Research Article

Nano Biomed Eng

The Effects of Nano Magnetic Graphene Oxide on *In Vivo* Maturation of Oocyte

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Received: Jun. 4, 2023; **Revised:** Jul. 10, 2023; **Accepted:** Aug. 13, 2023

Citation: M. Rahimi, T. Foroutan, F. Eini. The effects of nano magnetic graphene oxide on *in vivo* maturation of oocyte. *Nano Biomedicine and Engineering*, 2023, 15(4): 354−362.

http://doi.org/10.26599/NBE.2023.9290036

Abstract

Graphene oxide (GO) and Fe_3O_4 super paramagnetic material are good candidate for some applications such as drug delivery. It has been shown that combining Fe_3O_4 with graphene oxide increases the biological efficiency of GO. The use of novel assisted reproductive technologies such as gonadotropins injection has been able to help the fertility of infertile people, but the side effects of these methods and high costs are still problems. The aim of the present study was to investigate the effect of magnetic graphene oxide (MGO) on the *in vivo* maturation of mouse oocytes. Thirty 6–8-week old female Naval Medical Research Institute (NMRI) mice were treated with intra peritoneal (I.P) injection of MGO mixed with hormones. 12 h after I.P. injection of MGO mixed with PMSG and HCG, the number of metaphase II (MII) oocytes obtained from the left fallopian tubes was counted in each group. Also, immuno-cytochemical staining of glutathione and morphometric analysis of ovaries were studied. The results of this study showed that the simultaneous use of MGO, pregnant mare serum gonadotropin (PMSG), and human chorionic gonadotrophin (HCG) increases the number of MII oocytes and helps to increase maturation of oocytes. It could be concluded that MGO can increase the efficiency of super ovulating hormones due to increase in adsorption of serum hormones and growth factors.

Keywords: oocyte maturation; graphene oxide (GO); Fe_3O_4; *in vitro* fertilization (IVF)

Introduction

One of the most important causes of infertility (with a global prevalence of 10%–15%) is ovulation disorder. Many treatment methods including hormone therapy, *in vitro* maturation (IVM) of oocytes, *in vitro* fertilization (IVF), intracytoplasmic injection of sperm and freezing of embryos and gametes can helped to solve this problem [1–3]. Although gonadotropin injection is common to obtain more oocyte, the high stimulation of the ovary has side effects. Heavy costs and stimulation of polycystic ovary syndrome are among the consequences of this method [3, 4]. The quality and competence of IVM oocytes are significantly lower than those matured *in vivo*, and fewer of them enter metaphase II (MII) oocytes [5–7].

Although, during *in vitro* culture, the majority of immature oocytes reach the MII stage, this does not necessarily indicate proper developmental potential, and nuclear and cytoplasmic maturation must occur simultaneously. Also, the embryos resulted from the

IVM oocytes have a lower efficiency than normal embryos [6, 8].

On the other hand, the oocyte maturation ability to develop to the blastocyst stage in culture medium without supplements is lower compared to those matured *in vivo*. Therefore, the culture conditions play a key role in oocyte maturation, fertilization, embryo development, and may affect both nuclear and cytoplasmic maturation of the oocyte [9–11]. Oocyte manipulation during IVM is accompanied by the risk of increased reactive oxygen species (ROS) levels. Collection of oocytes and their fertilization in high environmental oxygen may produce large amounts of ROS that prevent embryonic development prior to implantation [12]. Therefore, discovering methods to increase oocyte maturation is one of the ways to improve ART.

Recently, some nano materials such as GO has attracted significant attention due to its excellent properties, including its large surface area, high electron transport capability, elasticity, thermal conductivity, drug delivery, tissue engineering, gene therapy, and biomedical applications [13]. The chemical functionalization of GO by magnetic iron oxide nanoparticles, Fe_3O_4, is a way to enhance its biocompatibility [14]. MNP was applied in various biomedical applications such as drug delivery, chemotherapy, and bio imaging [13]. Yang et al. [15] showed higher cytotoxicity of GO compared with GO mixed with MNP. Our previous studies showed the positive effects of I.P. injection of synthesized magnetic graphene oxide (MGO) on treatment of acute kidney and liver injuries [16–18]. The aim of the present study was to investigate the effects of I.P injection of MGO on in vivo maturation of mouse oocytes obtained from fallopian tube. Also, intra cellular levels of glutathione (GSH) as a key factor to assess the maturity of oocyte were examined.

