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Magnesium Glycinate Attenuates Cyclophosphamide-induced Ovarian Toxicity in Wistar Rats

Luqman Adepoju Hassan[1], Foluso Olamide Ojo[1*], Rasheed Tunde Lawal[2], [3] Omoola Oluwatosin Olasheu[3], Abdul-Raheem Folorunsho Ahmad[4], Kolade Pelumi Folorunso[1], Idowu Oluwagbemiga Coker[5], Sirajudeen Olasunkanmi Bashir[4], Mistura Bolaji Ajani[6], Tolulope Mary Onaolapo[7], Aishat Abidemi Olawale[1], Muhsinat Bisola Lawal[1], Oluwafemi Samuel Olaniyi[1]

[1]Dept of Anatomy, [2]Dept of Microbiology, University of Ilesa, Ilesa; [3]Dept of Anatomy, Kampala International University; [4]Dept of Biochemistry, [5]Dept of Pharmacology, University of Ilesa, [6]Dept of Radiography, University of Ilesa, Ilesa; [7] Dept of Nursing Science, Faculty of Nursing Sciences, Saint Peter's University, New Jersey, United States of America

***Corresponding Author:** Email: foluso_ojo@unilesa.edu.ng; Tel: +234 7062328364; ORCID: 0009-0005-4876-6966

ABSTRACT

Cyclophosphamide (CP) is an alkylating chemotherapy agent that causes severe, dose-limiting ovotoxicity, often leading to diminished ovarian reserve. This study investigated the protective efficacy of magnesium glycinate (MgG), a highly bioavailable organic magnesium chelate with antioxidant properties, against CP-induced structural and endocrine ovarian damage. Forty-eight adult female Wistar rats were divided into six groups (n=8): Control, MgG Low-Dose (22.8 mg/kg), MgG High-Dose (32.8 mg/kg), CP-Only (150 mg/kg i.p. on days 1, 3, and 5), and CP co-administered with low or high-dose MgG orally for 21 days. Serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were quantified via ELISA. Ovarian histoarchitecture, stromal fibrosis, and glycoprotein barrier integrity were evaluated utilizing Hematoxylin & Eosin, Masson's Trichrome, and Periodic Acid-Schiff staining. CP intoxication profoundly disrupted the hypothalamic-pituitary-ovarian axis, significantly suppressing serum FSH and LH levels ($p < 0.05$). Histologically, CP induced massive interstitial hemorrhage, vascular congestion, extensive stromal fibrosis, and the degradation of glycoprotein microenvironments (follicular basement membrane and zona pellucida). Co-administration of MgG dose-dependently attenuated these toxicities. The high-dose MgG regimen normalized LH concentrations, upregulated FSH secretion, prevented hemorrhagic necrosis, suppressed aberrant collagen cross-linking, and preserved the structural integrity of critical glycoprotein barriers and developing follicles. Magnesium glycinate successfully mitigates CP-induced ovotoxicity by rescuing vital gonadotropin secretion, suppressing pathological extracellular matrix remodeling, and preserving essential follicular microenvironments, positioning it as a promising adjunct therapy for fertility preservation during gonadotoxic chemotherapy.

Keywords: Cyclophosphamide; ovarian toxicity; magnesium glycinate; oxidative stress; fertility preservation

INTRODUCTION

Cyclophosphamide (CP) is a widely used nitrogen-mustard alkylating agent employed in multi-drug chemotherapy regimens and as an immunosuppressant in severe autoimmune disease. CP is administered as an inactive prodrug and requires hepatic bioactivation to form cytotoxic metabolites, principally phosphoramide mustard, which produces DNA crosslinks (notably at guanine N-7) and thereby disrupts DNA replication and triggers cell death [1]. Although clinically effective, CP is also associated with dose-limiting toxicities, including gonadotoxicity that can compromise reproductive potential [2].

The ovary is particularly susceptible to CP-related injury because female fertility depends on a finite pool of primordial follicles; damage to this reserve can result in diminished ovarian reserve, endocrine disruption, and premature ovarian insufficiency [3]. Mechanistic evidence indicates that CP ovarian toxicity is multifactorial and involves oxidative stress and depletion of antioxidant defenses, inflammatory signaling, mitochondrial dysfunction, DNA damage, and apoptotic loss of granulosa cells with subsequent follicular atresia [4,5]. In addition, experimental work supports a "follicular activation/burnout" model in which CP can abnormally recruit dormant primordial follicles into growth pathways, progressive reduction of the ovarian reserve [6].

While established fertility-preservation options such as oocyte/embryo cryopreservation and other assisted reproductive approaches exist, access remains uneven. In many low-resource settings, major barriers include

limited availability of trained teams/technology, inadequate insurance coverage, and high out-of-pocket costs, creating a strong rationale for evaluating low-cost, widely available adjuvant strategies that may reduce ovarian injury during gonadotoxic therapy [7].

