the text: see Page 6, line 174-186.

Comment 3: - *"Curcumin encapsulated in GO-based nanoparticles and functionalized with folic acid enhances specific targeting to cancer cells, utilizing the high glutathione concentration inside tumors to induce drug release." (line 147) What kind of tumors? What kind of study is this? Samples type?*

Reply 3: Thank you very much for your comments. The cell lines used in this study are Human Umbilical Vein Endothelial Cells (HUVEC) and Human colorectal cancer (HT29).

In this study, to augment the effects of 5-fluorouracil (5FU) and curcumin (Cur) on colon cancer, authors developed a folic acid- (FA-) modified co-delivery carrier based on graphene nanoparticles (GO-Alb-Cur-FA-5FU). The results demonstrated that folic acid-modified graphene oxide nanoparticles containing Cur and 5FU have delivered drugs to HT-29 cells in comparison with normal cells and may have a high capability to deliver drugs to HT-29 cells. Therefore, the surface modification of graphene oxide nanoparticles with folic acid could increase its capability and selectivity for active targeted delivery.

Changes in the text: see Page 7, line 207.

Comment 4: *-"Additionally, the combination of GO with anticancer drugs like doxorubicin shows significant antitumor activity by inducing apoptosis in CRC cells [50]"*

4a) How does this happen? Is there any hint in the mechanism?

4b) Also, expand on how GO-modified silver nanomaterials enhance the radiotherapy effect on CRC [50].

Reply 4:

4a) Thank you indeed for your comments. According to the Methods section of the manuscript by Banoon SR et al., different concentrations of graphene oxide (GO), doxorubicin (DOX), and graphene oxide plus doxorubicin (GO-DOX) were prepared. The viability of cells was assessed using the MTT test, and flow cytometry was performed after exposure to DOX, GO, and GO-DOX. The expressions of caspase 3, Bax, and ATG5 autophagy-related genes were analyzed using the RT-qPCR technique. The results showed an increase in apoptosis and autophagy following incubation with GO-DOX in HCT-116 cells. Furthermore, comparison of the DOX, GO, and GO-DOX groups with controls revealed that GO-DOX exerted a significantly higher inhibitory effect against cancer cells.

4b) First, I need to clarify that due to my oversight, the silver nanoparticles modified with graphene were mistakenly described as being modified with graphene oxide in our manuscript (the related content has been deleted from the manuscript). Here's how silver nanoparticles modified with graphene enhance the effects of radiotherapy for colorectal cancer (CRC): Metal nanoparticles have significant interaction cross-sections with electromagnetic waves due to their large surface area-to-volume ratio, which can be exploited in

cancer radiotherapy to locally enhance the radiation dose deposition in tumors. Authors developed a new type of silver nanoparticle composite, PEGylated graphene quantum dot (GQD)-decorated Silver Nanoprisms (pGAgNPs), that show excellent in vitro intracellular uptake and radiosensitization in radiation-sensitive HCT116 and relatively radiation-resistant HT29 colorectal cancer cells. Furthermore, following biodistribution analysis of intravenously injected nanoparticles in nude mice bearing HCT116 tumors radiosensitization was evaluated. Treatment with nanoparticles and a single radiation dose of 10 Gy significantly reduces the growth of colorectal tumors and increases the survival time as compared to treatment with radiation only.

Changes in the text: see Page 9, line 254-258.

Comment 5: - *"Jiang et al. also reported inhibition of ferroptosis as a mechanism of resistance to programmed cell death 1 (PD-1)/programmed death-ligand 1 (PD-L1) therapy [69]. I " Expand on this mentioning that these researchers found that cancer cells resistant to PD-1/PD-L1 therapy exhibited increased resistance to ferroptosis. Was this resistance associated with alterations in cellular metabolism and redox balance, leading to reduced levels of lipid peroxides and decreased susceptibility to ferroptotic cell death?*

Reply 5: The authors treated parental 4T1 (4T1-P) or Tyro3-overexpressing (Tyro3-OE) tumor-bearing mice with anti-PD-1 therapy and analyzed the treatment's effect on lipid peroxidation (a functional marker of ferroptosis). Through anti-PD-1 therapy, Tyro3-OE $CD45^-$ tumor cells exhibited lower levels of lipid ROS compared to

