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Primary Ovarian Insufficiency; Cyclophosphamide; Leuprolide; cGAS-STING pathway; H-151, Ovarian Reserve.

The Possible Protective Effects of leuprolide against Cyclophosphamide-Induced ovarian toxicity Via Modulation of the Cgas-STING Pathway in Rats.

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ABSTRACT: Premature Ovarian Insufficiency (POI) is defined as a premature loss of ovarian activity that results in low levels of estrogen, loss of follicles and infertility before age 40. The potent chemotherapeutic agent cyclophosphamide (CTX) causes significant gonadotoxic effects and early onset of follicular atresia. Recent discoveries suggest that the cyclic GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) pathway is an innate immune sensor of cytosolic double-stranded DNA (dsDNA) and is implicated in downstream inflammation and cellular injury during DNA damage.

Objective: This study aimed to assess the protective effect of Leuprolide acetate (GnRH agonist) in ovarian toxicity induced by CTX through modulating inflammation by the cGAS-STING pathway, and compare its results with the specific STING inhibitor, H-151.

Materials and Methods: Forty eight adult female Albino rats (6-8 weeks old) were divided into six different experimental groups (n=8 per group): Control, Cyclophosphamide , Leuprolide protective , Leuprolide with cyclophosphamide , STING Inhibitor group , and H-151 + Cyclophosphamide . Anti-Müllerian Hormone (AMH) and Autophagy progression was evaluated by the quantification of the hormone using ELISA. RT-qPCR was used to measure the gene expression of the downstream nuclear factor-kappa B (NF-ϰB). Immunohistochemical scoring and light microscopy were used to assess apoptotic indexes (Caspase and Bcl-2), and to determine the histopathological architectural changes.

Results: AMH reserve markers were significantly reduced (33.49±5.93) and cell stress mediated LC3B autophagy markers were significantly elevated (6.53±1.28), alongside with enhanced levels of apoptosis markers (Caspase score: 8, Bcl-2 score: 2) after CTX induction. The co-treatment with Leuprolide or H-151 before CTX induction significantly maintained high AMH reserves (74.20±11.84), and restored the baseline expression of homeostatic LC3B (1.39±0.25). In addition, combination therapies limited the fold change in NF-ϰB downstream of the fold, reduced the level of apoptotic signal through the activation of Caspase and maintained primary/secondary follicular morphometrics.

Conclusion: pre-Treatment with Leuprolide is protective against ovarian collapse induced by chemotherapy. This preservation is due to the inhibition of downstream cGAS-STING signalling pathway, which fails to cause chronic inflammatory damage and preserve normal follicular architecture.

Ethical Approval:

This study protocol and experimental handling of animals was approved by the Institutional Ethics Committee, College of Pharmacy, Mustansiriyah University (Approval Reference No. 72).

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INTRODUCTION

Premature Ovarian Insufficiency (also known as Premature Ovarian Failure (POF)) is a clinical condition in which the normal function of the ovary ceases before the age of 40. Pathologically, POI is characterized by either disrupted follicular development or accelerated follicular depletion resulting in severe hypoestrogenism, abnormal menstrual cycle dynamics, amenorrhea or oligomenorrhea, and an increase of serum follicle-stimulating hormone (FSH) levels to menopausal indices. In addition to structural infertility, chronic estrogen deficiency puts POI patients at systemic risk of structural defects, mostly osteoporosis, progressive metabolic syndrome and cardiovascular disease. [1]

Cyclophosphamide (CTX) is a broad-spectrum alkylating agent that is utilized in many clinical oncology protocols and in high doses for advanced autoimmune treatments. Its systemic gonadotoxic side effects, despite its therapeutic success, severely limit its integration. The initiation of double-stranded DNA breaks in highly mitotic granulosa cells, caused by the presence of CTX, results in the extensive process of follicular atresia, disruption of basic ovarian matrix morphology and in general, a decrease in reproductive lifespan . [2]

Recently, cyclic GMP-AMP synthase (cGAS)–stimulator of interferon genes (STING) has emerged as a key link between cell genomic damage and chronic innate inflammatory activation. When the cell ruptures or there is a lot of strain on the mitochondria, the host DNA is released into the cytosol as double stranded DNA. The misplaced dsDNA is recognized by the cytoplasmic enzyme cGAS which catalyzes the production of the secondary messenger cyclic GMP-AMP (cGAMP). This ligand activates STING, an endoplasmic reticulum-resident protein which then moves to the Golgi apparatus. STING activates TANK-binding kinase 1 (TBK1) and phosphorylates interferon regulatory factor 3 (IRF3), while also promoting the activation of nuclear factor-kappa B (NF-ϰB) transcription and release of pro-inflammatory cytokines and Type I interferons (IFN-α/β). [3]

In the setting of cytotoxic chemotherapy, cytoplasmic fragments that accumulate activate the cGAS-STING axis converting acute chemical stress into chronic tissue-damaging microenvironments. Treatment with leuprolide acetate (a synthetic gonadotropin-releasing hormone (GnRH) agonist) can cause complete pituitary desensitization when used continuously. This short-term inhibition of luteinizing hormone (LH) and FSH levels causes ovarian metabolism to become quiescent (non-cycling). This metabolic down-regulation would be expected to protect the granulosa cells from the systemic effects of circulating alkylating chemicals. It is still unclear, however, whether this protection is mediated by crosstalk between the cGAS-STING inflammatory pathway. Thus, the objectives of this study are to compare Leuprolide with the targeted small-molecule STING inhibitor H-151, in an adult rat in the context of hormonal, transcriptomic and morphometric indexes of CTX-induced injury. [4,5]

Study Objectives

to assess the protective role of leuprolide against Cyclophosphamide induced ovarian toxicity in rats by histopathological, molecular and biochemical assessments with special consideration to the cGAS–STING signaling pathway.