Magnesium is an essential element playing a role in numerous enzymatic and cellular processes relevant to redox balance and inflammation. Across experimental and clinical literature, magnesium status has been linked to inflammatory regulation and oxidative-stress biology, although effects on oxidative-stress biomarkers vary by context [8]. Magnesium glycinate (also described in the literature as magnesium (di)glycinate/bisglycinate) is an organic chelated form often selected for comparatively favorable absorption and gastrointestinal tolerability; classic human absorption work suggests that magnesium diglycinate may be absorbed, at least partly, via peptide-associated transport mechanisms in the intestine [9]. Despite these properties, the potential of magnesium glycinate to mitigate CP-induced ovarian toxicity has not been adequately characterized.

Therefore, the current study investigated the protective and therapeutic effects of magnesium glycinate against CP-induced ovarian toxicity in Wistar rats, with emphasis on endocrine (hormonal) assessment and histopathological evaluation of ovarian tissue architecture.

MATERIALS AND METHODS

Chemicals and reagents

Magnesium glycinate (250 mg tablets; Vitacost, New York, USA) and cyclophosphamide for injection (xGen Pharmaceuticals DJB, Inc., USA) were obtained commercially. Rat-specific Enzyme-Linked Immunosorbent Assay (ELISA) kits for the quantitative determination of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) were procured from strictly validated commercial suppliers. All histological stains (Hematoxylin, Eosin, Masson's Trichrome reagents, and Periodic Acid-Schiff reagents) and other laboratory chemicals utilized in this study were of the highest analytical grade.

Experimental animals and ethical approval

Forty-eight (48) adult female Wistar rats (eight weeks old; body weight 150–170 g) were obtained from certified animal breeders in Iwo, Osun State, Nigeria. The animals were housed in standard polypropylene cages (45 × 24 × 20 cm) under controlled laboratory conditions, including an ambient temperature of 22 ± 2°C, relative humidity of 50 ± 5%, and a 12-hour light/dark cycle. Standard pelleted rodent chow (Top Feeds®, Nigeria) and clean drinking water were provided *ad libitum*.

After a two-week period of acclimatization to the laboratory environment, the rats were randomly separated into six experimental groups (n = 8 per group). All experimental protocols were executed in strict compliance with the European Council Directive 2010/63/EU regarding the protection of animals used for scientific purposes.

Ethical approval was granted by the Animal Research Ethics Committee of the Faculty of Basic Medical Sciences, University of Ilesa, Osun State, Nigeria (Approval Code: UNILESA/FBMS/2025/04). The study design and reporting followed the ARRIVE guidelines (ARRIVE 2.0).

Experimental design and drug administration

Following acclimatization, the 48 female Wistar rats were allocated into six groups and treated for 21 days consecutively as follows:

Group A (Control): Received distilled water and normal saline orally and intraperitoneally (i.p.), respectively.

Group B (MgG Low Dose): Received a single dosage of magnesium glycinate at 22.8 mg/kg orally by gavage, daily.

Group C (MgG High Dose): Received a single dosage of magnesium glycinate at 32.8 mg/kg orally by gavage, daily.

Group D (CP Only): Received cyclophosphamide at 150 mg/kg i.p. on days 1, 3, and 5 to induce severe ovotoxicity.

Group E (MgG Low Dose + CP): Co-administered cyclophosphamide (150 mg/kg i.p. on days 1, 3, 5) and magnesium glycinate (22.8 mg/kg orally, daily).

Group F (MgG High Dose + CP): Co-administered cyclophosphamide (150 mg/kg i.p. on days 1, 3, 5) and magnesium glycinate (32.8 mg/kg orally, daily).

Magnesium glycinate dosing was adapted from established literature protocols [10]. Cyclophosphamide was dissolved in normal saline and administered to establish a cumulative ovotoxic insult model. Body weights were systematically recorded weekly using a calibrated electronic balance to monitor systemic toxicity.

Sample collection

On day 22, following an overnight fast, animals were humanely euthanized by cervical dislocation under light anesthesia. Blood samples were immediately collected via cardiac puncture into plain, non-heparinized tubes. The blood was left for 30 minutes at room temperature in order to allow it to clot, after which it was subsequently centrifuged at 3,000 rpm for 15 minutes to separate the serum. Serum samples were carefully aspirated and stored at -20°C for subsequent hormonal analyses. Immediately following blood collection, the ovaries were excised, cleared of adherent adipose tissue, washed in ice-cold physiological saline, and fixed in 10% neutral buffered formalin for histological processing.

Biochemical evaluation of reproductive hormones

Serum concentrations of the primary gonadotropins, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), were quantified using specific rat

ELISA kits in accordance with the manufacturer's rigorous operating instructions. Assays were based on the principle of competitive binding, and optical densities were measured spectrophotometrically using a microplate reader. The final hormone concentrations were calculated using standard curves generated concurrently with the samples and expressed as mIU/ml.

