

Research Article

How to cite this article:

Kabiri F, Foroutan T, Pashaei-Asl R, Pashaei-Asl M, Ganjian M, Vatanpour V. Magnetic graphene oxide improves the expression of some human cumulus cells biomarkers. *Advanced Pharmaceutical Bulletin*, doi: 10.34172/apb.47289

Magnetic graphene oxide improves the expression of some human cumulus cells biomarkers

Fahimeh Kabiri ¹, Tahereh Foroutan ^{1*}, Roghiyeh Pashaei-Asl ^{2,3}, Maryam Pashaei-Asl ^{4,5*}, Mohammad Ganjian ⁶, Vahid Vatanpour ⁷

¹ Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran.

² Interdisciplinary Research Development Center, Iran University of Medical Sciences, Tehran, Iran.

³ Department of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran.

⁴ Department of Reproductive Biology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran.

⁵ Women's Reproductive Health Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.

⁶ Department of Electronic Technology, TU Ilmenau, Ilmenau, Germany

⁷ Faculty of Chemistry, Kharazmi University, Tehran, Iran.

ARTICLE INFO

Keywords:

Cumulus cells,
Magnetic graphene oxide,
Apoptosis,
Antioxidant

Article History:

Submitted: March 19, 2026

Revised: July 18, 2026

Accepted: August 14, 2026

ePublished: September 26, 2026

ABSTRACT

Purpose: Developing non-invasive, accurate methods for selecting high-implantation embryos is crucial for assisted reproductive technologies (ART). Cumulus cells (CCs) are reliable indicators of oocyte quality. This study investigated the effects of magnetic graphene oxide (MGO) on the viability, apoptosis, oxidative status, and proliferation-related proteins of human CCs in vitro.

Methods: Human CCs were treated with MGO (1.5–400 µg/mL) for 24, 48, and 72 hours. Viability was assessed via MTT and Annexin V-FITC/PI flow cytometry. Expression of apoptosis-related (Bcl-2, Bax, cleaved/pro-caspase-3), antioxidant (Nrf2, SOD2), and cell-cycle (cyclin D1) proteins was analyzed using Western blotting. Additionally, Catalase (CAT) activity, Reduced glutathione (GSH), and total antioxidant capacity (TAC) were determined using colorimetric assays.

Results: MGO showed dose- and time-dependent effects, with low concentrations showing high biocompatibility at 48 hours. Flow cytometry revealed MGO significantly increased cell viability from 88.06±1.7% (control) to 94.58±2.88%, 92.87±0.43%, 97.09±2.69%, and 97.61±1.72% at 3.125, 12.5, 50, and 100 µg/mL, respectively ($p < 0.0001$), while total apoptosis decreased from 11.05±0.55% to 4.96±0.27%, 6.74±0.46%, 2.50±0.21%, and 2.16±0.10% ($p < 0.0001$). MGO upregulated Bcl-2 ($p < 0.001$), Nrf2 (>2-fold, $p < 0.0001$), SOD2 (>2.5-fold, $p < 0.0001$), and cyclin D1 (1.5-fold, $p < 0.01$), but downregulated Bax ($p < 0.05$) and cleaved caspase-3 ($p < 0.0001$). CAT, GSH, and TAC were significantly elevated ($p < 0.001$).

Conclusion: Low concentrations of MGO enhanced human CC viability, reduced apoptosis, and boosted antioxidant defenses and proliferation-related pathways. These findings suggest MGO's biomedical potential in cell survival and anti-apoptotic effects on reproductive medicine research. However, more comprehensive studies are needed to determine its long-term safety and efficacy.

*Corresponding Authors

Tahereh Foroutan, Email: foroutan@khu.ac.ir, ORCID: 0000-0002-1476-3928

Maryam Pashaei-Asl, Email: pashaimaryam@yahoo.com, ORCID: 0000-0002-4490-6627

1. Introduction

The use of assisted reproductive technologies (ART) has been introduced as an effective approach to the management of infertility. Currently, more than 4% of births are achieved through ART methods, but the overall success rate remains limited, with only about 35% of ART cycles resulting in live births.¹⁻³ Today, in addition to ART, various technologies such as stem cell therapy, regenerative medicine, the use of nanomaterials, and blastocyst artificial collapse prior to vitrification are used to enhance fertility.⁴⁻⁶ Single embryo transfer has become the preferred strategy in in vitro fertilization (IVF) programs to reduce the incidence of multiple pregnancies and associated risks such as preterm birth and their associated complications. Therefore, the accurate identification of embryos with high implantation potential is a major challenge in reproductive medicine. Traditionally, oocyte quality has been assessed based on morphological criteria, including the appearance of the cytoplasm, polar bodies, zona pellucida thickness, and meiotic spindle position.⁷ However, the low accuracy of these methods is considered to be one of their limiting factors.^{8,9} The bidirectional communication between oocytes and their surrounding cumulus cells plays a fundamental role in oocyte maturation and fertilization, as well as subsequent embryonic development.^{10,11} Cumulus cells accomplish this by secreting paracrine signals and regulatory molecules, thereby facilitating meiotic progression and influencing the organization of the cytoskeleton within the oocyte.¹¹ Recent research, emphasizing non-invasive indicators of oocyte competence, has paid special attention to cumulus cells as indirect indicators of oocyte quality.¹² Dysfunction or structural changes in cumulus cells are associated with impaired oocyte maturation and reduced developmental competence.

Evidence suggests that cumulus cell apoptosis is a reliable molecular marker of oocyte quality in ART. Clinical studies have shown that cumulus cell apoptosis is lower in pregnant women than in non-pregnant women.¹³ Similarly, laboratory animal models have linked increased DNA fragmentation and apoptotic activity to reduced oocyte viability and diminished fertility potential.¹⁴ Taken together, these findings support the use of cumulus cell apoptosis as a predictive marker for implantation success.¹⁵ Furthermore, interventions aimed at reducing apoptotic activity in cumulus cells increase oocyte growth potential and improve subsequent fertilization and embryo implantation outcomes.¹⁵

Nowadays, nanomaterials science has attracted much attention with diverse applications in biomedicine, including targeted drug delivery, biosensors, tissue engineering, cancer therapy, and infertility treatment.¹⁶ Among various nanomaterials, graphene-based compounds have been widely used due to their exceptional physicochemical properties. Graphene nanosheets with high electrical and thermal conductivity, mechanical strength, optical transparency, and favorable biocompatibility have been excellent candidates for medical and biological applications.¹⁷ Some of its derivatives, such as graphene oxide (GO), have been widely investigated for their potential to differentiate stem cells, enhance synaptic connectivity in neuronal cells, and improve the mechanical properties of biological tissues.¹⁸⁻²¹ GO has been reported to exhibit less cytotoxicity than graphene due to its surface functional groups.

Magnetic nanoparticles have been widely used in biomedical research due to their magnetic response, chemical stability, and low toxicity. Their applications include magnetic resonance imaging, thermotherapy, tissue repair, biosensors, and drug delivery systems.²²⁻²⁵ Magnetic graphene oxide (MGO) is a hybrid nanomaterial that possesses properties such as high surface area, magnetic responsiveness, and tunable chemical functionality.²⁶⁻²⁸ The aforementioned properties make this compound a promising candidate for biomedical applications.