METHODS

2.1. Experimental animals and ethical supervision.

Forty-eight healthy adult female Albino rats (6–8 weeks old) were used. The experimental groups were housed in the Laboratory of the College of Pharmacy, Al-Mustansiriyah University(Approval Reference No. 72). All procedures respected national and international guidelines for the care and use of laboratory animals. . The animals were kept in individual custom-built plastic cages in a 12-h light/dark cycle with precisely controlled ambient temperature (25±2∘C) and relative humidity (65±5%). All subjects had access to regular laboratory chow and fresh tap water. All experimental procedures were carried out according to institutional and international guidelines for the Care and Use of Laboratory Animals.

2.2. Experimental design and dosage regimens. [6]

After an acclimatization period, the 48 rats were randomly divided into six experimental groups (8 rats per group) and were treated for 29 days:

Group (Control C): Daily i.p. injection of vehicle (Dimethyl Sulfoxide, DMSO 0.5 mL/kg) was given for 29 consecutive days.

Group (Cyclophosphamide - M): eight rats ware received i.p. DMSO daily for 14 days. To generate the ovarian injury model, one intraperitoneal (i.p.) Cyclophosphamide loading dose (40 mg/kg) IP was given (Day 15) and then 8 mg/kg/day (i.p., maintenance dose) was given from Day 16 to Day 29.

Group (Leuprolide - L): eight rats ware treated with Leuprolide acetate (50 μg/kg/day) subcutaneously (s.c.) for 29 days.

Group (Leuprolide + Cyclophosphamide - D): they received preventive daily s.c. injections of Leuprolide (50 μg/kg/day) from Day 1 to Day 29. On Day 15 animals were given the Cyclophosphamide loading dose (40 mg/kg) IP and on a daily basis the maintenance dose of 8 mg/kg (i.p.) until Day 29.

Group (H-151 H):- The small-molecule STING inhibitor H-151 was given at 10 mg/kg/day (i.p.) for 29 days in group V (STING Inhibitor Baseline - D).

Group (H-151 + Cyclophosphamide - G) received daily i.p. injection of H-151 (10 mg/kg/day) for 29 days. Day 15: CTX loading (40 mg/kg) IP was given, and the daily maintenance injections of 8 mg/kg i.p. CTX were administered until Day 29.

2.3. Tissue Harvesting and Bio sample Processing

Animals were deeply anesthetized on Day 30 by injecting them intraperitoneal with a Ketamine (50 mg/kg) and Xylazine (5 mg/kg). Blood samples (~4 mL) were obtained through direct cardiac puncture, left to clot at room temperature, then centrifuged, and the serum was preserved at −80°C for biochemical analyses. Ovaries were quickly extracted and processed as required downstream for analytical purposes: one ovary was used for molecular stabilization for RT-qPCR, the other one was used for fixative solutions for histopathological and immunohistochemical rendering. [7]

2.4. Biochemical Analysis (ELISA)

Specialized quantitative enzyme-linked immunosorbent assays (ELISA) for Anti-Müllerian Hormone (AMH) which reflects the total primordial reserve pool viability and Microtubule-associated protein 1 light chain 3B (LC3B) which is an explicit measure of induction of cellular autophagy were used for quantitative measurement. serum elements were handled with great care strictly in accordance with the manufacturers' diagnostic procedures and total absorbance was measured using a microplate reader. [9]

2.5 Real-Time PCR (RT-qPCR) to measure NF-ϰB Expression.

For transcriptional validation, the ovarian tissue was briefly washed in ice-cold PBS to remove blood debris and was snap frozen in liquid nitrogen or kept in the RNA stabilization reagent (RNAlater) and stored at −80°C. Total RNA extraction, purity and complementary DNA (cDNA) synthesis was performed using commercial extraction systems. The fold-change expression profiles of Nuclear Factor Kappa B (NF-ϰB) were evaluated by quantitative PCR amplification using specific primer pair sets and normalizing the levels of expression of these specific genes to a housekeeping reference gene. [8]

2.6. Histopathological staining and processing.

Overlain tissues ware Fixed in 10% neutral buffered formalin, then dehydrated in ascending grades of ethanol, cleared in xylene, and embedded in solid paraffin blocks. Thin cross-sections (4-5 μm) were prepared using a rotary microtome, mounted on conventional glass slides and stained with conventional Hematoxylin and Eosin (H&E) procedures. Qualitative assessment of slides was performed under light microscope to monitor changes in structures, luteinization status and absolute development parameters of primary, secondary and mature follicles. [10]

2.7. Immunohistochemical (IHC) Assessment of the concentration of Caspase and Bcl-2 protein is analyzed by.

Paraffinized sections were mounted on charged slides, cleared and rehydrated for immunohistochemical evaluation. The antigen retrieval process was carried out using citrate buffer and then exposed to anti-apoptotic regulator Bcl-2 and anti-executioner apoptotic enzyme Caspase-3. Subsequent sections were developed using an antibody that was coupled to a horse-radish peroxidase-chromogen (DAB) matrix system. Immuno-positive dynamics were localized using light microscopy of specific brown granular cytoplasm/nuclear patterns. [11]

The Scoring System was used for semi-quantitative analysis. The total allocation index was calculated by adding the Percentage of Positive Cells Score (0-5) and the Staining Intensity Score (0-3) to give an overall expression range of 0 to 8:

. Percentage Score Criteria: 0 = No positive staining; 1 = <1/100 cells positive; 2 = 1/100 to 10/100 cells positive; 3 = 10/100 to 30/100 cells positive; 4 = 1/3 to 2/3 cells positive; 5 = >2/3 cells positive.