Histological and histochemical processing

Fixed ovarian tissues were dehydrated through a graded series of ethanol, cleared in xylene, and embedded in paraffin wax. Serial sections (5 µm thickness) were cut using a rotary microtome and mounted on positively charged glass slides.

Hematoxylin and Eosin (H&E) staining: Sections were deparaffinized, rehydrated, and stained with Mayer's hematoxylin and counterstained with eosin to evaluate general ovarian cytoarchitecture, including follicular development, vascular congestion, and interstitial hemorrhage.

Masson's Trichrome (MT) staining: To specifically assess the extent of CP-induced stromal fibrosis and collagen deposition, sections were stained utilizing a standard Masson's Trichrome protocol. Collagenous fibrous tissue was identified by distinct aniline blue staining against red-stained cytoplasm and erythrocytes.

Periodic Acid-Schiff (PAS) staining: To evaluate the integrity of critical glycoprotein barriers, sections were oxidized with periodic acid and treated with Schiff's reagent. This histochemical technique prominently highlighted the basement membranes and zonae pellucidae in a vivid magenta/deep pink.

All stained sections were cover-slipped using DPX mounting medium and examined using a Sellon-Olympus trinocular light microscope (XSZ-107E, China) at ×400 magnification. Photomicrographs were acquired using an integrated digital camera system.

Statistical analysis

All quantitative data are expressed as the mean ± standard error of the mean (SEM). Statistical evaluation was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test to ascertain statistically significant inter-group differences. All analyses were conducted utilizing GraphPad Prism software (version 9.0; GraphPad Software, San Diego, CA, USA). A *p*-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of magnesium glycinate on reproductive hormones (FSH and LH)

Serum concentrations of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) were quantified to assess endocrine function (Figures 1 and 2). Cyclophosphamide (CP) intoxication induced a significant reduction in serum FSH and LH levels compared to the control group ($p < 0.05$), indicating severe disruption of the hypothalamic-pituitary-gonadal axis. Administration of MgG alone significantly elevated basal LH secretion while moderately depressing FSH levels. Co-administration of MgG with CP effectively attenuated CP-induced endocrine suppression. Specifically, MgG co-treatment significantly increased LH concentrations in the CP-only group and significantly upregulated FSH secretion, preserving the gonadotropin milieu necessary for follicular maturation.

Effect of magnesium glycinate on ovarian histoarchitecture

Histological evaluation via Hematoxylin and Eosin (H&E) staining demonstrated that MgG dose-dependently mitigated CP-induced structural alterations (Figure 3). Control and MgG-alone ovaries exhibited normal histoarchitecture, featuring a well-defined stroma and multi-stage follicular development, including intact preovulatory follicles. In contrast, CP intoxication caused severe ovotoxicity, characterized by massive interstitial hemorrhage, vascular congestion, edematous vacuolation, and extensive architectural disruption. Co-administration of low-dose MgG provided partial protection, reducing hemorrhage and supporting limited follicular survival. High-dose MgG concurrent treatment effectively prevented CP-induced hemorrhagic necrosis, preserving normal ovarian architecture and stromal integrity.

Attenuation of cyclophosphamide-induced stromal fibrosis

Masson's Trichrome (MT) staining was utilized to assess stromal fibrosis and collagen deposition (Figure 4). Control and MgG-alone groups displayed physiological collagen distribution restricted to the theca externa and supportive stroma. CP intoxication precipitated profound stromal fibrosis, indicated by dense, irregular collagen bands replacing the healthy extracellular matrix, alongside pervasive interstitial hemorrhage. Low-dose MgG provided moderate anti-fibrotic attenuation. Conversely, high-dose MgG effectively suppressed CP-induced pathological collagen cross-linking, maintaining extracellular matrix integrity comparable to healthy controls.