The incorporation of magnetic properties into graphene oxide (GO) nanosheets, resulting in magnetic graphene oxide (MGO), enhanced biocompatibility and high chemical stability, and also the magnetic iron oxide (Fe_3O_4) component provides the nanocomposite with superparamagnetic properties, which facilitate the non-invasive, remote manipulation of the carrier under external fields, while simultaneously minimizing the mechanical stress often induced by traditional separation methods in delicate primary cell cultures.²⁹ Furthermore, the integration of these magnetic cores allows for real-time monitoring and tracking of the nanocarriers' internalization within the cells, providing a robust platform to assess their impact on the expression of developmental competence biomarkers without compromising cellular viability.³⁰

Cumulus cells surrounding the oocyte play a vital supporting role for the oocyte until fertilization and are considered a non-invasive biomarker for assessing the quality and developmental competence of the oocyte. The aim of the present study was to investigate the possible antioxidant role of MGO and also to evaluate its effect on the viability and apoptosis of human CCs.

2. Materials and Methods

2.1. Synthesis of the MGO

In the present experimental laboratory study, MGO with an average size of 200–400 nm was produced according to our previous study (supplementary section).²¹ The GO prepared from graphite and the $Fe_3O_4@GO$ composites were obtained by co-precipitation of $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4 H_2O$ in the presence of GO. The temperature was raised to 85°C, and ammonia solution was added to increase the pH to 10. A mixture of styrene and benzoyl peroxide was added under a stream of N_2 over a period of 10 min. The final mixture was homogenized by sonication at 0°C for another 30 min. Polymerization was carried out by increasing the temperature of the mixture in an oil bath to 80°C for 4 h under an inert nitrogen blanket. GO and MGO were characterized by X-ray diffraction (XRD, Philips Xpert MPD, Co K irradiation, $\lambda = 1.78897\text{\AA}$), Raman spectroscopy, scanning electron microscopy (SEM, Philips XL30 microscope with an accelerating voltage of 25 kV), vibrating sample magnetometry (VSM), energy-dispersive X-ray spectroscopy (EDX), dynamic light scattering (DLS), Zeta potential and transmission electron microscopy (TEM) (PHILIPS, EM208S, Netherlands), at 100 kV of acceleration voltage. The synthesized MGO was then used to treat isolated human CCs.

2.2. Study Design and Participants

This experimental in vitro study involved the analysis of follicular fluid from 40 women diagnosed with male factor infertility who had been referred to Al-Zahra Hospital in Tabriz, Iran, between August 2021 and March 2023.

2.3. Inclusion Criteria

Participants included women aged 20 to 38 with male-factor infertility. The study focused on CCs from women whose oocytes showed a maturation rate of more than 80% at the metaphase II stage.

2.4. Exclusion Criteria

Women who had pre-existing medical conditions such as diabetes or cardiovascular disease were excluded from participating in the study.

2.5. Sample Size

Follicular samples were collected from several women during the puncture procedure. Approximately 20 follicles were transferred to the cell culture medium. Experimental conditions were kept constant in all study groups. The MTT assay was performed after the third passage. For flow cytometry, TUNEL staining, and Western blotting experiments, sample collection and cell culture were repeated to ensure the accuracy of the experiments.

2.6. Isolation and Culture of Human Cumulus Cells

2.6.1. Follicular Aspiration

Follicular fluid was collected from patients undergoing vaginal oocyte retrieval as part of ICSI procedures. The retrieval process was performed 36 hours after hCG injection by ultrasound guidance and intravenous sedation. Using a stereomicroscope, CCs surrounding the oocyte-cumulus complexes were mechanically dissociated and transferred to the culture medium. Only CCs from MII stage oocytes were selected for experiments.

2.6.2. Primary Culture of Cumulus Cells

The extracted CCs were washed twice in enzyme-free culture medium and centrifuged at 200 g for 10 minutes at room temperature. Viability and cell number were assessed using the trypan blue exclusion method. For subsequent experiments, the cells were cultured until the third passage.

2.7. Cell Viability Assessment Using MTT Assay

A volume of 100 μ L of a cell suspension containing 10,000 CCs in DMEM/F12 medium supplemented with 10% FBS was added to 96-well plates. After a 24-hour attachment period, MGO was added in concentrations ranging from 1.5 to 400 μ g/mL. Control wells received the medium alone. The plates were incubated at 37°C in 5% CO₂ for 24, 48, and 72 hours. After incubation, 20 μ L of MTT solution (5 mg/mL in PBS) was added to each well, and the plates were incubated for an additional 4 hours. During this period, viable cells reduced MTT to purple formazan crystals. The crystals were dissolved with 100 μ L of DMSO, and the optical density (OD) at 570 nm was measured using an ELISA reader (BioTek Instruments, Winooski, VT). Each experiment was conducted in triplicate.³¹

2.8. Detection of Cell Apoptosis via Annexin V-FITC/PI Staining

Approximately 3×10^5 CCs/well were seeded into 6-well plates for 24 hours before being treated with various MGO concentrations (3.12, 12.5, 50, and 100 μ g/mL). The control group received no treatment. After 48 hours, apoptosis was assessed using the ApoFlowEx® FITC kit (Exbio, Czech Republic). Both treated and untreated cells were trypsinized, washed with PBS, and resuspended in annexin-binding buffer. Cells were stained with 5 μ L Annexin V and 5 μ L propidium iodide and incubated in the dark at room temperature for 15 minutes. Finally, the samples were analyzed via flow cytometry.

2.9. TUNEL Assay

The terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay is designed to detect apoptotic cells undergoing extensive DNA degradation during the late stages of apoptosis. This method relies on the ability of TdT to label blunt ends of double-stranded DNA breaks independently of a template. For this purpose, an enhanced FITC TUNEL kit (Elabscience, USA) was used. Briefly, cells treated with 50 µg/ml of MGO for 48 hours and control cells were fixed with a polyformaldehyde fixative buffer at room temperature (15-25°C) for 15-60 minutes. The cells were then washed three times with PBS, each wash lasting 5 minutes. Following this, 1× Proteinase K was added and incubated at 20-37°C for 15-30 minutes. The slides were then placed in Triton X-100 on an ice bath for 2 minutes. After washing the cells three times with PBS (5 minutes each), the TdT Equilibration Buffer (5×) (E-CK-A334A) was diluted with ddH₂O to create a 1× TdT Equilibration working buffer. Then, 100 µL of the 1× TdT Equilibration working buffer was added to each slide and incubated at room temperature for 30 minutes. Subsequently, 50 µL of TdT Enzyme working solution was added to each slide and incubated at 37°C for 60 minutes in a humidified chamber. The cells were then washed three times with PBS (5 minutes each). DAPI was added and incubated at room temperature for 5 minutes, followed by four washes with PBS. Finally, the slides were examined, and images were captured using a fluorescence microscope (Olympus BX50). To quantify apoptosis, TUNEL-stained images were analyzed using ImageJ (ImageJ Software, National Institutes of Health, Bethesda, MD, USA).

2.10. Western blotting

Cumulus cells were cultured and treated with MGO at a final concentration of 50 µg/ml for 48 hours. Following treatment, cells were harvested by scraping and lysed in RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with protease and phosphatase inhibitor cocktails (Cell Signaling Technology, Danvers, MA, USA) on ice for 30 minutes. The lysates were centrifuged at 14,000 × g for 15 minutes at 4°C to collect the supernatant containing the total protein. Protein concentration was quantified using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

Western blot analysis was performed as described previously.³¹ Equal amounts of total protein (30 µg per sample) were denatured with Laemmli sample buffer and separated by SDS-PAGE using 10–12.5% polyacrylamide gels at 100 V for approximately 90 minutes. Proteins were then transferred onto 0.45 µm polyvinylidene fluoride (PVDF) membranes (Millipore Sigma, Burlington, MA, USA) using a wet transfer system (Bio-Rad Laboratories) at 100 V for 2 hours at 4°C. After transfer, the membranes were blocked with 5% non-fat dry milk (Bio-Rad Laboratories) in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature with gentle agitation to minimize non-specific binding.