Intensity Score Criteria: (0 = Negative, 1 = Weak staining, 2 = Moderate staining, 3 = Strong staining)

2.8. Statistical Analysis

The data was analyzed with SPSS software. The quantitative endpoints were recorded as Mean ± Standard Deviation (SD). One-way Analysis of Variance (ANOVA) was used to validate differences among multi-group variances with Tukey's post hoc testing being used to determine intra-group differences. A p-value of <0.05 was used as the criterion for statistical significance**.** [12]

RESULTS

3.1. The quantification of Ovarian Reserve (OR) with Serum AMH Profiles.

The findings of the serum AMH tracking revealed significant follicular destruction in the Cyclophosphamide induction group (Group M) with the mean AMH level reaching a low point in the study (33.49±5.93) versus normal AMH level in the healthy Control group (92.95±8.76). Robust AMH baseline was maintained with both Leuprolide (Group L) and H-151 as monotherapies. Most importantly, the AMH level of 77.35±10.16 was well maintained in the protective application of Leuprolide against CTX (Group H), which was very significant in preserving the follicular pool. This protection outperformed the H-151 + CTX combination (Group D, 55.94±13.51) and the G group (44.79±7.78),in Table 1. And figure 1

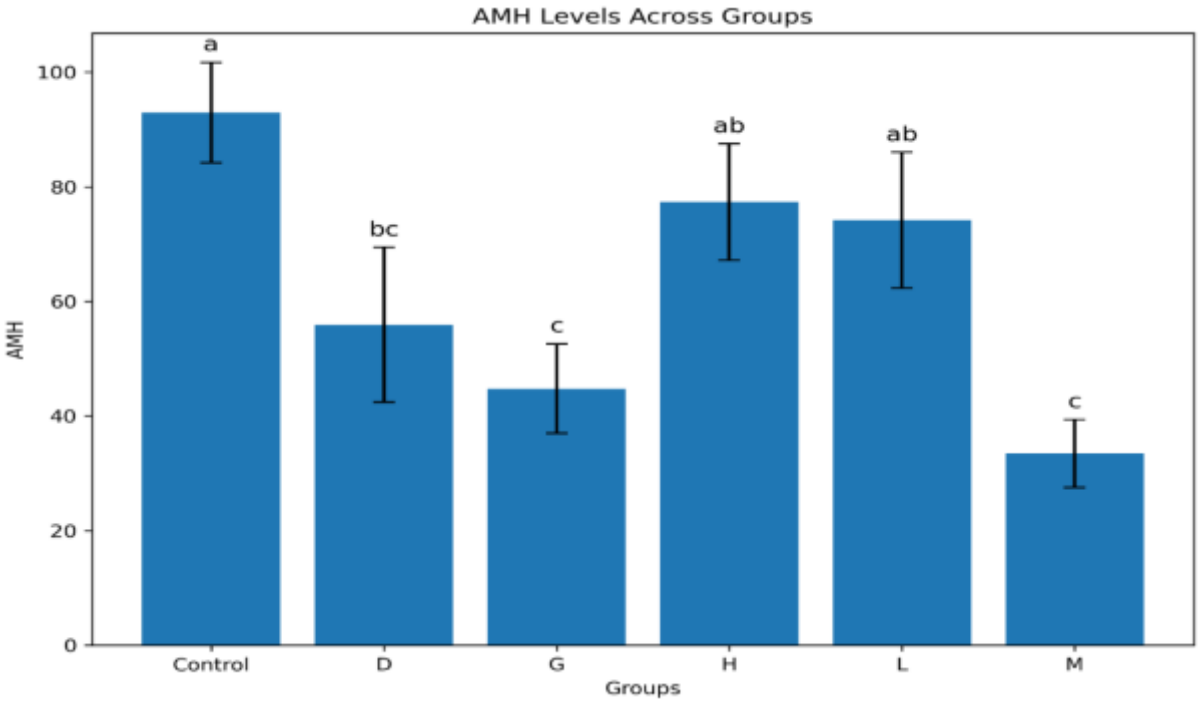


Figure 1 the effect of serum AMH hormone on ovarian function

3.2. Assessment of the induction of Cellular Autophagy (LC3B Markers).

LC3B was evaluated using ELISA and the normal baseline turnover of autophagy was seen in Control group (1.70 ± 0.16). The level of LC3B accumulation was significantly increased by CTX toxicity (Group M: 6.53±1.28, P<0.0001), which suggests that the cells were stressed and accelerated macroautophagy. However, the combined treatment of Leuprolide did not allow this increase to occur: in the two experimental groups treated with Leuprolide, LC3B expressions were respectively 1.39±0.25 (Group L) and 1.34±0.16 (Group H) which are not statistically different from the healthy value. Both the STING

inhibitor arms (Groups G and D) maintained at 2.09±0.63 and 2.08±0.33 respectively, were very similar to this down-regulation. Table 2: Autophagy Marker (LC3B) Quantifications