4T1-P CD45− tumor cells. Adding anti-PD-1 significantly increased the lipid ROS levels in 4T1-P, but not in Tyro3-OE cells. Furthermore, the authors treated 4T1-P and Tyro3-OE 4T1 cells in vitro with the ferroptosis inducer erastin and the ferroptosis inhibitor ferrostatin-1 (Fer-1). Compared to the parent cells, Tyro3 overexpression inhibited erastin-induced lipid ROS, and when Fer-1 blocked ferroptosis, the reduction in cell death and lipid ROS mediated by Tyro3 overexpression was not observed. The authors further confirmed the reduction of lipid peroxidation in Tyro3-OE tumor cells using malondialdehyde (MDA) assay. The PI3K/AKT signaling pathway, located downstream of TAM kinases, can increase NRF2 transcriptional activity. The authors found that TYRO3-OE-mediated NRF2 transcriptional activation was abolished by the AKT inhibitor MK2206 or by a dominant-negative AKT mutant plasmid. Moreover, in the presence of MK2206, the reduction in lipid peroxidation mediated by TYRO3 was no longer observed. These results suggest that TYRO3 inhibits ferroptosis through the AKT/NRF2 axis.

Changes in the text: see Page 10-11, line 308-312.

Comment 6: *-Define IKE (line 222)*

Reply 6: We appreciate your attention to the fact that the abbreviation "IKE" was introduced without its full designation initially in our document. This oversight has been addressed in the revised manuscript. Specifically, "IKE" stands for imidazole ketone erastin, which is an analog of the ferroptosis inducer, erastin.

Changes in the text: see Page 10, line 303-304.

Comment 7: *-You mentioned that the review will cover the physicochemical properties of GO, the mechanisms of its integration with ferroptosis, and the challenges and opportunities of novel nanomaterials like GO. Not much information about the challenges is present in the text.*

Reply 7: Thank you for your feedback. We acknowledge the need for more detailed information regarding the challenges associated with GO in the review. And we have supplemented and improved this section in the manuscript.

The clinical application of GO still faces challenges such as its in vitro cytotoxicity, in vitro and in vivo toxicity in mammals, poor solubility in aqueous solutions, and the development of new methods for large-scale synthesis of GO. For instance, Das et al. treated human umbilical vein endothelial cells (HUVEC) with GO and rGO of the same size, finding that GO exhibited greater toxicity than rGO and caused severe DNA damage and a significant increase in intracellular ROS [1]. Guo, Qing et al. discovered that exposure to graphene oxide led to significant weight loss, developmental delays, reduced mobility, and shortened lifespan in w1118 fruit flies. Further research by them suggested that this toxic effect might be related to severe damage to the fruit fly's intestine, primarily due to oxidative stress triggered by excessive accumulation of ROS [2]. Rhazouani, Asmaa et al. assessed the toxicity of graphene oxide in male mice through intraperitoneal injection at different doses (2 mg/kg and 5 mg/kg) over five days. While behavioral tests in mice showed no significant abnormalities, histopathological analysis of liver sections indicated that graphene oxide caused liver inflammation [3]. Additionally, Liao KH et al. studied the cytotoxicity of graphene and

graphene oxide (350 nm) on the normal components of human red blood cells, finding that severe hemolysis was the result of strong electrostatic interactions between the red blood cell membrane lipid bilayer and the graphene surface, leading to membrane disruption [4]. These findings underscore the need for further research to address issues related to the toxicity and biocompatibility of GO before its clinical translation can be advanced.

Reference

[1] DAS S, SINGH S, SINGH V, et al. Oxygenated Functional Group Density on Graphene Oxide: Its Effect on Cell Toxicity [J]. Particle & Particle Systems Characterization, 2013, 30.

[2] GUO Q, YANG Y, ZHAO L, et al. Graphene oxide toxicity in W1118 flies [J]. Sci Total Environ, 2022, 805: 150302.

[3] RHAZOUANI A, GAMRANI H, ED-DAY S, et al. Sub-acute toxicity of graphene oxide (GO) nanoparticles in male mice after intraperitoneal injection: Behavioral study and histopathological evaluation [J]. Food and Chemical Toxicology : an International Journal Published For the British Industrial Biological Research Association, 2023, 171: 113553.

[4] LIAO K-H, LIN Y-S, MACOSKO C W, et al. Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts [J]. ACS Appl Mater Interfaces, 2011, 3(7): 2607-15.

Changes in the text: see Page 15, line 386-408.

Comment 8: *-A table to summarize the main ways that graphene oxide and ferroptosis can be used in the diagnosis and treatment of cancer is*

needed, categorized by type of study, types of samples used, and main findings with references. Also, your review could benefit from having a figure that shows the mechanisms of Ferroptosis in CRC.