Experimental

Natural flake graphite powder was supplied by Qingdao Dingding Graphite Products Factory (Laixi, Shandong, China). H_2SO_4 (98%), H_2O_2 (30%), hydrochloric acid (HCl, 37%), and $KMnO_4$ were purchased from Sigma-Aldrich Co.

Preparation of MGO nano-hybrid

MGO was synthesized according to our previous studies [17–19].

Characterization

As-prepared samples were characterized using X-ray diffraction (XRD, Philips Xpert MPD, Co K irradiation, 1.78897 A), scanning electron microscopy (SEM) (Philips XL30 microscope with an accelerating voltage of 25 kV), and dynamic light scattering (DLS, Horiba SZ-100) analyses.

Animal model and oocyte collection

6–8-week old female NMRI mice (n = 30; 25–30 g) were kept under controlled conditions (12-h:12-h light/dark cycle, 22 °C). All animal protocols were approved by the Kharazmi University Animal Ethics Committee based on the University guidelines. The study groups included the control group not receiving any treatment; the control group receiving hormone (10 IU PMSG and 10 IU HCG); the sham group receiving distilled water followed by 10 IU HCG; the group receiving 10 IU PMSG + 10 μg/mL nano MGO + 10 IU HCG; and the group receiving 10 IU PMSG + 10 μg/mL nano MGO + 10 IU HCG + 5 μg/mL nano MGO. All the experiments were repeated three times (n = 6). 12 h after HCG administration, the mice were killed by cervical dislocation and the oocytes were collected at MII stage from fallopian tubes and then cultured in TCM-199.All animal protocols were approved by the Kharazmi University Animal Ethics Committee based on the University guidelines.

Intracellular measurement of GSH levels

MII oocytes obtained from control and treatment groups were stained with GSH antibodies. 35–40 denuded MII oocytes from control and treatment groups were incubated in phosphate buffer saline (PBS) and then were washed with PBS/Poly(vinyl alcohol) (PVA) drops, placed under mineral oil and observed using a fluorescence microscope.

Statistical analysis

The statistical analyses were conducted using Graph Pad Prism 8, One-way ANOVA. The Tukey test was used to evaluate the differences between the groups. $p < 0.05$ was considered statistically significant.

Results and Discussion

MGO synthesis

Herein, MGO were fabricated using previous method [18, 20]. Figure 1 shows the schematic representation