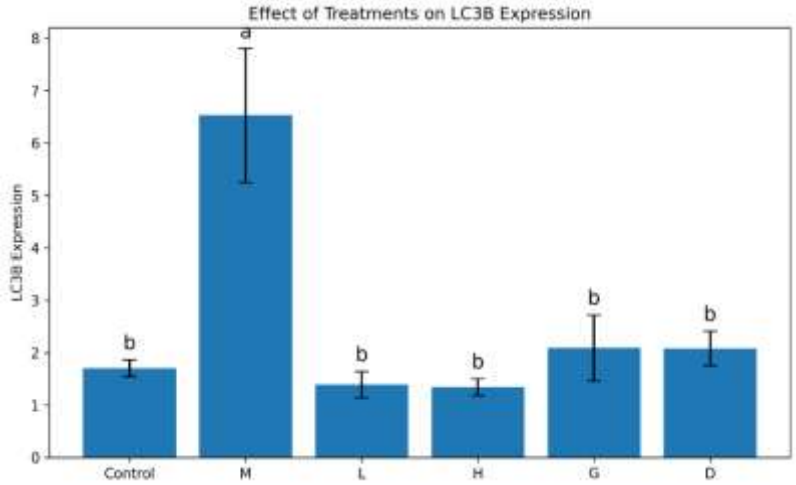
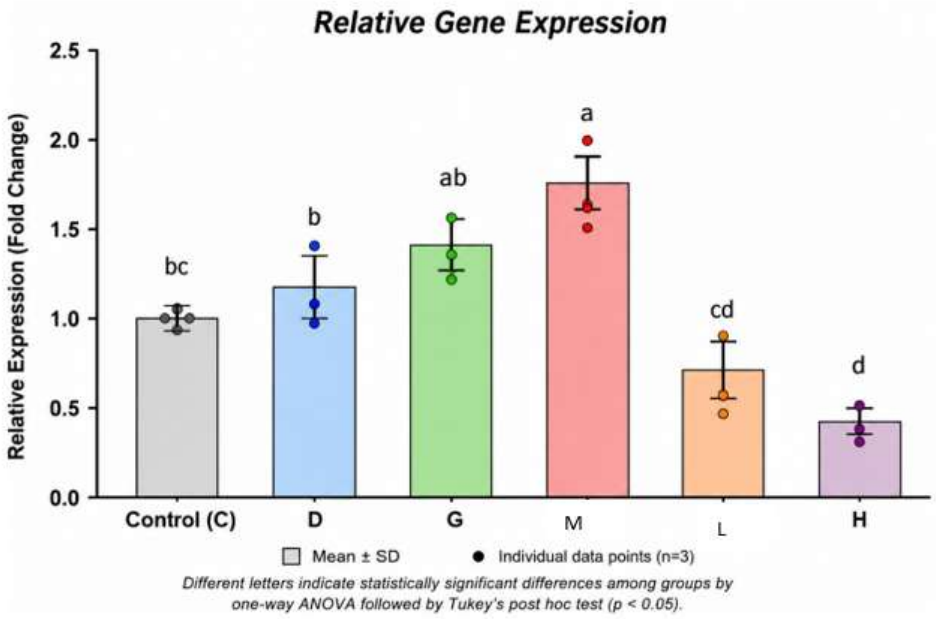


Figure 2 the effect of serum LC3B on overlain function

3.3. The expression of inflammatory transcription factor NF-ϰB.

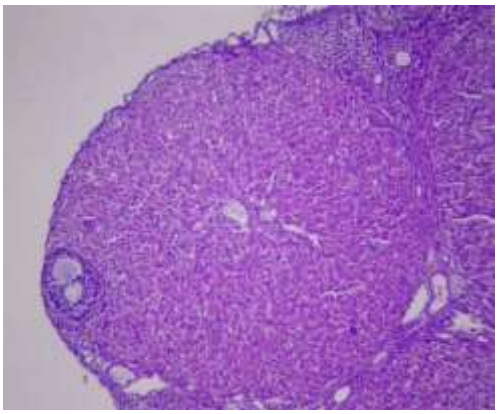
Downstream inflammatory signaling was significantly different as revealed by transcriptional profiling by the use of RT-qPCR (F=13.24, P=0.0072). Group L had a higher fold change of the homeostatic value (1.766±0.225) that was gradually brought down in the combination protocols and in the baseline suppressions. The baseline index, which is the value obtained when the cells are not treated, was an ultra-low value for group H (0.439±0.032), and remained at intermediate values for the chemical STING inhibitor groups (Group G: 1.425 ± 0.091; Group D: 1.203 ± 0.320). The relative NF-ϰB gene expression levels for all experimental groups are summarized in Table 3.

Similarly, targeted STING inhibition via H-151 limited apoptotic cascade activation, as summarized in Table 4.

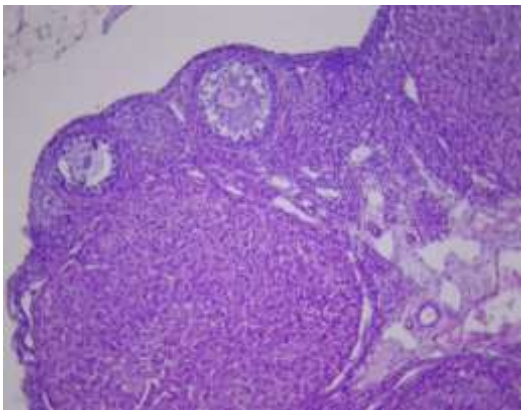


3.4. Light Microscopy (H&E) – Structural Histopathology (Qualitative)

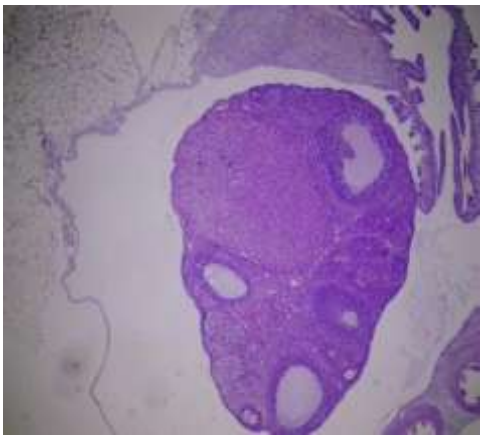
The histopathological examinations of the Control cohort revealed good structural morphology with well-preserved stromal architecture, active luteinization and good distribution of mature follicles with normal oocytes. In group M (Cyclophosphamide) sections, there was extensive tissue degradation with a significant loss of functional follicles, wrinkling of follicles and damaged boundaries of the granulosa. Primary, secondary and mature Graafian systems were observed in protective interventions performed with Leuprolide (Group L) and H-151 (combination) containing drugs, where the follicular borders were clearly defined and the dynamics of expulsion of the oocytes were clear.