The PVDF membranes were subsequently incubated overnight at 4°C with primary antibodies diluted in TBST. The antibodies used were: rabbit anti-Bcl-2 (1:1000 dilution, Cat. No. sc-492), rabbit anti-Bax (1:1000 dilution, Cat. No. sc-7480), rabbit anti-caspase-3 (1:1000 dilution, Cat. No. sc-136219), rabbit anti-Nrf-2 (1:1000 dilution, Cat. No. sc-365949), rabbit anti-SOD2 (1:1000 dilution, Cat. No. sc-137254), rabbit anti-cyclin D1 (1:1000 dilution, Cat. No. sc-8396), rabbit anti-p53 (1:1000 dilution, Cat. No. sc-126), and mouse anti-actin (1:3000 dilution, Cat. No. sc-517582) as an internal loading control. All primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Following primary antibody incubation, the membranes were washed three times with TBST for 10 minutes per wash. The blots were then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (goat anti-rabbit IgG HRP, Cat. No. sc-2054; or goat anti-mouse IgG HRP, Cat. No. sc-2005; Santa Cruz Biotechnology, Inc.) diluted 1:3000 in TBST for 1 hour at room temperature. After secondary antibody incubation, the membranes were washed again three times with TBST for 10 minutes each.

Enhanced chemiluminescence (ECL) detection reagent (Amersham, GE Healthcare, UK) was applied to the membranes for 1 minute at room temperature. Protein bands were visualized using an Amersham Imager 600 system (GE Healthcare) and exposed to chemiluminescence-sensitive film (Kodak, USA). Densitometric analysis of the bands was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The relative expression levels of target proteins were normalized to the expression of β -actin.

2.11 Colorimetric assessment of CAT, GSH, and TAC

Reduced glutathione (GSH) levels, catalase (CAT) activity, and total antioxidant capacity (TAC) were evaluated using established colorimetric methods. GSH was determined using Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid), DTNB], based on the formation of the yellow-colored 5-thio-2-nitrobenzoic acid, with absorbance measured at 412 nm. Catalase activity was measured based on the decomposition of hydrogen peroxide, and residual H_2O_2 was quantified by the ammonium molybdate method at 405 nm. TAC was assessed by the ferric reducing antioxidant power (FRAP) assay, in which antioxidants reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions, producing a colored ferrous-tripyridyltriazine complex measured at 593 nm. Cumulus cells were treated with 12.5 μ g/ml of MGO. After 48 hours the levels of CAT, GSH, and TAC were assessed using colorimetric assays.

2.11. Ethical considerations

This study received ethics approval from the Ethics Committee of Kharazmi University (Code: IR.KHU.REC.1400.006). In addition, permission to conduct the research at Al-Zahra Hospital was granted by the Ethics Committee of Tabriz University of Medical Sciences. Written informed consent was obtained from all participants for the use of CCs in this study.

2.12. Statistical analysis

Graphs and statistical analyses were performed using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). The effects of different MGO doses on cell viability across three time points were analyzed using repeated measures ANOVA. One-way ANOVA was used to examine the effects of varying MGO doses at the same time point. Differences between the control and MGO-treated groups were evaluated using Student's *t*-test. All data were presented as mean $\pm$ SEM. Statistical significance was defined as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3. Results

3. 1. Microscopic image of cultured cumulus cells

Images of CCs were taken using an inverted microscope at early stages and different passages of culture (Figure 1. A).

3. 2. Dose- and time-dependent effects of MGO on cumulus cell viability

The impact of magnetic graphene oxide (MGO) nanosheets on the viability and morphological integrity of human cumulus cells (CCs) was evaluated across various concentrations (1.5 to 400 µg/ml) and exposure durations (24, 48, and 72 hours) (Figure 1. B). The MTT assay revealed a distinct dose- and time-dependent response. After 24 hours treatment, low concentrations of MGO (specifically 1.5 µg/ml) exhibited a mild stimulatory effect on cell viability. However, a significant reduction in cell survival was observed at higher doses; viability dropped sharply to 34.76 % at 12.5 µg/ml and reached a minimum of 22.48 % at 200 µg/ml and 21.53% at 400 µg/ml compared untreated control ($p < 0.0001$). At 48 hours exposure, low doses of MGO (up to 12.5 µg/ml) maintained high biocompatibility, with cell viability remaining comparable to or slightly higher than the control group. Nevertheless, further increases in MGO concentration triggered significant cytotoxicity, with viability decreasing progressively to 49.23% at 100 µg/ml, 35.93% at 200 µg/ml and 30.08% at 400 µg/ml ($p < 0.01$, $p < 0.0001$ and $p < 0.0001$). By 72 hours, the cells demonstrated a tolerance to low-dose treatments (1.5, 3.125, ~~12.5~~ and 25 µg/ml), where viability remained above the control. Cell viability fell to 39.85% at 400 µg/ml ($p < 0.001$). Based on the MTT assay results, 48 hours was selected as the optimal treatment duration for assessing the effects of MGO in subsequent experiments.