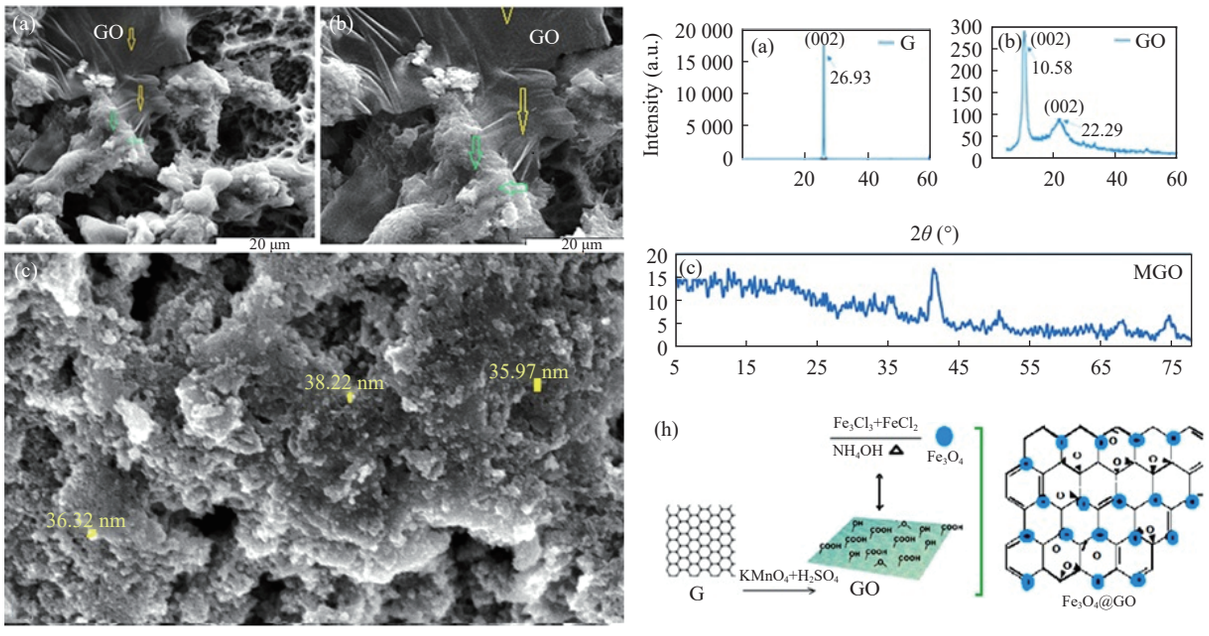

Fig. 1 SEM images of GO and MGO in different magnifications. **(a)** and **(b)** SEM images of the interaction between GO edges (yellow arrow) and MSCs and growth factors derived from stem cells (green arrow). **(c)** SEM image of MGO. XRD patterns of pristine graphite powder (G), GO, and MGO **(d)**. Schematic representations of the synthesis of GO and MGO (Fe_3O_4@GO) **(h)**.

of synthesis of the MGO and also its characterization includes scanning electron microscope (SEM) images in different magnifications, X-ray Diffraction (XRD) patterns of pristine graphite powder, GO, and MGO and the schematic representation of MGO.

Morphometric study

Our previous study showed that MGO injection is safe and non-toxic at low concentration (lower than 12 μg/mL [19]. The results of the present study showed an increase in the length and width of the ovaries in the hormone, hormone + MGO, and even MGO groups compared to the control group (Table 1, Fig. 2). An increase in angiogenesis is especially evident in the MGO group (arrow). The number of primordial, primary, secondary, and Graafian follicles in the groups treated with hormone that mixed with MGO showed an increase compared to the control groups (Fig. 3).

Histological study

Also, treating with MGO alone caused the increase of follicles compared to the control groups (Fig. 3). MII oocytes with one polar body and a round zona pellucid was significantly increased in the PMSG, HCG + MGO and PMSG+ MGO groups compared to control and sham groups ($p < 0.001$). The number of MII oocytes with one polar body and a round zona pellucid was significantly increased in the PMSG, HCG + MGO, and PMSG + MGO groups compared to control and sham groups ($p < 0.001$). The comparative details of all groups have been shown in Fig. 4 and Table 2.

Cytoplasmic levels of GSH in oocytes

To study the mechanisms in which MGO affects the maturation of oocytes in laboratory rats, the content of glutathione (GSH) in fallopian tube oocytes were measured. Intra cytoplasmic GSH was stained using fluorescent cell tracker dye in matured oocytes in three groups and then imaged with an Inverted Fluorescent Microscope at 370 nm. Data on each oocyte was quantitatively assessed by ImageJ

Table 1 The mean and SD of length and width of ovary of control and treated with hormones and MGO groups

Parameter (μm)	Goups				
	Control (mean ± SD)	Control + hormone (mean ± SD)	Sham (mean ± SD)	PMSG + MGO (mean ± SD)	PMSG&HCG +MGO (mean ± SD)
Length of ovary	26.16 ± 1.32	33.12 ± 1.41	25.88 ± 1.91 #	*** 35.87 ± 1.10 #	*** 37.34 ± 1.17 ##
Width of ovary	15.33 ± 1.63	20.10 ± 0.61	15.61 ± 1.47 #	*** 22.53 ± 0.92 #	*** 24.44 ± 1.30 #