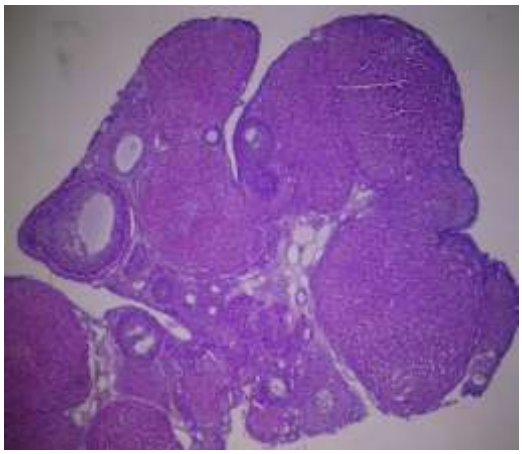
Group C



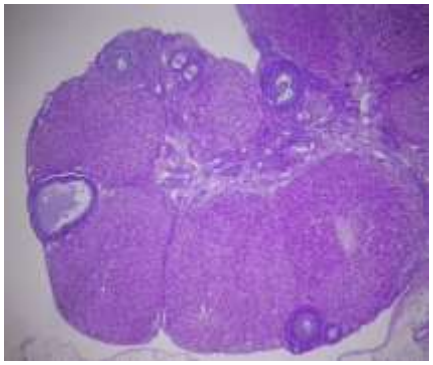
Group M



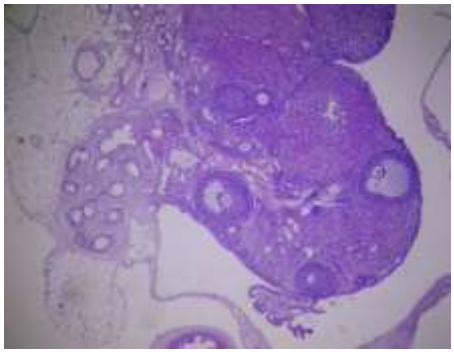
Group L



Group H



Group D



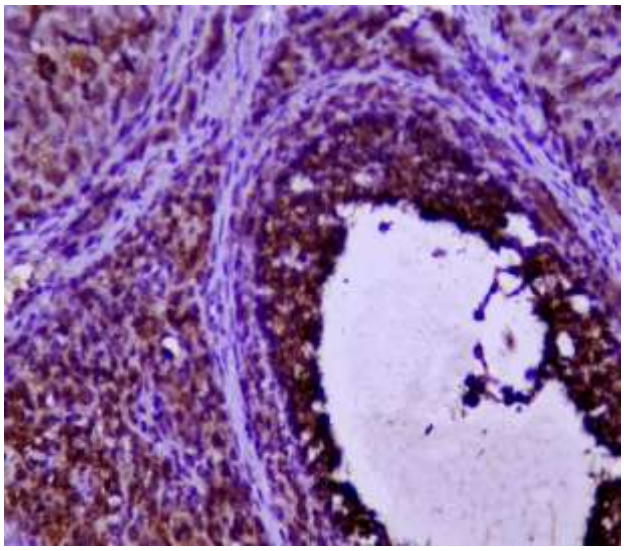
Group G

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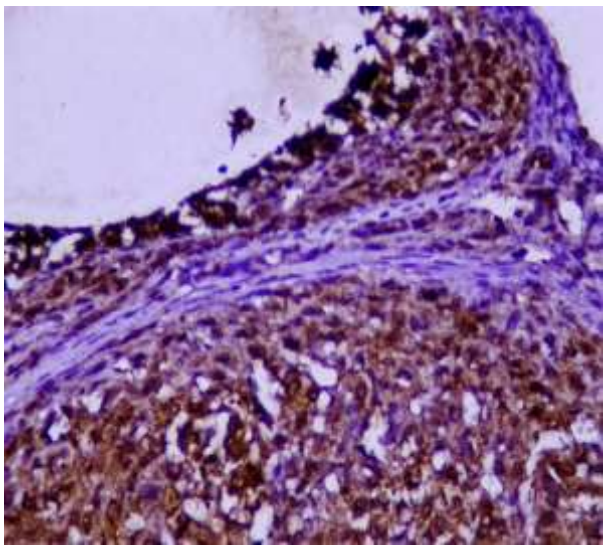
3.5. Immunohistochemical Quantification of Apoptotic Cascade Regulators

Semi-quantitative Allred analysis of Caspase-3 and Bcl-2 proteins confirmed that CTX treatment pushed ovarian tisue toward apoptosis. The CTX injury group (Group M) exhibited intense cytoplasmic/nuclear Caspase-3 expression, reaching an Allred score of 8, while its anti-apoptotic Bcl-2 expression dropped to a study low of 2. Leuprolide protective monotherapy (Group L) effectively reversed this apoptotic profile, reducing the Caspase-3 score to 4 and restoring Bcl-2 levels to 5. Similarly, targeted STING

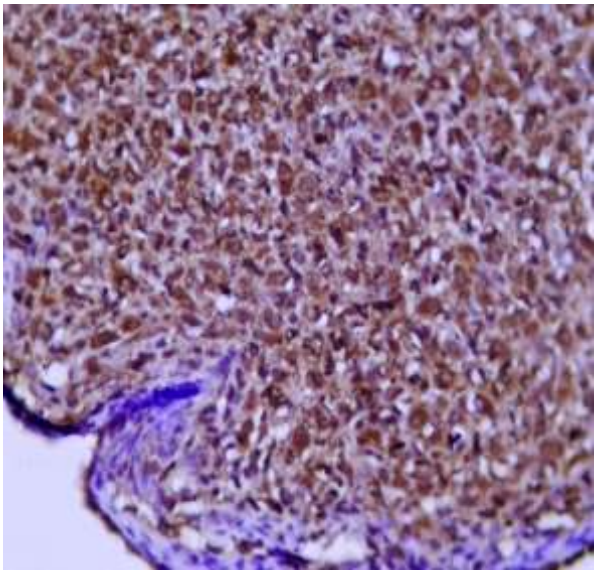
A- Immunohistochemistry for Caspase-3:



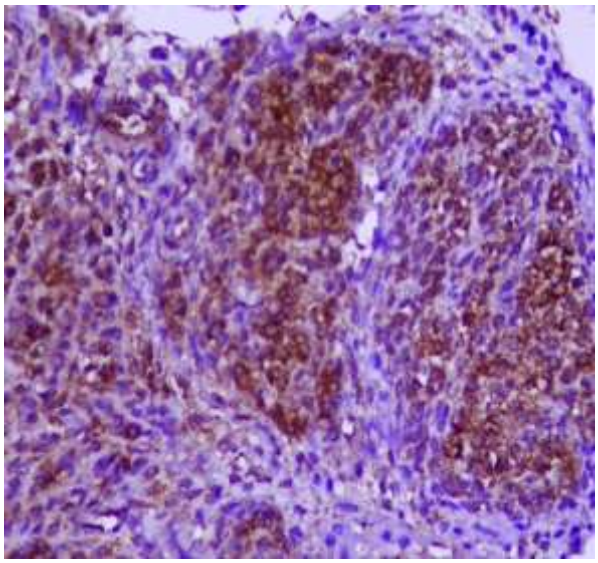
Group C



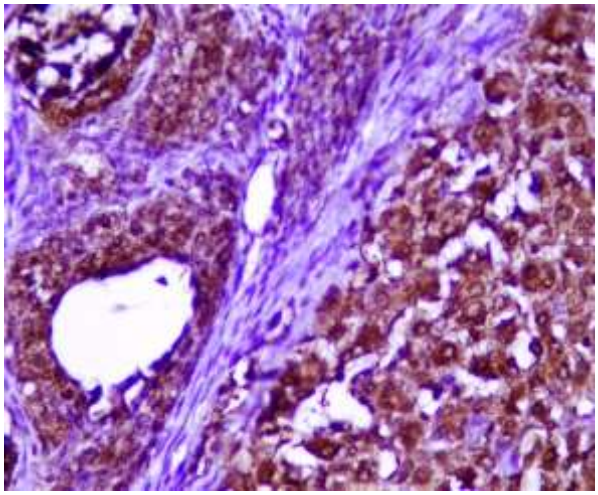
Group M



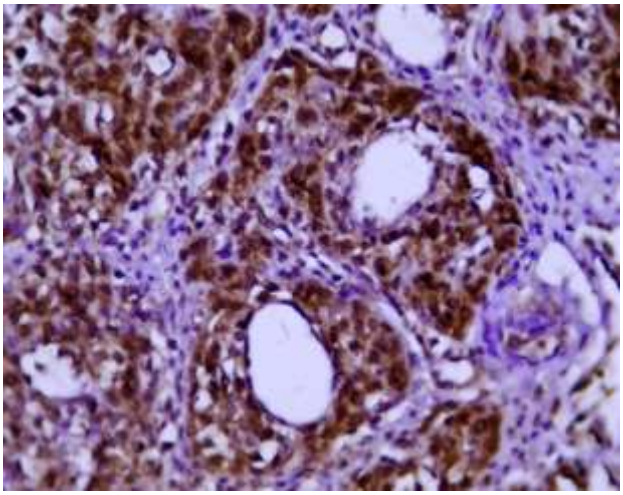
Group L



Group H



Group D



Group G

Caspase Score 4 (IHC)(X400) was demonstrated in control group ovary by Section.

G2 M Induction group: Section showed caspase score 8 in Induction group (IHC)(X400).

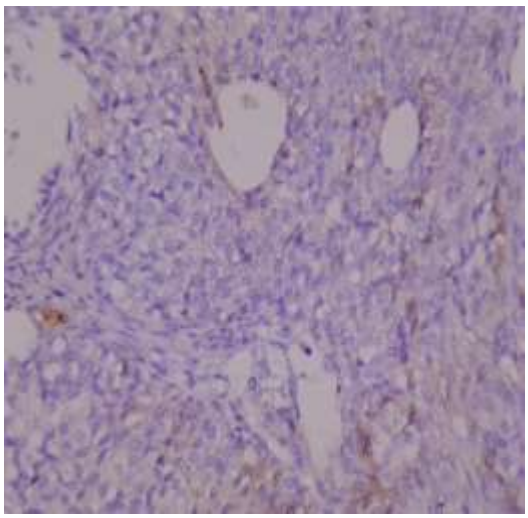
G3 (L) Protective group: Section of caspase score 4, in protective group (IHC) (X400).

G4 (H) Protective group: Section showed ovarian tissue caspase score 4 (IHC)(X400).]

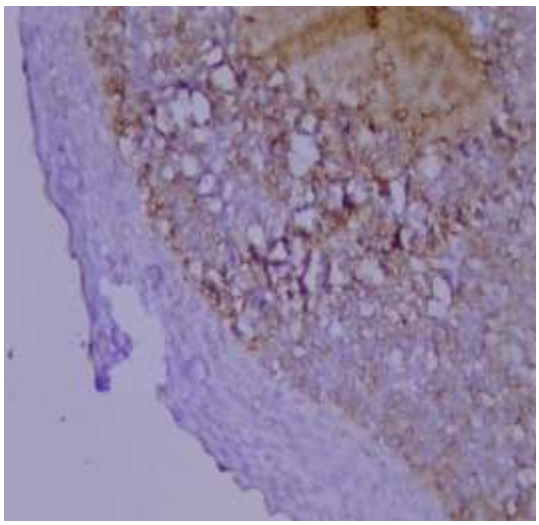
G5 (D): Section of ovary caspase score = 6 (IHC)(X400).