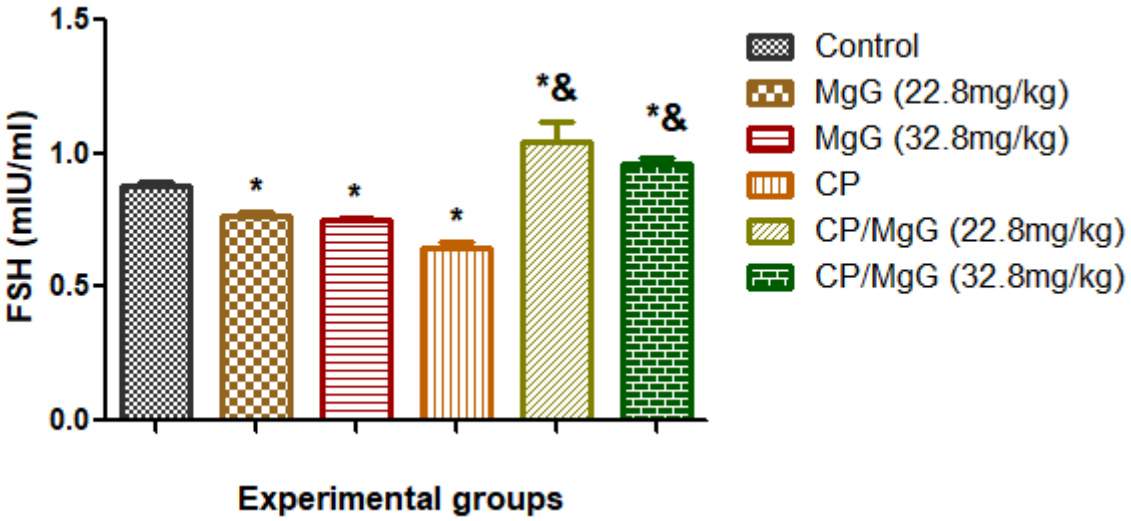

Figure 1: Effect of magnesium glycinate on serum concentrations of Follicle Stimulating Hormone (FSH) in cyclophosphamide-intoxicated Wistar rats.

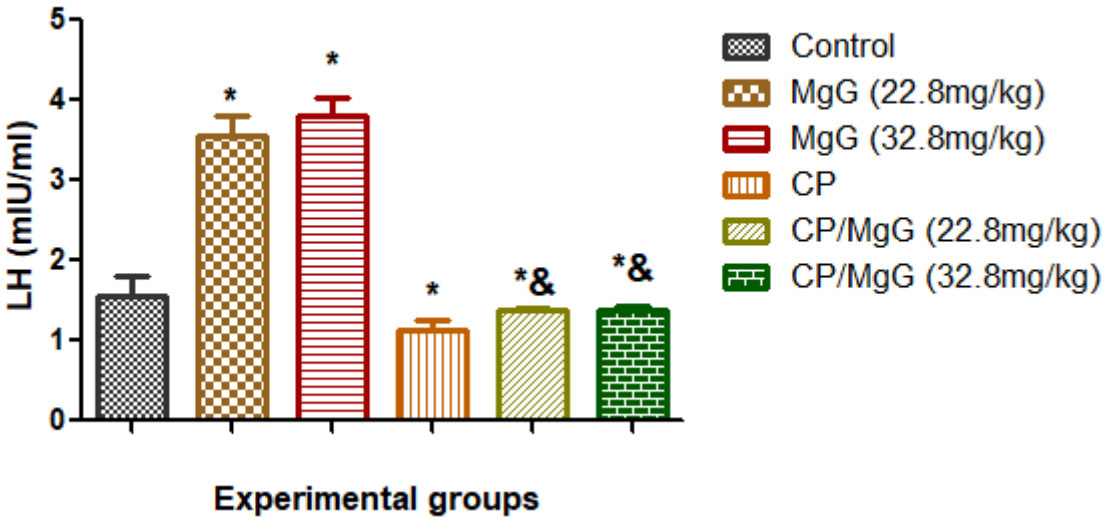

Figure 2: Effect of magnesium glycinate on serum concentrations of Luteinizing Hormone (LH) in cyclophosphamide-intoxicated Wistar rats.