Reply 8: Thank you for your thorough review and constructive suggestion, which is very helpful for improving our article. We have taken your advice into consideration and prepared a table that summarizes the main applications of graphene oxide and ferroptosis in cancer diagnosis and treatment. The table is categorized by the type of study, the types of samples used, the main findings, and the respective references. Please see the table below for details:

Table. Graphene Oxide and Ferroptosis in CRC Diagnosis and Treatment

Type of Study	Types of Samples Used	Main Findings	Reference(s)
GO and Early Diagnosis	LoVo, HCT116 cell and LoVo cells in human; human blood; human fecal	Chen H et al. developed a GO-based fluorescent aptasensor that provides a simple, one-step, and highly sensitive approach for the detection of mCRC cells; developed a new biosensor based on a GO nanocomposite for the precise measurement of CEA in CRC biomarker detection; Alustiza M et al. developed a novel non-invasive diagnostic method for CRC using magnetic GO to extract volatile organic compounds from feces, serving as a potential screening technique for CRC and precancerous lesions	[1-3]
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GO and Photother	KM12C cell	He S et al. developed a two-dimensional graphene oxide-template gold nanosheet (GO@SiO_2@AuNS)	[6]

Type of Study	Types of Samples Used	Main Findings	Reference(s)
apy		hybrid, which effectively killed colorectal cancer cells (KM12C) under NIR irradiation.	
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Ferroptosis Triggers Tumor Cell Death	colorectal cancer stem cell; HCT116 cell and BALB/c mice; HCT116, SW480 cell	LV X and others found that IFNγ is a key cytokine capable of blocking intestinal stem cells, and they verified its role in killing colorectal cancer stem cells through triggering GPX4-dependent ferroptosis; ZHAO Y et al. discovered that both in vitro and in vivo, downregulation of CAPG can significantly inhibit CRC cells. Interfering with CAPG can block the cell cycle at the G1 phase and induce apoptosis and ferroptosis in CRC cells by upregulating the P53 pathway; LIU X et al. discovered that TRIM36-mediated FOXA2 promotes the progression of CRC by inhibiting the Nrf2/GPX4 ferroptosis signaling pathway.	[9-11]
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abbreviation GO: Graphene Oxide; mCRC: metastatic colorectal cancer ; CEA: carcinoembryonic antigen; FA-: folic acid-; Alb: Albumin; 5FU: 5-fluorouracil; Cur: curcumin; NIR: near-infrared; MGO: magnetic graphene oxide; PEG: polyethylene glycol; CET: cetuximab; DOX: doxorubicin; ROS: reactive oxygen species; CAPG: Capping Actin

Protein; FOXA2: forkhead box transcription factor A2; IDA: trans-3-indoleacrylic acid; CDK1: cyclin-dependent kinase 1.

Reference

[1] CHEN H, ZHANG S, HSIAO Y-C, et al. Graphene Oxide and Fluorescent-Aptamer-Based Novel Aptasensors for Detection of Metastatic Colorectal Cancer Cells [J]. Polymers, 2022, 14(15).

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[3] ALUSTIZA M, RIPOLL L, CANALS A, et al. A novel non-invasive colorectal cancer diagnostic method: Volatile organic compounds as biomarkers [J]. Clin Chim Acta, 2023, 542: 117273.

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[5] LU Y-J, LIN P-Y, HUANG P-H, et al. Magnetic Graphene Oxide for Dual Targeted Delivery of Doxorubicin and Photothermal Therapy [J]. Nanomaterials (Basel, Switzerland), 2018, 8(4).

[6] HE S, LI J, CHEN M, et al. Graphene Oxide-Template Gold Nanosheets as Highly Efficient Near-Infrared Hyperthermia Agents for Cancer Therapy [J]. International Journal of Nanomedicine, 2020, 15: 8451-63.

[7] SHEN J, DONG J, SHAO F, et al. Graphene oxide induces autophagy and apoptosis via the ROS-dependent AMPK/mTOR/ULK-1 pathway in colorectal cancer cells [J]. Nanomedicine (Lond), 2022, 17(9): 591-605.

[8] KRASTEVA N, KEREMIDARSKA-MARKOVA M, HRISTOVA-PANUSHEVA K, et al. Aminated Graphene Oxide as a Potential New Therapy for Colorectal Cancer [J]. Oxidative Medicine and Cellular Longevity, 2019, 2019: 3738980.

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[10] ZHAO Y, MA R, WANG C, et al. CAPG interference induces apoptosis and ferroptosis in colorectal cancer cells through the P53 pathway [J]. Mol Cell Probes, 2023, 71: 101919.