G6 (G): Section of ovary Caspase score = 6 (IHC)(X400)

. **B- Immunohistochemistry for Bcl-2:**

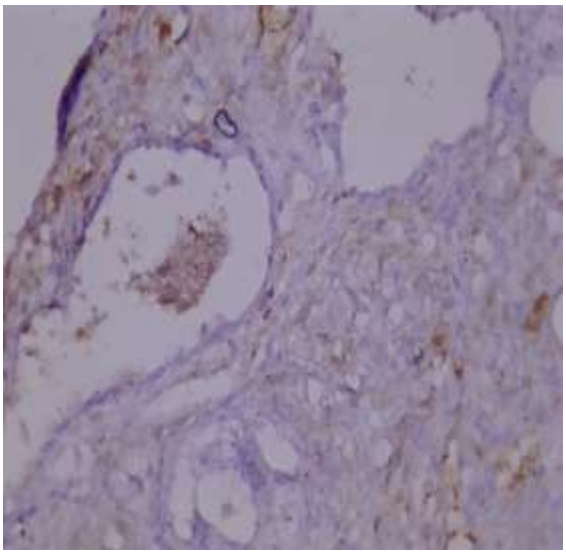


Group C

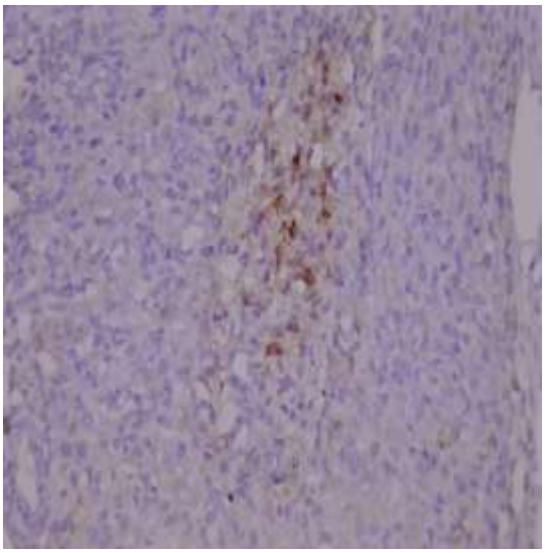


Group M

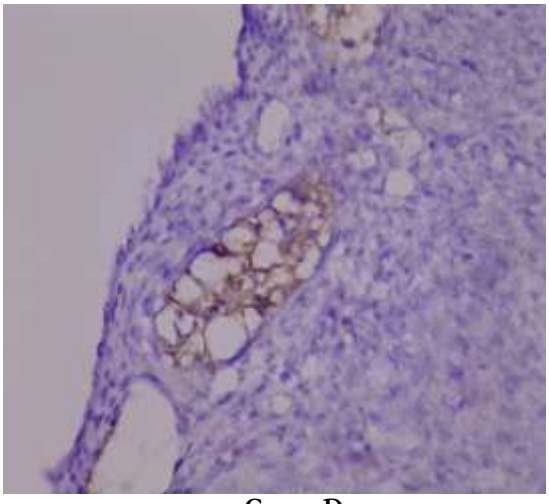
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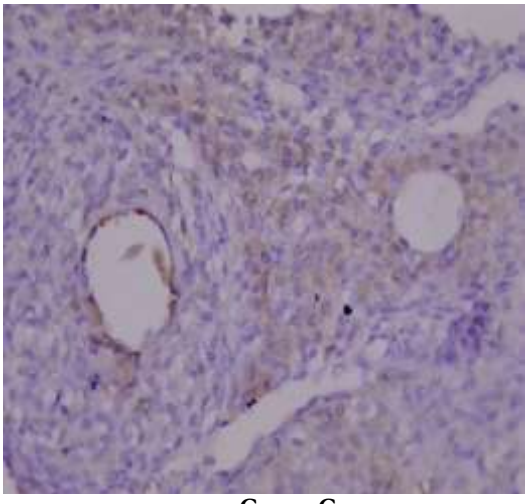
Group L



Group H



Group D



Group G

DISCUSSION

Highly ovary toxic drugs like alkylating agents like cyclophosphamide cause depletion of the follicles and result in acute clinical POI. Traditionally, such damage has been attributed to only direct cross-linking of DNA. Recent evidence, however, has shown that the cGAS-STING innate immune pathway plays a central role in this pathology, and that when cellular damage is caused by the release of DNA, it initiates an inflammatory response that has a destructive, chronic effect. [13]

In this study that is a rapid depletion of the functional ovarian reserve following administration of CTX, as evidenced by a sudden decrease in the levels of AMH in serum. AMH is only produced by granulosa cells in the preantral and small antral follicles and is a good indicator of the total reproductive reserve. The decrease in AMH observed here is consistent with the reported AMH reduction in H&E stained tissues where the follicular layers were reported to be damaged, and there was extensive degradation of the stroma. On the molecular level, activation of the cGAS-STING axis is responsible for this tissue damage. After exposure to CTX, host dsDNA enters the cytoplasm, causing downstream signaling which greatly upregulates inflammatory transcription factor NF-κB, thereby stimulating activity of the apoptotic pathways. [14]

Our data indicate that ongoing treatment with Leuprolide is effective and will help to protect this damage, allowing AMH levels to be maintained and follicular architecture to remain healthy. Leuprolide puts the ovaries into a metabolic rest state, thus

decreasing follicular recruitment and the sensitivity of granulosa cells to alkylating agents. Importantly, Leuprolide also inhibits downstream inflammatory signaling, a process similar to that of the specific STING inhibitor H-151. This treatment strongly reduced the expression of Caspase-3, increased the anti-apoptotic protein Bcl-2 and maintained the autophagy markers (LC3B) at their normal, basal, level. The results show that Leuprolide can protect the primordial follicle pool from chemotherapeutic damage by blocking pathological cGAS-STING activation, which attenuates chronic inflammation. [15]

CONCLUSION

The present study shows that cyclophosphamide causes a marked ovarian damage involving a decrease in ovarian reserve, an increase in autophagy, activation of inflammatory signaling, increase in apoptosis and alteration of ovarian histological architecture. Repeatedly leuprolide acetate significantly diminished these negative effects by maintaining AMH levels, normalizing the expression of LC3B, decreasing NF-κB activation, decreasing the expression of Caspase-3, increasing the expression of Bcl-2, and normalizing follicular morphology. The protective effects of leuprolide were similar to those of the selective STING inhibitor H-151, indicating that modulation of the cGAS – STING signaling pathway is a part of its mechanism of action. In summary, these results suggest that leuprolide could be a potential therapeutic approach to prevent ovarian toxicity during cyclophosphamide treatment and are consistent with a role for cGAS–STING signalling in chemotherapy-related ovarian toxicity. The findings of this study should be confirmed in clinical trials and further investigated to explain the molecular

What is already know on this topic

1-Cyclophosphamide is a known cause of premature ovarian insufficiency through damage to the DNA, follicular depletion and apoptosis.