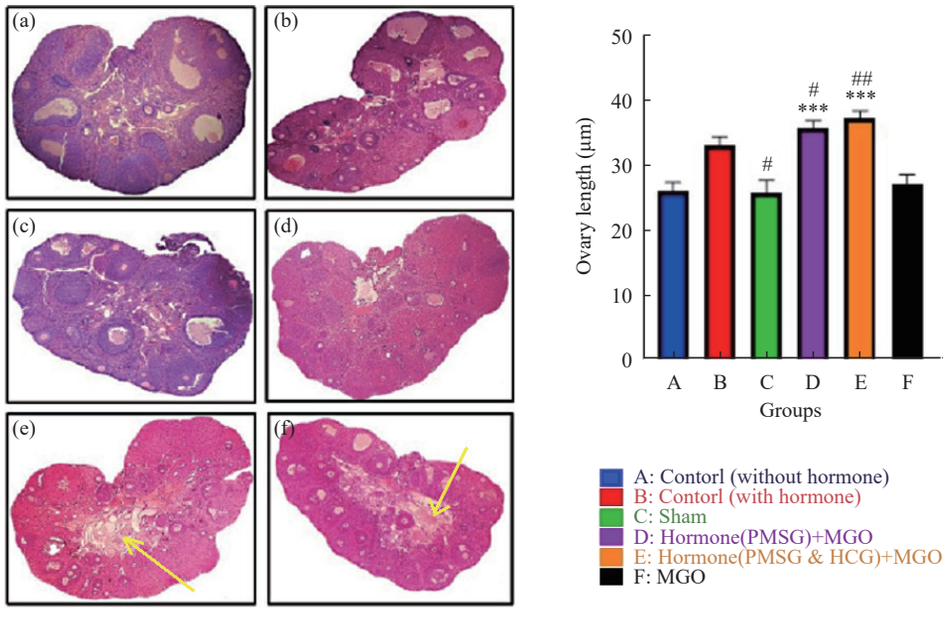

Fig. 2 left: Histological images from ovarian length in control **(a, b)**, treated with hormone mixed MGO **(c)** and **(d)**, and MGO **(e, f)** groups. The increase in angiogenesis is especially evident in the MGO group (arrow). Right: the length of ovaries in different groups (H & E, ×400). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ show significant differences between the control without hormone and the other groups. # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ show significant differences between the control with hormone and the other groups.

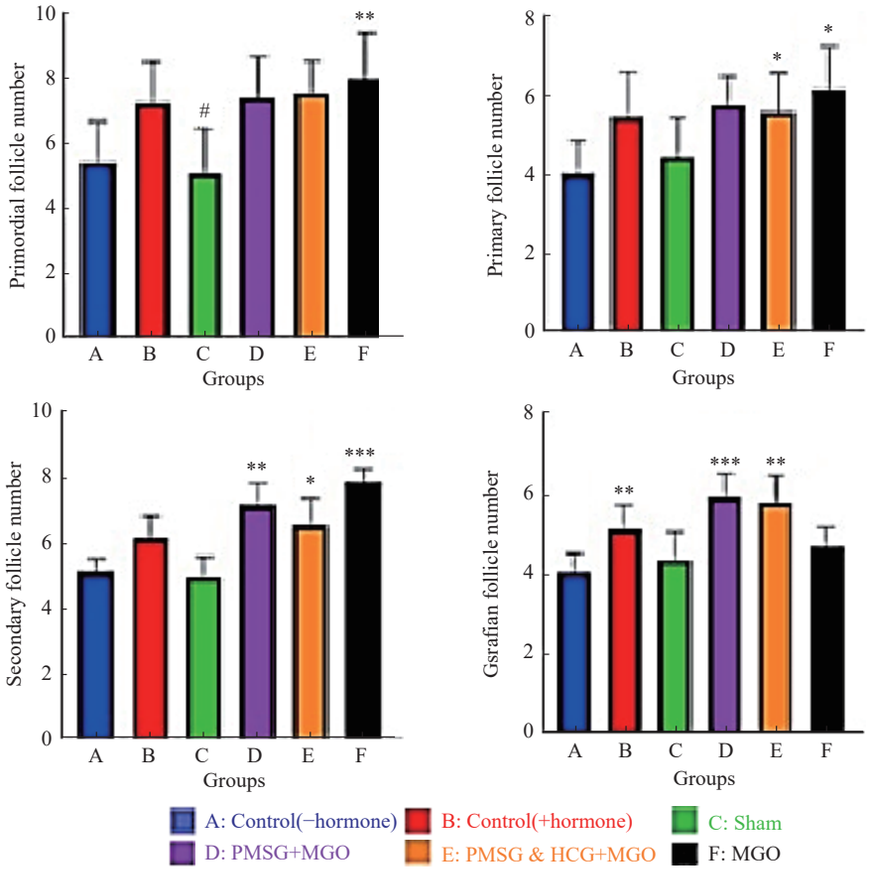

Fig. 3 Comparison of the number of primordial, primary, secondary, and Graafian follicles in different groups.* indicates a significant difference between the control group without hormones and the other groups. # indicates the difference between the control group with hormone and the treated groups. * and # show differences between the groups at $p < 0.05$, and *** show differences between the groups at $p < 0.001$.