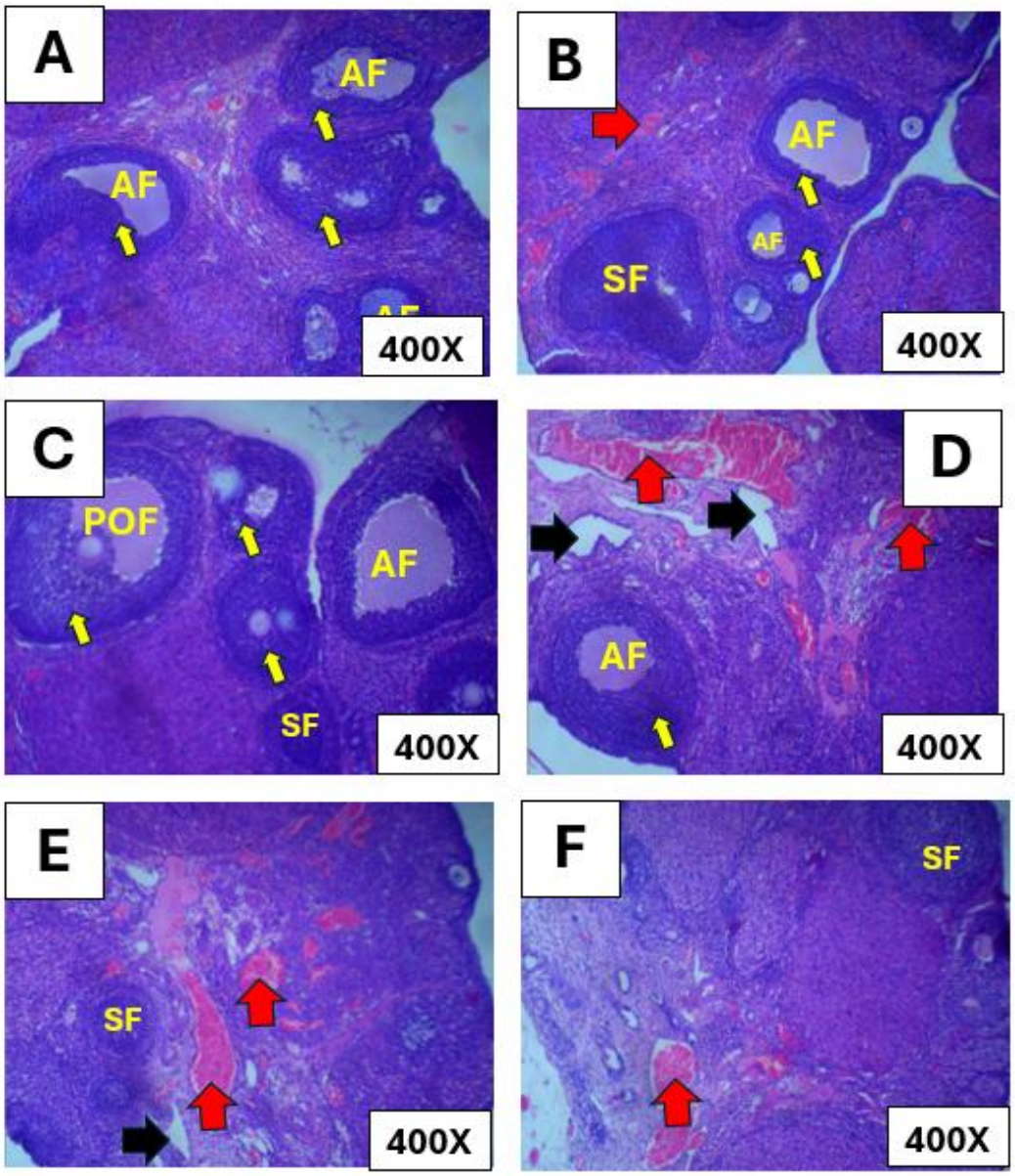

Figure 3: Histopathological evaluation of ovarian tissue (H&E stain, 400X). Groups A, B, and C (Normal Control, MgG Low Dose, and MgG High Dose), respectively, exhibited normal ovarian histoarchitecture with healthy antral follicles (AF) and intact basement membranes (BM), indicating an absence of toxicity. Group D (CP-intoxicated) displayed a severe toxicity characterized by massive interstitial hemorrhage (red arrows), vascular congestion (BV), profound edematous spaces, and loss of tissue architecture (black arrows). (E) MgG Low Dose + CP group showed a partial cytoprotection with mild to moderate vascular congestion (red arrows) and localized edematous spaces (black arrow). While group F **(**MgG High Dose + CP) demonstrated robust preservation of ovarian stroma (OS) and multiple healthy secondary follicles (SF), closely resembling the control architecture.