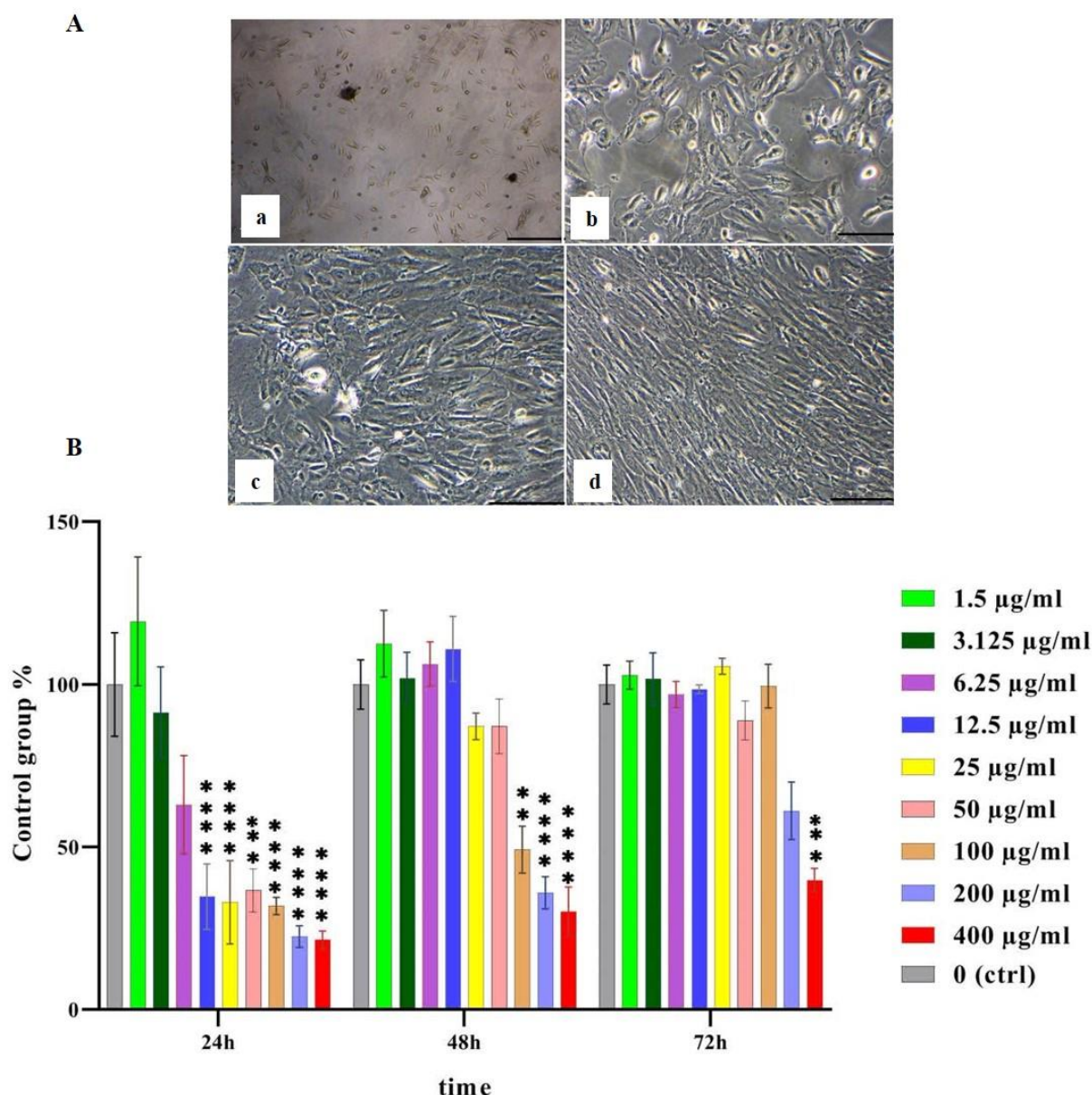


Figure 1. MTT assay: A: Light inverted microscopic images of cultured human CCs at different passages (a-d) and their viability after MGO treatment (B). (a) Primary culture, (b) first passage, (c) second passage, and (d) third passage. Scale bar: 100 μ m. B: different concentrations of MGO on human CCs viability at 24, 48, and 72 hours. Repeated measures Two-way ANOVA were performed to do statistical analysis. Data were expressed as mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001

3. 3. MGO reduces apoptosis in cumulus cells

To evaluate the effect of MGO on cell survival and death, we performed flow cytometry using Annexin V-FITC and Propidium Iodide (PI) double staining. This assay allows the distinction between viable, apoptotic, and necrotic cells based on phosphatidylserine exposure and membrane integrity. As shown in Figure 2, MGO treatment shifted the cell population toward the viable quadrant compared with the control group. According to Table 1, cell viability increased significantly after treatment with MGO at concentrations of 3.125, 12.5, 50, and 100 μ g/mL, rising from $88.06 \pm 1.10\%$ in the control group to $94.58 \pm 0.60\%$, $92.87 \pm 0.75\%$, $97.09 \pm 0.46\%$, and $97.61 \pm 0.26\%$, respectively (p < 0.0001). In parallel, total apoptosis, including both early and late stages, decreased markedly from $11.05 \pm 0.98\%$ in the control group to $4.96 \pm 0.47\%$, $6.74 \pm 0.80\%$, $2.50 \pm 0.35\%$, and $2.16 \pm 0.19\%$ in the treated groups (p < 0.0001). Necrotic cells also remained low across all MGO-treated groups

and were significantly reduced compared with the control. Overall, these findings suggest that MGO exerts a clear cytoprotective effect by enhancing cell viability and suppressing apoptosis and necrosis.

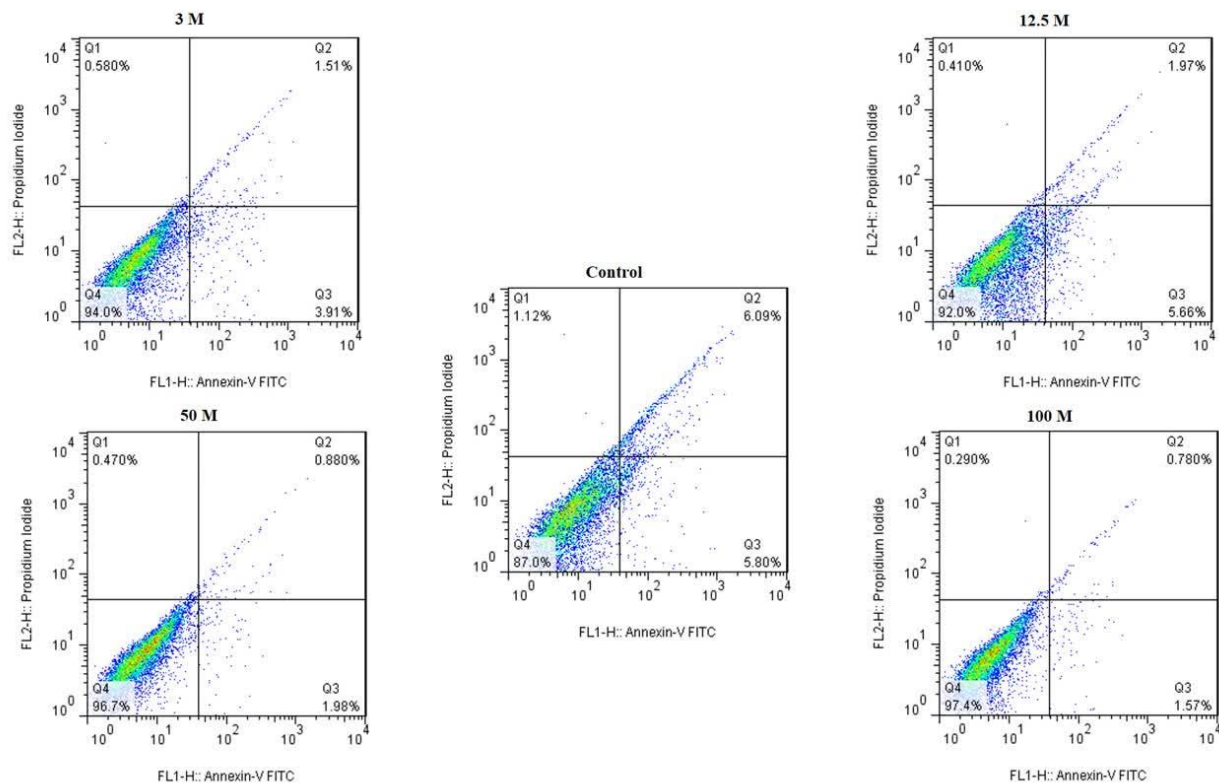


Figure 2. Flow cytometry quantitative analysis of CCs after 48 hours of treatment with MGO in different concentrations (0, 3.125, 12.5, 50, 100 µg/ml.).

Table 1: Flow cytometry results measuring the levels of apoptosis and necrosis in cumulus cells under the MGO treatment.

MGO concentration	3.125µg/ml	12.5 µg/ml	50 µg/ml	100 µg/ml	Control	P-value
Viability (%)	94.58±2.88 ****	92.87±0.43 ****	97.09±2.69 ****	97.61±1.72 ****	88.06±1.7	$p < 0.0001$
Apoptosis (%) (early + late)	4.96±0.27 ****	6.740±0.46 ****	2.50±0.21 ****	2.16±0.10 ****	11.05±0.55	$p < 0.0001$
Necrosis (%)	0.45±0.85 *	0.41±0.07 *	0.42±0.88 *	0.25±0.49 **	0.88 ± 0.24	$p < 0.05$ $p < 0.01$

Values (Mean ± SEM) are from three independent experiments (n = 3). ANOVA (one way) was used to do statically analysis. P-value indicates the level of significance between each concentration of MGO on the control group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$

3. 4. Evaluation of apoptosis by DAPI and TUNEL staining methods

DAPI is a fluorescent nuclear color that stains the cell nucleus and fragmented chromatin, allowing for the observation and examination of nuclear morphological changes can be observed and examined under a fluorescent microscope. As figure 3 illustrates, in apoptotic cells, the nucleus becomes condensed and fragmented, which can be detected by DAPI staining. DNA fragmentation is a characteristic feature of apoptosis. TUNEL is a specific staining method for apoptosis that detects DNA breaks caused by apoptotic processes. Therefore, in the present

study, DAPI and TUNEL staining were used simultaneously to demonstrate apoptosis, and images of their overlap were taken.