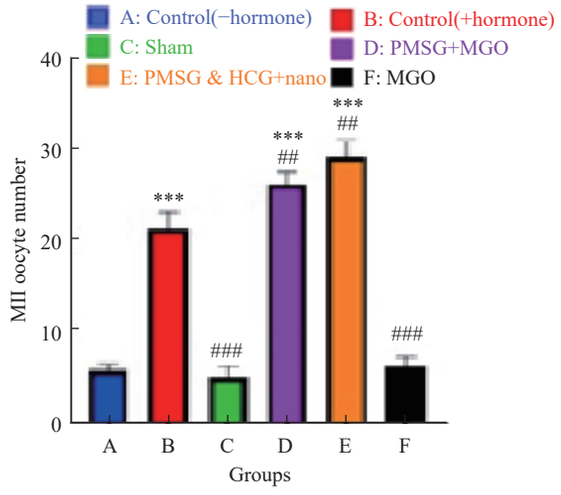

Fig. 4 The number of MII oocytes obtained from various groups. The number of MII oocytes in the hormones + MGO groups (***p < 0.001) was significantly increased as compared with control group. There is a significant difference between the control + hormone group and sham and MGO groups (###p < 0.001). There is also a significant difference between the control + hormone group and the hormones + MGO groups (##p < 0.01).

(National Institutes of Health, USA). In each group, 30–40 oocytes were studied in three replications. All tests were conducted in darkness. The data is given in Table 3 and Figs. 5 and 6. A significant increase in intra cytoplasmic GSH, which indicates the cytoplasmic maturation of oocyte, was observed in the hormone groups (Figs. 5 and 6, Table 3).

Our results showed that intraperitoneal injection of MGO increases the cytoplasmic GSH of oocytes. This result indicates the induction role of MGO on maturation and development of oocytes. GSH as an antioxidant and a key factor to assess the maturity of oocyte in animals prevents damage to cellular components caused by ROS [21]. GSH plays the role of improving cell activities by reducing ROS. In other words, the decrease in intracytoplsmic GSH is accompanied with an increase of reactive oxygen species (ROS) production [22]. It seems that the role of MGO in increasing the number and maturation of oocytes is induced by reducing the amount of ROS in the cells. Perhaps the increase in the maturity of oocytes by MGO is through the reduction of ROS induced by GSH. Based on this result while injection of MGO increasing the cellular GSH, it causes an increase in the number of ovarian follicles compared to the control group without hormones. Previous study showed that supplementation of culture medium with an antioxidant increased cytoplsmic GSH in pronuclear cells [23]. On the other hand, it has been shown that MGO improves the biocompatibility of cells [17, 18]. It could be concluded that MGO improves the activity of ovarian cells and facilitates their maturation by increasing the GSH in the cells and subsequently reducing the fat in the ovarian cells. It seems that MGO induces an increase in the expression of cytoplasmic GSH and thereby facilitates oocyte maturation.

There are conflicting reports about the toxicity of graphene derivatives. Some of studies believe that GO derivatives has possible toxic effects on different tissue. Holmannova et al. reported that carbon nanoparticles have a negative effect on reproduction and offspring development. Also, a deleterious effect of carbon nanoparticles on sperm and oocytes has been reported [24]. On the other hand, it has been shown that GO effects on toxicity of cells is dose-dependent, while adding some substances to it reduce its toxicity [25]. Asghar et al. described that some carbon nanotubes induced ROS production in human

Table 2 The mean and SD of MII oocytes obtained from the fallopian tube in control and treated with hormones and MGO groups

Parameter	Groups				
	Control (mean ± SD)	Control+ hormone (mean ± SD)	Sham (mean ± SD)	PMSG + MGO (mean ± SD)	PMSG&HCG + MGO (mean ± SD)
Metaphase II oocyte	5.83 ± 0.75	21.33 ± 1.96 ***	5.16 ± 1.16 ###	26.33 ± 1.36 ## ****	29.33 ± 1.75 ## ****

Significant difference between control group (without hormone) and the other groups; *p < 0.05, **p < 0.01, ***p < 0.001. Significant difference between control group (with hormone) and the other groups; #p < 0.05, ##p < 0.01, ###p < 0.001.