2-The cGAS–STING pathway is involved in the induction of inflammation and tissue damage after chemotherapy-induced DNA damage.

What this study adds

In addition to its known effects on ovarian suppression, this study shows that leuprolide also can prevent ovarian toxicity by modulating the cGAS–STING/NF-κB signaling pathway caused by cyclophosphamide.

It is the first direct comparison that demonstrates that both treatments maintain ovarian reserve and decrease apoptosis and preserve ovarian histological integrity

Competing interests

The authors declare that they do not have competing interests.

Authors' contributions

Ruaa Allawy Hasan conceived the study, conducted the experimental work, performed animal handling and sample collection, carried out laboratory analyses, analyzed and interpreted the data, prepared the figures and tables, and drafted the initial version of the manuscript.

Prof. Dr. Intesar Tarik Numan supervised the study, contributed to the study design and methodology, interpreted the experimental findings, critically revised the manuscript for important intellectual content, and approved the final version of the manuscript for publication.

Both authors read and approved the final manuscript and agreed to be accountable for all aspects of the work, ensuring the accuracy and integrity of the study.

Tables and figures :-

List all tables and figures included in the manuscript, with their detailed legend:.

Table 1 : Serum Anti-Müllerian Hormone (AMH) concentrations among the experimental groups, presented as mean ± SD with Tukey's post hoc multiple comparison letters indicating statistically significant differences.

Table 2 : Quantitative analysis of LC3B expression as an autophagy marker in ovarian tissue across the experimental groups, expressed as mean ± SD and analyzed using one-way ANOVA followed by Tukey's post hoc test.

Table 3: Relative NF-κB gene expression levels determined by RT-qPCR in ovarian tissues of the different experimental groups, presented as fold change (mean ± SD) with statistical group comparisons.

Table 4: Semi-quantitative immunohistochemical (Allred) scoring of apoptotic markers (Caspase-3 and Bcl-2) in ovarian tissues, including percentage of positive cells, staining intensity, and total Allred scores for each experimental group.

Figure 1 : Serum Anti-Müllerian Hormone (AMH) levels in the different experimental groups. Data are presented as mean ± SD. Different letters above the bars indicate statistically significant differences among groups ($p < 0.05$).

Figure 2 : Effet of cyclophosphamide, léporide, and H-151 treatments on ovarien LC3B expression. Results are presented as mean ± SD, with different letters indicating significant differences among groups ($p < 0.05$).

Figure 3 : Relative NF-κB gene expression (fold change) in ovarian tissue measured by RT-qPCR across the experimental groups. Data are expressed as mean ± SD, and statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test.

Figure 4 : Representative hematoxylin and eosin (H&E)-stained ovarian sections from the experimental groups illustrating histopathological changes in ovarian architecture, follicular integrity, stromal organization, and follicular development.

Figure 5 : Representative immunohistochemical staining of Caspase-3 expression in ovarian tissues from the experimental groups, demonstrating differences in apoptotic activity (×400 magnification).

Figure 6 : Representative immunohistochemical staining of Bcl-2 expression in ovarian tissues from the experimental groups, illustrating anti-apoptotic protein expression and follicular preservation (×400 magnification).

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Include all the tables here, listed numerically

Table1 Serum Anti-Müllerian Hormone (AMH) concentrations among the experimental groups, presented as mean ± SD with Tukey's post hoc multiple comparison letters indicating statistically significant differences

Group	Mean ± SD	Tukey Letters
Control	92.95 ± 8.76	a
H	77.35 ± 10.16	ab
L	74.20 ± 11.84	ab
D	55.94 ± 13.51	bc
G	44.79 ± 7.78	c
M	33.49 ± 5.93	c

Table2. Quantitative analysis of LC3B expression as an autophagy marker in ovarian tissue across the experimental groups, expressed as mean ± SD and analyzed using one-way ANOVA followed by Tukey's post hoc test

Experimental Group	LC3B Expression (Mean ± SD)	Tukey Homogeneity Letter
Group c	1.70±0.16	b
Group - H	1.34±0.16	b
Group - L	1.39±0.25	b
Group - D	2.08±0.33	b
Group G	2.09±0.63	b
Group - M	6.53±1.28	a

Table 3. Relative NF-ϰB gene expression levels determined by RT-qPCR in ovarian tissues of the different experimental groups, presented as fold change (mean ± SD) with statistical group comparisons

Group	Mean ± SD	Tukey Homogeneity Letter
D	1.203 ± 0.320	b
G	1.425 ± 0.091	ab
L	1.766 ± 0.225	cd
M	0.739 ± 0.225	a
H	0.439 ± 0.032	d
C	0.000 ±1,000	bc

Table 4: Semi-quantitative immunohistochemical (Allred) scoring of apoptotic markers (Caspase-3 and Bcl-2) in ovarian tissues, including percentage of positive cells, staining intensity, and total Allred scores for each experimental group

Experimental Group	Caspase Positive Cell %	Caspase Intensity	Aggregate Caspase Score	Bcl-2 Positive Cell %	Bcl-2 Intensity	Aggregate Bcl-2 Score
G1: C	35% (+1)	2	4	60% (+1)	2	7
G2: (M)	90% (+3)	3	8	5% (+1)	1	2
G3 (L)	40% (+1)	2	4	38% (+1)	1	5
G4: H)	35% (+1)	2	4	28% (+1)	1	4
G5: (D)	60% (+3)	2	6	25% (+1)	2	5
G6: (G)	65% (+3)	2	6	20% (+1)	1	4