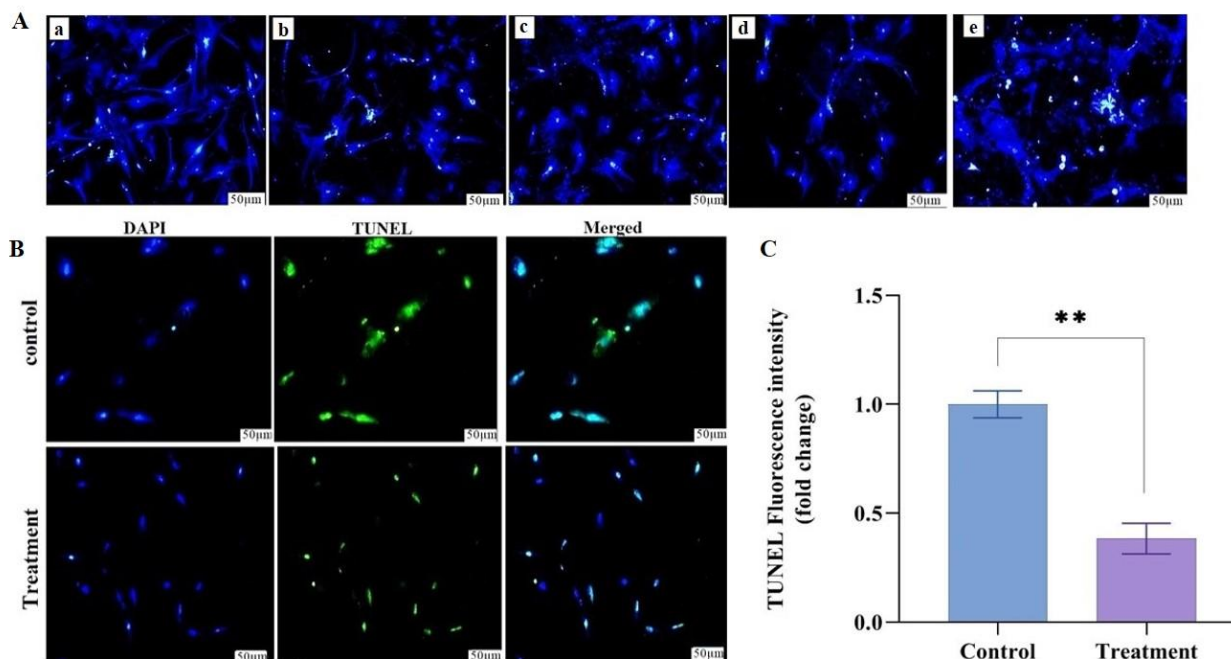


Figure 3. DAPI and TUNEL staining: (A) Effects of MGO on CCs after 48 hours with DAPI at different concentration (a-e) and (B) TUNEL staining at 50 µg/ml concentration. (C) The quantified results of the TUNEL staining. Student's t-test was used to do statically analysis. Data were expressed as mean $\pm$ SEM. ** $P < 0.01$. (A) 0.0, (B) 3.125 µg/ ml, (C) 12.5 µg/ ml, (D) 50 µg/ ml, (E) 100 µg/ ml

3. 5. Protein expression levels by Western Blotting

3. 5.1. MGO increases cell viability by reducing mitochondrial apoptosis proteins

To investigate apoptosis, the expression levels of intrinsic or mitochondrial apoptosis pathway proteins, including Bcl-2, Bax, pro-caspase-3 and cleaved caspase-3, were measured using Western blotting. The results showed that MGO reduced the level of apoptosis. The levels of Bax ($p < 0.05$) and cleaved caspase-3 ($p < 0.0001$) were significantly decreased, while the level of Bcl-2 was increased ($p < 0.001$) (Figure 4).

3.5.2. In CCs, the levels of Nrf2 and SOD-2 proteins level are increased by MGO

The results in figure 4 shows that the expression of Nrf2 and SOD2 proteins, which are components of the oxidative stress response pathway, increased significantly after treatment with 50 µg/ml MGO. As shown in Figure 4, Nrf2 and SOD2 protein levels were significantly increased by MGO by more than 2-fold and 2.5-fold, respectively, compared with the control group ($p < 0.0001$) (Figure 4).

3. 5.3. MGO increases cell proliferation by increasing the expression of cyclinD1 protein

In this study, we investigated the cyclin D1, a cell cycle regulatory protein. MGO significantly increased cyclin D1 expression by 1.5-fold compared with the control group ($p < 0.01$) (Figure 4).