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[13] CUI W, GUO M, LIU D, et al. Gut microbial metabolite facilitates colorectal cancer development via ferroptosis inhibition [J]. Nat Cell Biol, 2024, 26(1): 124-37.

[14] HE Y, LING Y, ZHANG Z, et al. Butyrate reverses ferroptosis resistance in colorectal cancer by inducing c-Fos-dependent xCT suppression [J]. Redox Biol, 2023, 65: 102822.

[15] ZENG K, LI W, WANG Y, et al. Inhibition of CDK1 Overcomes Oxaliplatin Resistance by Regulating ACSL4-mediated Ferroptosis in Colorectal Cancer [J]. Adv Sci (Weinh), 2023, 10(25): e2301088.

Changes in the text: see Page 12-14, line 363-372.

Reviewer C

Comment 1: We have completed the peer review process, and I regret to inform you that we are unable to accept your manuscript for publication in its current form.

Limited Novelty: While your manuscript addresses an important topic, it primarily summarizes existing literature without providing significant novel insights or perspectives. To enhance the originality of your manuscript, consider incorporating more critical analysis and synthesizing the literature to identify gaps and opportunities for future research.

Reply 1: Thank you for your comprehensive review and careful consideration of our manuscript. We acknowledge and agree with your suggestion that more novel insights or perspectives are needed. In response to your feedback, we have made revisions to incorporate more critical analysis and literature synthesis, along with relevant summarization. Please refer to the "Conclusions and Future Prospects"

section of our manuscript, where we have elaborated on the challenges faced by graphene oxide (GO) in clinical applications, such as its cytotoxicity in vitro and its toxicity both in vivo and in vitro in mammals. We emphasize that further research is necessary to address the issues related to GO's toxicity and biocompatibility before its clinical translation can be advanced. Additionally, I have deepened the manuscript by exploring the correlation between GO and the mechanisms of iron-deficiency anemia, providing insights into the potential future directions for GO and iron-deficiency anemia in the field of cancer diagnosis and treatment. The details are as follows:

Changes in the text: see Page 15-17, line 383-408, 418-436, 438-463.

Comment 2: *Incomplete Coverage: The abstract suggests a comprehensive review of the potential applications of ferroptosis and Graphene Oxide (GO) in colorectal cancer (CRC) therapy. However, it lacks specific details on the methodologies employed in the literature search and the criteria for selecting studies for inclusion. Providing transparency in these aspects will strengthen the credibility of your review.*

Reply 2: Thank you for your valuable feedback and for highlighting the need for transparency in our review process. We acknowledge the importance of providing specific details on the methodologies employed in the literature search and the criteria for selecting studies for inclusion.

Partial literature search strategies are as follows:

Search: (Graphene[Title/Abstract] OR Graphene Oxide[Title/Abstract]) AND (Colorectal Neoplasm[Title/Abstract] OR Colorectal Cancer[Title/Abstract] OR Colorectal Tumor[Title/Abstract] OR Colorectal Carcinoma[Title/Abstract])

Search: (Ferroptosis[All Fields]) AND (Colorectal Neoplasm[All Fields] OR Colorectal Cancer[All Fields] OR Colorectal Tumor[All Fields] OR Colorectal Carcinoma[All Fields])

Search: (Graphene[All Fields] OR Graphene oxide[All Fields]) AND (Ferroptosis[All Fields])

Search: (Colorectal Neoplasm[Title] OR Colorectal Cancer[Title] OR Colorectal Tumor[Title] OR Colorectal Carcinoma[Title]) AND (Chemotherapy[Title] OR Radiotherapy[Title] OR Photothermal Therapy[Title] OR Gene Therapy[Title])

Inclusion criteria for the study: Research that is directly related to the review topic; when involving the latest research progress or technologies, recent literature should be cited to ensure the timeliness of the information. At the same time, classic or fundamental studies should also be considered; studies in languages other than English are excluded.

Changes in the text: see Page 3, line91.

Comment 3: *Limited Scope: The review focuses primarily on the potential applications of ferroptosis and GO in colorectal cancer therapy. However, it does not sufficiently explore the correlations between these*

two topics or provide a comprehensive analysis of how they could be integrated synergistically for enhanced therapeutic outcomes.

Reply 3: Firstly, thank you for your valuable feedback on our review. We appreciate your insight regarding the limited scope of our discussion. We acknowledge the importance of exploring the correlations between ferroptosis and GO and providing a comprehensive analysis of their synergistic integration for enhanced therapeutic outcomes. We have made the following modifications and additions to the Discussion section at the end of the manuscript.