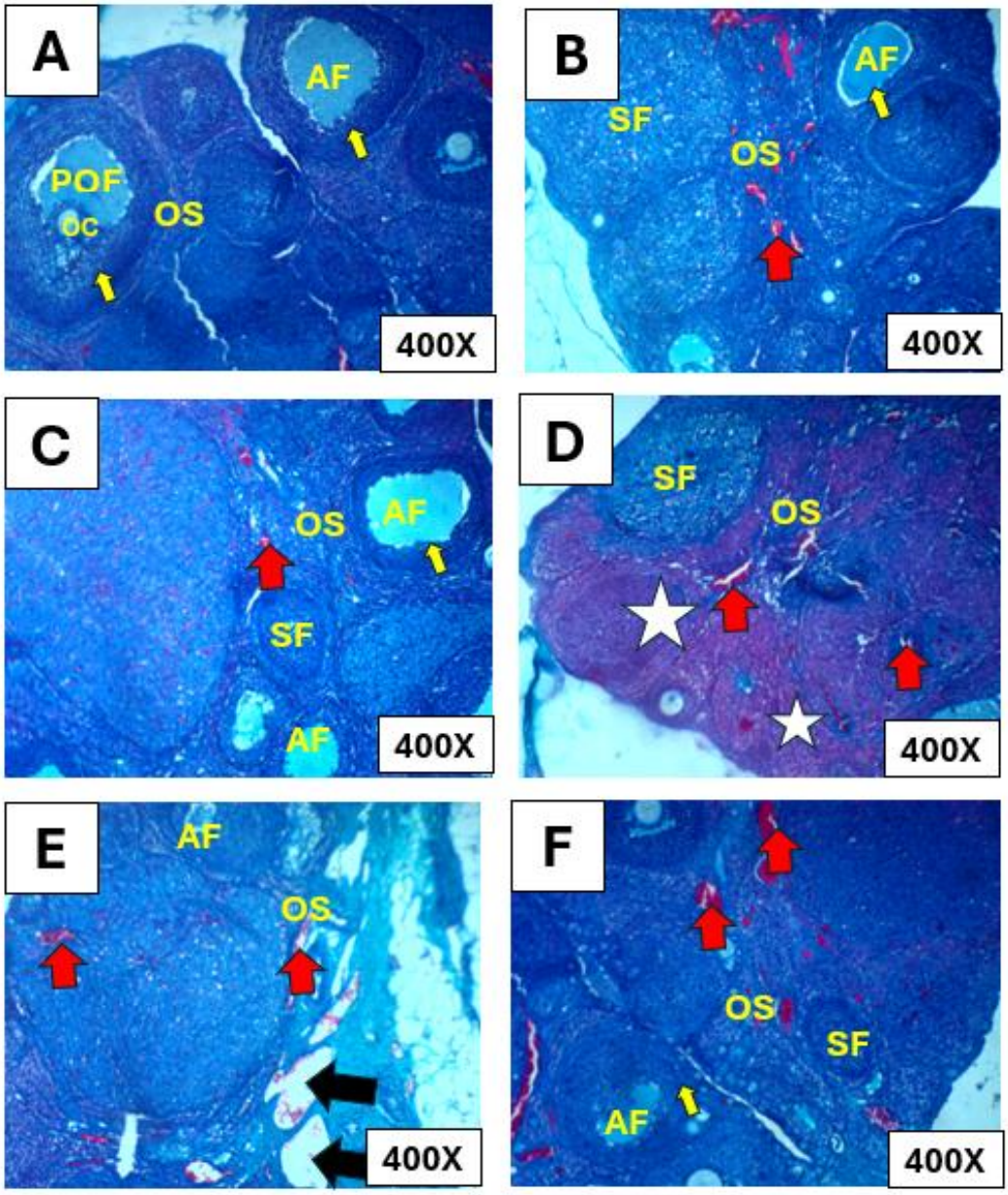

Figure 4: Assessment of ovarian stromal fibrosis (Masson's Trichrome stain, 400X). Collagen fibers stain blue, while cytoplasm and erythrocytes stain red. **(A-C)** Control and MgG-treated groups display delicate, orderly collagen distribution within the normal stroma (OS) and around healthy follicles (POF, AF, SF). **(D)** CP-intoxicated group reveals extensive pathological alterations, denoted by white stars, highlighting areas of massive interstitial hemorrhage (red arrows), admixed with disorganized, dense fibrotic scarring. **(E)** MgG Low Dose + CP group exhibits partial anti-fibrotic protection, though localized hemorrhage (red arrows) and tissue tearing (black arrows) remain evident. **(F)** MgG High Dose + CP group shows marked attenuation of CP-induced fibrosis, with normalized collagen distribution and preservation of the supportive stromal matrix (OS) around developing follicles (AF, SF).

Preservation of critical glycoprotein barriers

Periodic Acid-Schiff (PAS) staining evaluated the integrity of glycoprotein-rich microanatomical barriers (Figure 5). Control and MgG-treated ovaries exhibited intact, continuous PAS-positive basement membranes and distinct zonae pellucidae surrounding healthy oocytes. CP exposure induced severe degradation of these barriers, resulting in the loss of basement membrane boundaries and widespread structural smearing. MgG co-administration dose-dependently mitigated this degradation. Specifically, high-dose MgG preserved the glycoprotein integrity of both the follicular basement membranes and the zona pellucida, effectively preventing CP-induced barrier dissolution.