Table 3 The mean and SD of intracytoplasmic GSH content in matured oocytes obtained from the fallopian tube in control and treated with hormones and MGO groups.

Parameter (nmol GSH /mg protein)	Groups		
	Control + hormone (mean ± SD)	PMSG + MGO (mean ± SD)	PMSG&HCG + MGO (mean ± SD)
Intracytoplasmic GSH	185.90 ± 1.81	193.36 ± 1.74###	197 ± 1.61###

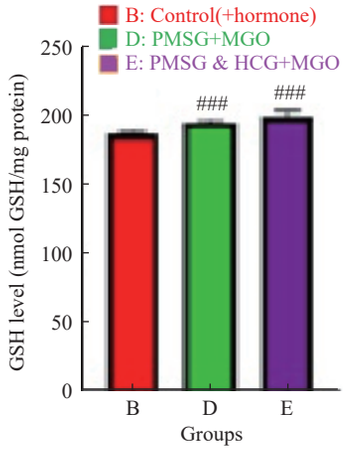

Fig. 5 Intra cytoplasmic GSH content in matured oocytes. There is a significant increase between PMSG + MGO and PMSG+HCG+MGO (p <0.001).

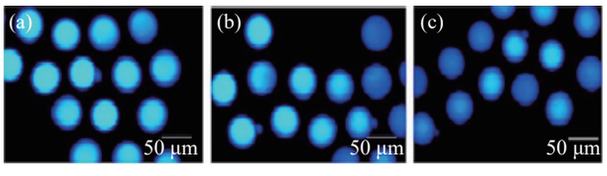

Fig. 6 The use of fluorescent staining to measure the GSH content in MII oocytes in the experimental groups. The injection of nano in two steps exerted the highest influence on the GSH content. There is a significant difference between the PMSG and HCG groups and the control + hormone group. **(a)** Control + hormone, **(b)** PMSG + MGO, **(c)** PMSG + HCG + MGO; Magnification: 100×.

spermatozoa at a concentration of 25 µg/mL, while the presence of reduced GOs did not increase oxidative stress [26]. Indeed, lower doses and shorter exposures are more effective in reducing the levels of these genotoxicity markers [27]. Importantly, GOs at a dose of 1–25 µg/mL did not affect sperm viability [28]. The dose used in the present study was not only non-toxic but also had positive effects in increasing GSH level. Human reproductive cells, in the study by Aminzadeh et al., were exposed to carbon nanoparticles and did not attenuate sperm viability. Interestingly, reduced GOs are more biocompatible and did not induce tissue to the damage of reproductive system [29]. In this way, GOs can alter the epigenetic signaling involved in the self-protection mechanism against GOs toxicity [30]. It has been reported that GO diversities not only reduced reproductive capacity and altered gonad development in some organisms but also induced germ cells. Kong et al. showed that graphene did not affect the reproductive capacity, indicating that the functionalization of carbon nanomaterials may be beneficial of improving biocompatibility [31].