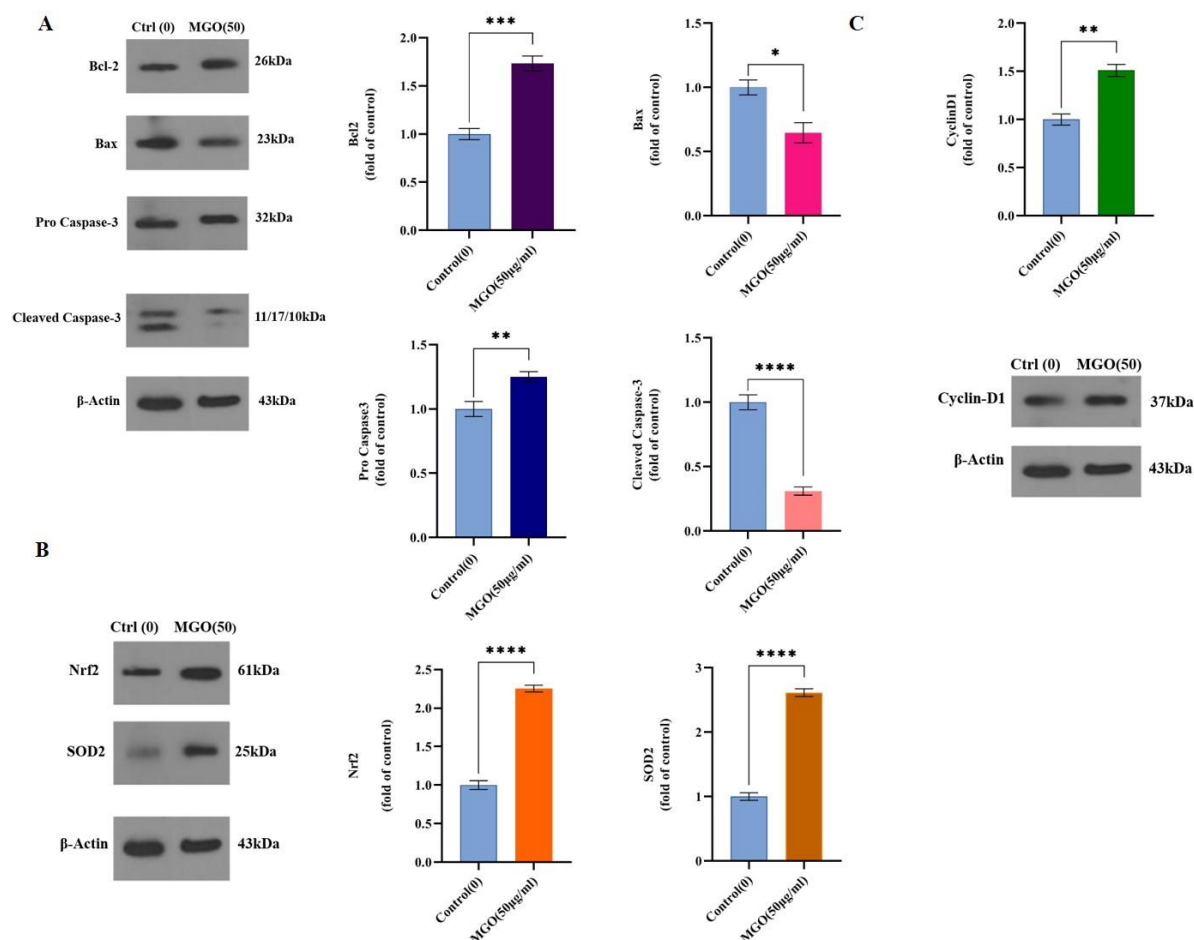


Figure 4. Western blot analysis of selected proteins. (A) Confirmation of two-dimensional gel electrophoresis results by western blotting of Bcl-2, Bax, pro-caspase-3 and cleaved caspase-3 proteins using β -actin as an internal control (left), and quantification of the western blot results (right). (B) Confirmation of two-dimensional gel electrophoresis results by western blotting of Nrf2²¹ and SOD2 using β -actin as an internal control (left), and quantification of the western blot results (right). (C) Confirmation of two-dimensional gel electrophoresis results by western blotting of cyclin D1 using β -actin as an internal control (down), and quantification of the western blot results (up). Data were expressed as mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3. 6. Colorimetric assessment of CAT, GSH, and TAC

Figure 5 shows CAT activity was significantly increased in the MGO (12.5 μ g/mL) group compared with the control group ($p < 0.001$). GSH level was also increased in the MGO-treated group ($p < 0.001$). Similarly, TAC was elevated in the MGO group ($p < 0.001$) (Figure 5). Given that the 50 μ g/ml concentration of MGO, like the 12.5 μ g/ml concentration, was determined to be non-toxic by the MTT assay, these findings are likely applicable to this concentration as well.

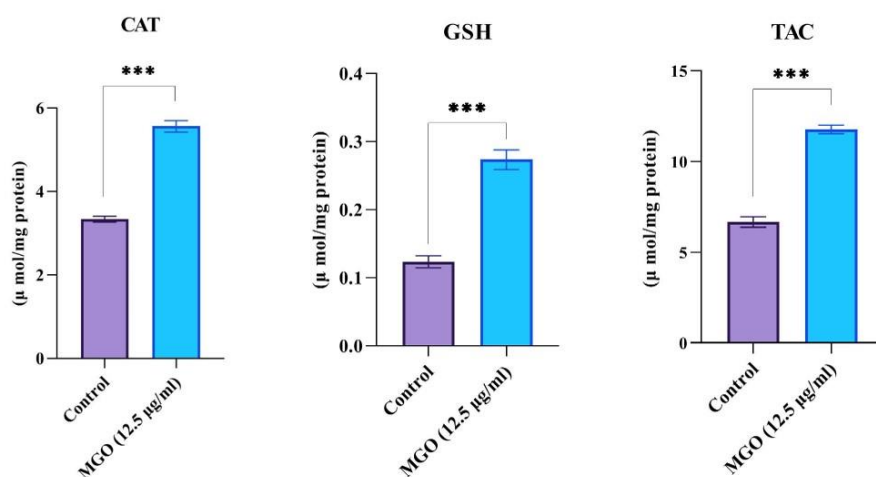


Figure 5. Colorimetric assessment of CAT, GSH, and TAC in the control and MGO-treated (12.5 µg/mL) groups. The results showed that CAT activity, GSH level, and TAC were increased in the MGO-treated group compared with the control group. Student's t-test was used for statistical analysis. Data were expressed as mean $\pm$ SEM. *** $P < 0.001$.

4. Discussion

In recent years, the benefits of using nanomaterials in cell imaging, targeted drug delivery, biosensors, cancer therapy, immunotherapy, and tissue regeneration have been demonstrated. A concentration of 10 µg/ml MGO was able to induce toxicity in adipose-derived mesenchymal stem cells, causing survival inhibition, dose-dependent inhibition of cell proliferation, and cell cycle arrest in the G1/G0 phase.²⁸ Urbas et al. examined the compatibility of L929 cell line treated with GO and MGO at different concentrations and concluded that the best biocompatibility was observed with MGO at a concentration of 12.5 µg/ml, while GO showed the greatest reduction in cell survival rate at a dose of 100 µg/ml for GO (1-3 µM).³² In their opinion, the difference in nanoparticle size does not lead to a change in cell viability. In the present study, exposure of human CCs to MGO at a concentration of 1.5 µg/ml for 24 hours initially increased proliferation; however, cell viability subsequently declined significantly, reaching 34.76 % at 12.5 µg/ml and 22.48 % at 200 µg/ml. After 48 hours of exposure, MGO at 12.5 µg/ml led to increased growth and proliferation in cumulus cells; at higher concentrations, viability decreased to 49.23% at 100 µg/ml, 35.93% at 200 µg/ml and 30.08 at 400 µg/ml. After 72 hours, cell viability at certain concentrations (1.5 µg/ml, 3 µg/ml and 25 µg/ml) exceeded that of the control group (100%), and then gradually decreased, reaching 39.85% at concentration of 400 µg/ml, respectively ($p < 0.001$).

Previous studies showed that after 24 hours of simultaneous incubation with Fe₃O₄/GO, a portion of HBE cells underwent apoptotic cell death, and the number of dead cells increased at higher concentrations (100 and 200 µg/ml), indicating the induction of apoptosis of HBE cells at high concentrations of Fe₃O₄/GO.³³ In the present study, MGO treatment reduced the apoptosis rate of CCs at concentrations of 3.125, 12.5, 50 and 100 µg/ml to 4.96%, 6.47%, 2.50% and 2.16%, which was lower than the control level 11.05%, and further experiments were performed to clarify the underlying mechanism. Western blot results of key apoptotic pathway proteins Bcl-2, Bax, pro-caspase-3 and cleaved caspase-3 showed that treatment with 50 µg/ml of MGO for 48 hours reduced CCs apoptosis. The expression level of Bcl-2 protein in MGO-treated cells was significantly higher than that in the control group ($p < 0.001$), while the expression of Bax protein ($p < 0.05$) and cleaved caspase-3 protein ($p < 0.0001$) were significantly less than the control group.