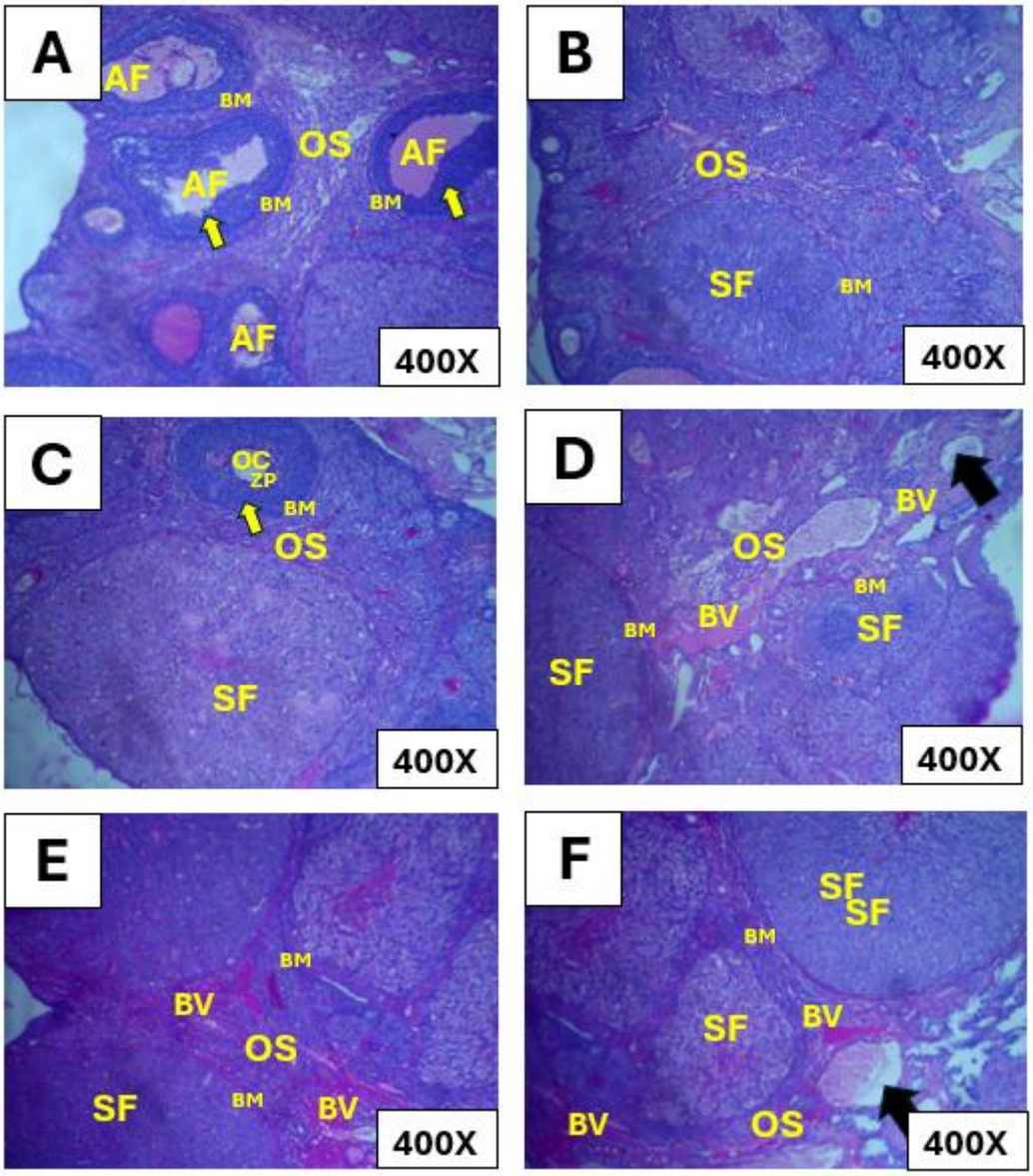

Figure 5: Evaluation of glycoprotein barrier integrity (Periodic Acid-Schiff stain, 400X). PAS-positive structures (glycoproteins) stain vivid magenta. **(A-C)** Control and MgG-treated groups present crisp, continuous basement membranes (BM) delineating the follicles (AF, SF, POF). Notably, Panel C highlights an intensely PAS-positive, intact zona pellucida (ZP) surrounding the oocyte (OC). **(D)** The CP-intoxicated group exhibits severe degradation of critical glycoprotein barriers, characterized by the loss of distinct basement membrane boundaries, widespread architectural smearing (black arrows), and significant erythrocyte pooling (red arrows). **(E)** MgG Low Dose + CP group shows moderate structural preservation, though some disruption persists (red arrows). **(F)** MgG High Dose + CP group demonstrates excellent preservation of glycoprotein integrity, retaining distinct PAS-positive basement membranes around surviving follicles and minimizing CP-induced barrier degradation.

DISCUSSION

The results from this study indicate that cyclophosphamide (CP) induces severe, multi-level endocrine and ovarian toxicity in adult female Wistar rats, and that co-administering magnesium glycinate (MgG) mitigates these effects in a dose-dependent manner. Histological evaluation of CP-treated tissues revealed extensive degradation, including massive interstitial hemorrhage, vascular congestion, stromal fibrosis, and the breakdown of glycoprotein-rich microenvironments. Alongside these structural changes, CP exposure led to significant reductions in circulating follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels. Together, these findings support a model of CP-induced ovotoxicity characterized by acute vascular damage, endocrine disruption, follicular apoptosis, and extracellular matrix (ECM)

remodeling. They also demonstrate the antioxidant, anti-inflammatory, and cytoprotective properties of MgG in countering these pathological processes [11-15].

The mechanisms underlying this ovotoxicity depend on the hepatic bioactivation of CP into reactive metabolites, specifically acrolein and phosphoramide mustard[11]. As a potent alkylating agent, phosphoramide mustard induces DNA cross-linking, which leads to the apoptotic death of metabolically active and highly proliferative follicular somatic cells [16]. Simultaneously, the highly reactive electrophile acrolein causes severe oxidative damage by depleting intracellular glutathione (GSH) stores and increasing reactive oxygen species (ROS) production [12,16]. This combined genotoxic and oxidative stress disrupts mitochondrial function and triggers intrinsic apoptotic pathways, resulting in granulosa cell death, follicular atresia, and subsequent depletion of the ovarian reserve [12,15].