Despite the need to overcome numerous challenges, the rapid development of nanomaterial science, particularly the advancements in graphene oxide and its substrates, has turned many impossibilities into possibilities, especially in cancer diagnosis and treatment. Furthermore, it's worth mentioning that combining GO with ferroptosis-inducing therapies presents an intriguing path that remains largely unexplored. In the future, GO could be further modified to directly influence the proliferation, migration, and invasion of tumor cells, such as those in CRC, through mechanisms like ferroptosis. Additionally, research into GO's drug-carrying and releasing capabilities could be expanded to include ferroptosis inducers, utilizing GO's delivery potential to enhance ferroptosis induction in cancer cells. Moreover, addressing the toxicity of GO and improving its biocompatibility is crucial for its effective integration into cancer therapy. In summary, while GO and the ferroptosis mechanism offer significant opportunities to advance cancer treatment, their integration necessitates a thorough understanding of their interactions, meticulous management of potential toxicities, and innovative strategies to leverage their combined potential to improve

cancer treatment outcomes. This area warrants further research and exploration by the scientific community.

Changes in the text: see Page 17, line 445-463.

Comment 4: *Insufficient Focus on Clinical Translation: Your manuscript briefly touches upon the clinical implications of ferroptosis and GO in CRC therapy, but it would benefit from a more in-depth discussion on the challenges and opportunities for translating these findings into clinical practice.*

We appreciate the effort you have put into this manuscript and encourage you to address the aforementioned concerns in a revised version. Please ensure that the revised manuscript emphasizes originality, critical analysis, and clinical relevance to enhance its suitability for publication in our journal.

Reply 4: Thank you for your valuable feedback on our manuscript. We acknowledge the need for a more detailed exploration of the clinical translation of ferroptosis and GO in CRC therapy. In the revised version, we have expanded on the challenges and opportunities in translating these findings into clinical practice, ensuring a thorough analysis of their clinical relevance. We aim to enhance the originality and critical analysis in the manuscript to meet the publication standards. The specific modifications are as follows:

The clinical application of GO still faces challenges such as its in vitro cytotoxicity, in vitro and in vivo toxicity in mammals, poor solubility in aqueous solutions, and the development of new methods for large-scale synthesis of GO. For instance, Das et al. treated human umbilical vein

endothelial cells (HUVEC) with GO and rGO of the same size, finding that GO exhibited greater toxicity than rGO and caused severe DNA damage and a significant increase in intracellular ROS [1]. Guo, Qing et al. discovered that exposure to graphene oxide led to significant weight loss, developmental delays, reduced mobility, and shortened lifespan in w1118 fruit flies. Further research by them suggested that this toxic effect might be related to severe damage to the fruit fly's intestine, primarily due to oxidative stress triggered by excessive accumulation of ROS [2]. Rhazouani, Asmaa et al. assessed the toxicity of graphene oxide in male mice through intraperitoneal injection at different doses (2 mg/kg and 5 mg/kg) over five days. While behavioral tests in mice showed no significant abnormalities, histopathological analysis of liver sections indicated that graphene oxide caused liver inflammation [3]. Additionally, Liao KH et al. studied the cytotoxicity of graphene and graphene oxide (350 nm) on the normal components of human red blood cells, finding that severe hemolysis was the result of strong electrostatic interactions between the red blood cell membrane lipid bilayer and the graphene surface, leading to membrane disruption [4]. These findings underscore the need for further research to address issues related to the toxicity and biocompatibility of GO before its clinical translation can be advanced.

Additionally, we have added a table to summarize the main applications of oxidized graphene and ferroptosis in cancer diagnosis and treatment, categorized by type of study, type of sample used, and the main findings of the referenced literature.

Reference

[1] DAS S, SINGH S, SINGH V, et al. Oxygenated Functional Group Density on Graphene Oxide: Its Effect on Cell Toxicity [J]. Particle & Particle Systems Characterization, 2013, 30.

[2] GUO Q, YANG Y, ZHAO L, et al. Graphene oxide toxicity in W1118 flies [J]. Sci Total Environ, 2022, 805: 150302.

[3] RHAZOUANI A, GAMRANI H, ED-DAY S, et al. Sub-acute toxicity of graphene oxide (GO) nanoparticles in male mice after intraperitoneal injection: Behavioral study and histopathological evaluation [J]. Food and Chemical Toxicology : an International Journal Published For the British Industrial Biological Research Association, 2023, 171: 113553.

[4] LIAO K-H, LIN Y-S, MACOSKO C W, et al. Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts [J]. ACS Appl Mater Interfaces, 2011, 3(7): 2607-15.

Changes in the text: see Page 12-15, line 363-372, 386-408.