Tang and colleagues found that GO at certain concentrations induced Nrf2 translocation from the cytoplasm to the nucleus in MG-63 cells but not in K7M2 cells.³⁴ Our findings demonstrated that treatment with 12.5 µg/ml of MGO led to a significant increase in CAT activity, GSH levels, and TAC ($p < 0.001$), which is in complete alignment with the upregulated expression of Nrf2 and SOD2 proteins. The Nrf2 transcription factor serves as a master regulator of cellular antioxidant defense. This suggests the engagement of the Nrf2–ARE antioxidant signaling axis is essential for maintaining redox homeostasis.³⁵ While the concurrent increase in SOD2 and CAT levels aligns with an enhanced cellular antioxidant capacity that may prevent cumulus cell apoptosis, direct evidence of Nrf2 nuclear translocation or transcriptional activation remains to be established. Oxidative stress could stimulate Nrf2 nuclear translocation, where it binds to antioxidant elements in the DNA promoter, initiating the transcription of antioxidant genes such as SOD2 and heme oxygenase-1. Therefore, while these findings indicate the involvement of oxidative stress-responsive mechanisms, further studies utilizing immunofluorescence or gene silencing are required to definitively confirm direct Nrf2 pathway activation. By stimulating the transcription of its downstream target genes—including SOD2, CAT, and enzymes involved in GSH biosynthesis, it effectively supports the intracellular antioxidant network. Consequently, the concurrent elevation of TAC, GSH, and CAT levels observed in our study substantiates the active downstream function of the Nrf2 signaling pathway. Furthermore, given that the 50 µg/ml concentration was classified as non-toxic based on the MTT assay results, it is highly probable that these protective effects and the activation of the Nrf2/SOD2 axis are maintained at this concentration as well. These data suggest that at optimal and safe concentrations, MGO nanoparticles effectively enhance the free radical scavenging capacity and oxidative stress resistance of cumulus cells. This is achieved by orchestrating the Nrf2 pathway and upregulating SOD2 expression, together with enriching the levels of other key enzymatic and non-enzymatic antioxidants.

While studies have investigated the anticancer and antiproliferative effects of GO, there is no data on the effect of graphene derivatives on cyclin D1 protein expression. In the present study, the expression of cyclin D1 was evaluated after treatment with MGO. The increase in cyclin D1 expression after treatment with 50 µg/ml of MGO for 48 hours showed that this concentration of MGO was not only nontoxic to cumulus cells but also increased cell proliferation, which is consistent with previous results in this study. The increase in cyclin D1 in MGO-treated cells, together with the increase in the expression of the anti-apoptotic protein Bcl-2 and the decrease in the expression of pro-apoptotic markers, indicates a protective and proliferative cellular response. These results indicate that MGO induces a controlled level of oxidative stress that remains within the threshold of cellular antioxidant tolerance. The activation of Nrf2 and SOD2 as antioxidant biomarkers suggest the stimulation of the cellular antioxidant response and enhanced compensatory capacity. The concurrent increase in SOD2 and CAT implies the generation of a specific amount of ROS that has been effectively neutralized by the cellular defense system. Elevated ROS levels are not always detrimental; under certain conditions, they can have a stimulatory effect, strengthen cellular resilience and promote survival. The upregulation of Bcl-2 and downregulation of Bax and caspase-3 indicate a shift toward cell survival rather than apoptosis. Since the antioxidant system was activated without exceeding the cellular tolerance limit, the cells likely entered a compensatory phase, potentially leading to proliferation. This finding supports the potential application of these nanomaterials in strategies for drug delivery, apoptosis reduction, and cell survival.

Limitations

This study has several limitations. First, all experiments were performed *in vitro*; therefore, the findings cannot be directly extrapolated to the *in vivo* reproductive microenvironment or to clinical outcomes of ART. Second, since no experiments were performed on oocytes or embryos, the effects of MGO should not be extrapolated to improved oocyte competence, fertilization potential, or embryonic development. Third, the methods evaluated were limited and did not fully cover other potentially relevant pathways such as DNA damage, epigenetic changes, aging, mitochondrial dysfunction, or long-term ultrastructural changes. These aspects should be investigated in future *in vitro* and *in vivo* studies to provide a more comprehensive understanding of the biological and translational significance of the present findings. For example; stating that future studies are required to directly map the molecular mechanisms of Nrf2 signaling in this context. Finally, the experimental design lacked a positive control for apoptosis (e.g., H_2O_2 treatment). While our findings were validated across multiple independent assays (flow cytometry, TUNEL, and Western blotting), future studies should incorporate pro-apoptotic controls to better distinguish between the reduction of spontaneous culture-induced apoptosis and direct cytoprotection against external oxidative stressors.

Conclusion

Homeostasis of the cumulus-oocyte complex is critically dependent on bidirectional signaling pathways between the oocyte and surrounding cumulus cells. Physiological disruption of these paracrine/autocrine interactions usually triggers apoptotic cascades in cumulus cells through the activation of the mitochondrial pathway. MGO at low concentrations (e.g., 50 $\mu\text{g/ml}$) exhibits cellular protective properties by inducing nontoxic oxidative stress, thereby activating endogenous antioxidant defense mechanisms. Exposure of cells to MGO results in controlled ROS production and facilitates the nuclear translocation of the transcription factor Nrf2 and subsequently increases the expression of phase II detoxifying enzymes (e.g., SOD2, CAT, glutathione peroxidase). In addition to activating proteins related to the antioxidant pathway, MGO may also increase cell viability and proliferation, as supported by the increased expression of cyclin D1. Further studies are needed to explore the use of MGO in cell survival.

Funding

There was no funding.

Acknowledgment

We extend our gratitude to the Infertility Center at Al-Zahra Hospital, Tabriz, Iran, for their support.

Conflict of Interest

The authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author.

References

1. Ardehali A, Williams C, Monagle S, Cheung A, Graham A, Rychel V, et al. Effect of Assisted Reproductive Technologies in Women With Cardiovascular Disease. *Can J Cardiol* 2026. doi: 10.1016/j.cjca.2026.03.048
2. Ozgur K, Humaidan P, Coetzee K. Segmented ART - The new era in ART? *Reprod Biol* 2016;16(2):91-103. doi: 10.1016/j.repbio.2016.04.001
3. Mertens J, Belva F, van Montfoort APA, Regin M, Zambelli F, Seneca S, et al. Children born after assisted reproduction more commonly carry a mitochondrial genotype associating with low birthweight. *Nat Commun* 2024;15(1):1232. doi: 10.1038/s41467-024-45446-1
4. Pooyanfar F, Foroutan T, Dashtizad M. Effects of blastocyst artificial collapse prior to vitrification on hatching and survival rates and the expression of klf4 gene in mouse embryos. *Vet Res Forum* 2018;9(1):87-92.
5. Salehi PM, Foroutan T, Javeri A, Taha MF. Extract of mouse embryonic stem cells induces the expression of pluripotency genes in human adipose tissue-derived stem cells. *Iran J Basic Med Sci* 2017;20(11):1200-6. doi: 10.22038/ijbms.2017.9464
6. Eini F, Foroutan T, Bidadkosh A, Barin A, Dehghan MM, Tajik P. The effects of freeze/thawing process on cryopreserved equine umbilical cord blood-derived mesenchymal stem cells. *Comparative Clinical Pathology* 2012;21(6):1713-8. doi: 10.1007/s00580-011-1355-8
7. Huyghe S, Verest A, Thijssen A, Ombelet W. The prognostic value of perfollicular blood flow in the outcome after assisted reproduction: a systematic review. *Facts Views Vis Obgyn* 2017;9(3):153-6.
8. Lemseffer Y, Terret ME, Campillo C, Labrune E. Methods for Assessing Oocyte Quality: A Review of Literature. *Biomedicines* 2022;10(9). doi: 10.3390/biomedicines10092184
9. Jafarzadeh E, Pashaei-Asl R, Hakimi P, Pashaiasl M. The Effect of Letrozole and N-Acetylcysteine on the Expression Levels of Genes Involved in Glucose Metabolism in Patients with Polycystic Ovary Syndrome: A Clinical Trial Study. *J Reprod Infertil* 2025;26(2):81-9. doi: 10.18502/jri.v26i2.19433
10. Fang Y, Shang W, Wei DL, Zeng SM. Cited2 protein level in cumulus cells is a biomarker for human embryo quality and pregnancy outcome in one in vitro fertilization cycle. *Fertil Steril* 2016;105(5):1351-9.e4. doi: 10.1016/j.fertnstert.2015.12.137
11. Coticchio G, Dal Canto M, Mignini Renzini M, Guglielmo MC, Brambillasca F, Turchi D, et al. Oocyte maturation: gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. *Hum Reprod Update* 2015;21(4):427-54. doi: 10.1093/humupd/dmv011
12. Ruvalo G, Fattouh RR, Bosco L, Bruculeri AM, Cittadini E. New molecular markers for the evaluation of gamete quality. *J Assist Reprod Genet* 2013;30(2):207-12. doi: 10.1007/s10815-013-9943-y
13. Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. *J Assist Reprod Genet* 2001;18(9):490-8. doi: 10.1023/a:1016649026353
14. González-Marín C, Gosálvez J, Roy R. Types, causes, detection and repair of DNA fragmentation in animal and human sperm cells. *Int J Mol Sci* 2012;13(11):14026-52. doi: 10.3390/ijms131114026
15. Ruvalo G, Bosco L, Pane A, Morici G, Cittadini E, Roccheri MC. Lower apoptosis rate in human cumulus cells after administration of recombinant luteinizing hormone to women undergoing ovarian stimulation for in vitro fertilization procedures. *Fertil Steril* 2007;87(3):542-6. doi: 10.1016/j.fertnstert.2006.06.059