The glutathione level increases during oocyte maturation so that its level in the MII is three times of that in the germinal vesicle (GV) stage. Since the increase of GSH during oocyte maturation is important, therefore, increasing its level induced by MGO injection can be a sign of the role of MGO in increasing the number of MII oocytes. The method of super ovulation induced by PMSG and HCG for increasing the number of released oocytes is widely used in IVF clinics. Following the hormonal stimulation of ovulation, the amount of hormones secreted by the ovary, especially estradiol and progesterone, exceeds the physiological limit. Our results revealed the positive induction effect of magnetic GO on maturation of oocytes. In other word, we showed that co-administration of MGO and ovulation stimulating hormones increased the number of oocytes in the fallopian tubes and intracytoplasmic GSH of oocytes. Drugs will be more effective if they can meet physiological needs of body at appropriate times and positions. Today, nanomaterials are used in the design of drug delivery systems to overcome the defects and disadvantages of conventional pharmaceutical formulations, reduce drug consumption and/or increase drug effectiveness. Unique surface chemistry, high biocompatibility and low toxicity make GO as a good candidate for drug delivery systems [15]. The effectiveness of GO and its derivatives may vary by polymers and other nanomaterials and thus it is possible to increase the efficiency of drugs delivery [15]. The changes made in the GO can increase its mechanical strength, facilitate its entrance into the cell and also reduce its toxicity. GO has advantages over graphene such as its hydrophilic functional groups that can increase drug efficacy. It has also been found that the efficacy of GO hybridized with biocompatible polymers may be increased for targeted drug delivery if it is combined with tumor-specific antibodies and drugs [32]. For example, it has been shown that *in vivo* and *in vitro* treatment with GO-polyethylene glycol completely destroy prostate cancer tumors in mice with a fewer complications than the drug used alone [33]. It seems that due to the some properties such as drug delivery and absorbing proteins by GO and its derivatieves, the binding and entry of serum hormones and injected hormones into the cells is facilitated, and as a result, the rate of proliferation and maturation of oocytes increases. Another possible mechanism to justify the increase of oocyte maturation by graphene can be

covalent binding of GSH on magnetic nanoparticles such as MGO [34]. Also, Pham and colleagues studied the positive effects of MGO on separation and absorption of bovine serum proteins [13]. Chen et al. showed the separation/isolation and pre-concentration of protein species by graphene/GO and their derivatives [35]. On the other hand, the MNP has been used in various biomedical applications such as drug delivery [36]. According to its mentioned properties, it seems MGO by absorbing and binding to hormones and blood proteins and then delivering those to follicular cells, causes the proliferation and maturation of oocytes.

It has been reported that in the presence of GO, adhesion proteins such as vinculin and fibronectin and growth factors such as BMP are more efficiently adsorbed on the surface of cells [37]. The glutathione level in the matured oocytes under *in vivo* conditions is much higher than its level in the matured oocytes under *in vitro* conditions [38, 39]. To protect cells against oxidative stress during *in vitro* maturaion, the glutathione level in the oocyte is significantly decreased compared to oocytes matured under *in vivo* conditions [30, 32]. The GSH concentration in the oocyte after *in vitro* maturation can be considered as an index of the cytoplasmic maturation of the oocyte [40, 41]. Our results showed an increase in angiogenesis of ovaries, which is followed by an increase in the number of active follicles. Our previous paper also confirmed the increase in angiogenesis induced by MGO [19]. The results of the present research showed that addition of MGO to PMSG significantly incerased the level of GSH in the oocytes compared to the control group. Modification of drugs by substances with two different poles will increase their efficiency. Targeted drug delivery by using GO and its derivatives has advantages over the other nanoparticles. There is a positive synergistic of nano with hormone to increase ovulation.

Conclusion

We have shown the effects of ovulation-stimulating hormones can be increased by I.P. MGO injection. This nanomaterials could increase the efficiency of PMSG in the ovarian tissue and lead to increase the number of MII oocytes. The advantages of GO and Fe_3O_4 could be more powerful in bioanalytical fields when they combined together.The property of increasing oocyte maturation by MGO can be explained by three possible mechanisms: (1) the chemical combination of these substances could help to the higher hormones release capability and targeted oogenesis stimulating efficiency, (2) HCG and PMSG absorption by MGO surface and their improvement delivery into the cells, and (3) similarity of mechanism of MGO to hormones to increase ovulation. It could be concluded that MGO are a good candidates for hormone drug delivery.

CRediT Author Statement

Mitra Rahimi: Data curation, writing–review, and editing. **Tahereh Foroutan**: Conceptualization, investigation, methodology, project administration, supervision, visualization, writing–original draft, writing–review & editing. **Fatemeh Eini**: Data curation , investigation.

Acknowledgments

The authors would like to thank Dr. Naser Salsabili for his valuable assistance. The study would not have become possible without the support provided by Kharazmi Universty, Tehran, Iran.

Conflict of Interest

The authors declare that no competing interest exists.

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