A major component of the observed toxicity was the severe alteration of ovarian histoarchitecture. Hematoxylin and eosin (H&E) staining of CP-exposed tissues displayed extensive follicular atresia, edematous vacuolation, and severe interstitial hemorrhage. The presence of pronounced hemorrhage and vascular congestion suggests that CP-induced oxidative stress heavily impacts the ovarian microvasculature, leading to endothelial dysfunction and the loss of capillary integrity [12]. The resulting structural collapse of the follicle disrupts the essential paracrine communication between the oocyte and surrounding somatic cells, which is necessary for follicular survival. Co-administering MgG, particularly at higher doses, prevented this hemorrhagic necrosis and maintained the viability of multiple secondary and preovulatory follicles. Because magnesium is critical for regulating vascular tone, maintaining endothelial function, and protecting cellular membranes from lipid peroxidation [13], the preserved morphology in MgG-treated subjects likely results from a combination of reduced microvascular damage and decreased granulosa cell apoptosis.

In addition to cellular and vascular damage, CP initiated pathological ECM remodeling. Masson's trichrome staining showed marked stromal fibrosis in the CP-treated groups. Fibrosis compromises the follicular niche by altering tissue stiffness and disrupting cell-matrix interactions, which directly impairs folliculogenesis [14]. This fibrotic response is generally stimulated by inflammatory and oxidative signaling that upregulates mediators like transforming growth factor-beta (TGF-β), leading to fibroblast activation and excessive collagen deposition[17]. High-dose MgG supplementation significantly reduced this collagen accumulation. This suggests that MgG interferes with the oxidative stress and inflammation pathways before fibroblast activation, preventing abnormal ECM cross-linking and maintaining the stromal framework necessary for normal follicular growth [13].

The protection of the structural microenvironment was also evident in periodic acid-Schiff (PAS) stained sections, which showed extensive degradation of essential carbohydrate-rich structures, specifically the zona pellucida and follicular basement membrane in CP-treated ovaries [18]. An intact basement membrane is required to regulate nutrient transport and separate the avascular granulosa layer from the vascularized theca layer. Similarly, the zona pellucida provides a critical glycoprotein matrix necessary for early embryonic protection, sperm binding, and oocyte competence [19]. The breakdown of these structures is likely mediated by direct oxidative damage as well as the inflammatory activation of matrix metalloproteinases (MMPs)[20]. Co-administration of MgG effectively maintained these PAS-positive boundaries, demonstrating that preserving the extracellular microenvironment is as critical to ovoprotection as maintaining cellular viability [19].

The histological condition of the ovary is closely linked to its endocrine output. The marked decrease in circulating FSH and LH levels following CP exposure indicates a severe disruption of the hypothalamic-pituitary-ovarian (HPO) axis [11]. The absence of corpora lutea and preovulatory follicles in the H&E sections of the CP-only group aligns with this lack of a biochemical LH surge. Additionally, CP exposure alters the ultrastructure of pituitary gonadotrophs, which contributes to central hypogonadotropism [21]. The suppressed hormone levels thus result from both intraovarian steroidogenic failure and direct central pituitary toxicity [15,22]. The ability of MgG to elevate gonadotropin levels and stimulate an LH surge is supported by the corresponding reappearance of healthy preovulatory follicles in the tissue sections. However, as this study did not measure ovarian reserve markers like anti-Müllerian hormone (AMH) or ovarian steroids such as estradiol, determining the exact timeline and contribution of central pituitary versus peripheral ovarian recovery requires further investigation [15].

The dose-dependent effectiveness of MgG, which was optimal at the high-dose level, aligns with established pharmacological principles. During severe toxicological exposure, endogenous antioxidant systems are rapidly depleted, requiring sufficient exogenous cofactors to restore redox balance [13]. Magnesium glycinate is an organic amino acid chelate, a formulation chosen because organic magnesium salts exhibit greater systemic bioavailability than their inorganic counterparts [23]. Additionally, the glycine component possesses independent anti-inflammatory and cytoprotective

properties, primarily through the modulation of inflammatory pathways such as NF-κB [24]. The combined effects of magnesium on vascular and redox stability, alongside glycine's anti-inflammatory actions, explain the effective preservation of endocrine function and ovarian histoarchitecture observed in this study. These results indicate that MgG serves as a viable adjunct therapeutic option for mitigating chemotherapy-induced reproductive toxicity.

CONCLUSION

Cyclophosphamide induces severe, multi-level ovotoxicity in female Wistar rats. However, co-administration of magnesium glycinate, particularly at the high-dose regimen, robustly mitigates these pathological alterations, making magnesium glycinate a highly promising adjunct therapeutic candidate for the preservation of ovarian structural integrity and reproductive endocrine function during gonadotoxic chemotherapy.

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