16. Ramal-Sanchez M, Fontana A, Valbonetti L, Ordinelli A, Bernabò N, Barboni B. Graphene and Reproduction: A Love-Hate Relationship. *Nanomaterials (Basel)* 2021;11(2). doi: 10.3390/nano11020547
17. Jiang J, Zhang C, Gao C, Zhang H, Cai G, Zheng Y, et al. Magnetic graphene oxide functionalized composite nanofibrous stem cell-based neural scaffolds. *J Nanobiotechnology* 2026;24(1). doi: 10.1186/s12951-026-04127-5
18. Li D, Müller MB, Gilje S, Kaner RB, Wallace GG. Processable aqueous dispersions of graphene nanosheets. *Nat Nanotechnol* 2008;3(2):101-5. doi: 10.1038/nnano.2007.451
19. Guo W, Zhang X, Yu X, Wang S, Qiu J, Tang W, et al. Self-Powered Electrical Stimulation for Enhancing Neural Differentiation of Mesenchymal Stem Cells on Graphene-Poly(3,4-ethylenedioxythiophene) Hybrid Microfibers. *ACS Nano* 2016;10(5):5086-95. doi: 10.1021/acsnano.6b00200
20. Ettorre V, De Marco P, Zara S, Perrotti V, Scarano A, Di Crescenzo A, et al. In vitro and in vivo characterization of graphene oxide coated porcine bone granules. *Carbon* 2016;103:291-8. doi: 10.1016/j.carbon.2016.03.01
21. Foroutan T, Kassaei MZ, Salari M, Ahmady F, Molavi F, Moayer F. Magnetic Fe(3) O(4) @graphene oxide improves the therapeutic effects of embryonic stem cells on acute liver damage. *Cell Prolif* 2021;54(11):e13126. doi: 10.1111/cpr.13126
22. Huh YM, Jun YW, Song HT, Kim S, Choi JS, Lee JH, et al. In vivo magnetic resonance detection of cancer by using multifunctional magnetic nanocrystals. *J Am Chem Soc* 2005;127(35):12387-91. doi: 10.1021/ja052337c
23. Miller M, Prinz G, Cheng S-F, Bounnak S. Detection of a micron-sized magnetic sphere using a ring-shaped anisotropic magnetoresistance-based sensor: A model for a magnetoresistance-based biosensor. *Applied Physics Letters* 2002;81(12):2211-3. doi: 10.1063/1.1507832
24. Kabiri F, Foroutan T, Pashaiasl M. Magnetic graphene oxide increases the biocompatibility and nuclear factor erythroid 2-related factor 2 antioxidant of human cumulus cells: A lab-trial study. *Int J Reprod Biomed* 2024;22(9):709-16. doi: 10.18502/ijrm.v22i9.17475
25. Shakoori Z, Pashaei-Asl R, Pashaiasl M, Davaran S, Ghanbari H, Ebrahimie E, et al. Biocompatibility study of P (N-isopropylacrylamide)-based nanocomposite and its cytotoxic effect on HeLa cells as a drug delivery system for Cisplatin. *Journal of Drug Delivery Science and Technology* 2022;71:103254. doi: 10.1016/j.jddst.2022.103254
26. Qiu Y, Wang Z, Owens AC, Kulaots I, Chen Y, Kane AB, et al. Antioxidant chemistry of graphene-based materials and its role in oxidation protection technology. *Nanoscale* 2014;6(20):11744-55. doi: 10.1039/c4nr03275f
27. Pashaei-Asl R, Motaali S, Ebrahimie E, Mohammadi-Dehcheshmeh M, Ebrahimi M, Pashaiasl M. Delivery of doxorubicin by Fe(3)O(4) nanoparticles, reduces multidrug resistance gene expression in ovarian cancer cells. *Pathol Res Pract* 2024;263:155667. doi: 10.1016/j.prp.2024.155667
28. He Y, Shi F, Hu J, Li H, Chen X, Yuan L, et al. Magnetic graphene oxide nanocomposites induce cytotoxicity in ADSCs via GPX4 regulating ferroptosis. *Ecotoxicol Environ Saf* 2024;269:115745. doi: 10.1016/j.ecoenv.2023.115745
29. Makhzarza SA, Cirillo G, Vittorio O, Valli E, Voli F, Farfalla A, et al. Magnetic Graphene Oxide Nanocarrier for Targeted Delivery of Cisplatin: A Perspective for Glioblastoma Treatment. *Pharmaceuticals (Basel)* 2019;12(2). doi: 10.3390/ph12020076

30. Mai S, Inkielewicz-Stepniak I. Graphene Oxide Nanoparticles and Organoids: A Prospective Advanced Model for Pancreatic Cancer Research. *Int J Mol Sci* 2024;25(2). doi: 10.3390/ijms25021066.
31. Pashaei-Asl R, Pashaiasl M, Ebrahimie E, Lale Ataei M, Paknejad M. Apoptotic effects of human amniotic fluid mesenchymal stem cells conditioned medium on human MCF-7 breast cancer cell line. *Bioimpacts* 2023;13(3):191-206. doi: 10.34172/bi.2022.23813
32. Urbas K, Aleksandrak M, Jedrzejczak M, Jedrzejczak M, Rakoczy R, Chen X, et al. Chemical and magnetic functionalization of graphene oxide as a route to enhance its biocompatibility. *Nanoscale Res Lett* 2014;9(1):656. doi: 10.1186/1556-276x-9-656
33. Zhang Y, Zhang Y, Yang Z, Fan Y, Chen M, Zhao M, et al. Cytotoxicity Effect of Iron Oxide (Fe(3)O(4))/Graphene Oxide (GO) Nanosheets in Cultured HBE Cells. *Front Chem* 2022;10:888033. doi: 10.3389/fchem.2022.888033
34. Tang X, Qu Y, Deng S-L, Tan Y-Z, Zhang Q, Liu Q. Fullerene-regulated graphene oxide nanosheet membranes with well-defined laminar nanochannels for precise molecule sieving. *Journal of Materials Chemistry A* 2018;6(45):22590-8. doi: 10.1039/C8TA08261H
35. Ghareghomi S, Habibi-Rezaei M, Arese M, Saso L, Moosavi-Movahedi AA. Nrf2 Modulation in Breast Cancer. *Biomedicines* 2022;10(10). doi: 10.3390/biomedicines10102668

