

**Impairment of Insulin Signalling Pathway: AMPK $\alpha$ /SIRT1 and Pathophysiological Involvement of Gut Microbiota in Monosodium Glutamate Induced Polycystic Ovary Syndrome with Palliative Role of Flaxseed.**

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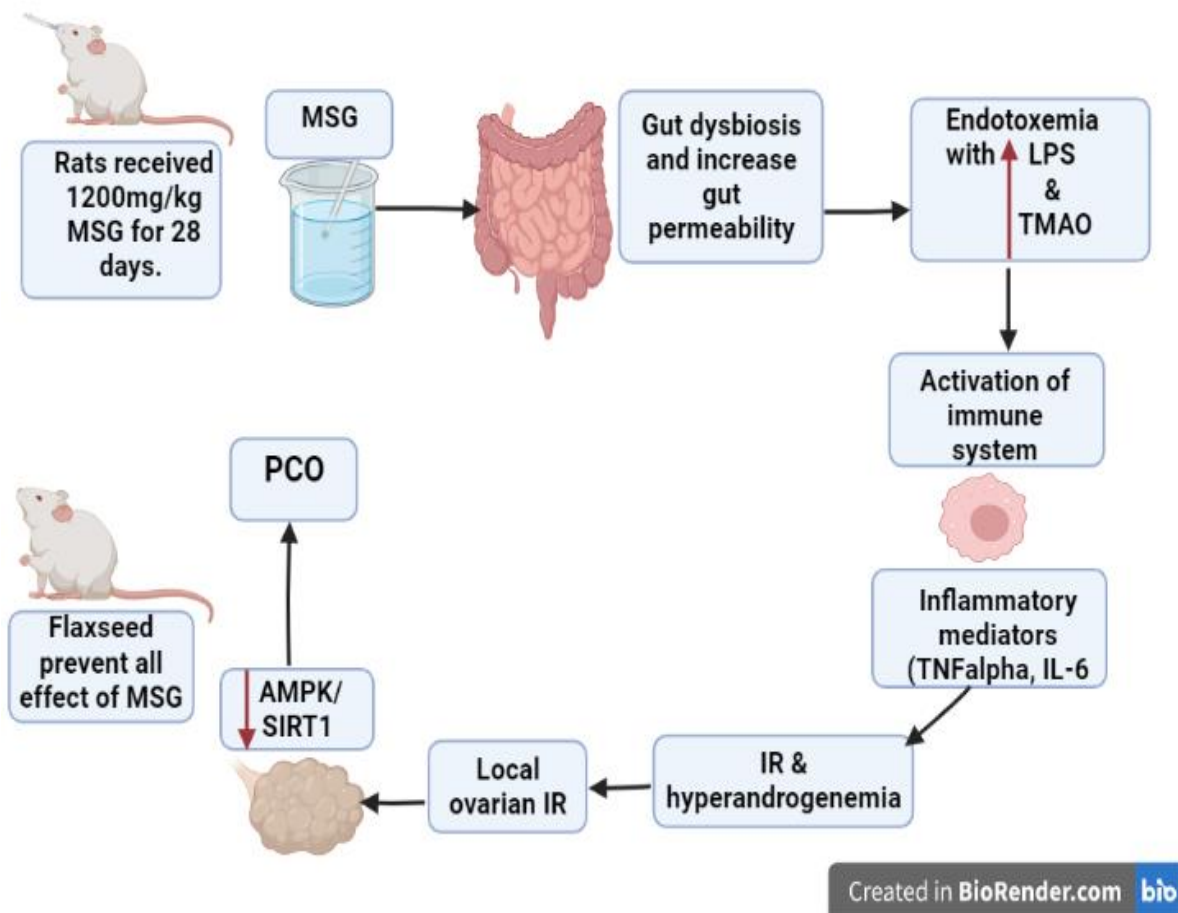
**Keywords**

- Microbiota
- Monosodium glutamate
- MSG-induced
- Dyslipidemia
- Intestinal dysbiosis

**Abstract**

**Aim:** This study aims to investigate the role of gut microbiota in polycystic ovarian syndrome (PCOS) induction by monosodium glutamate (MSG) with the study of the protective effect of flaxseeds. **Methods:** 24 female rats were divided into three groups: Group 1: distilled water; Group 2: MSG; and Group 3: flaxseed + MSG. Finally, body and ovarian weights, HOMA-IR, plasma (gonadal hormones, triglycerides, HDL, LDL, adiponectin, TNF- $\alpha$ , IL-6, and lipopolysaccharides), intestinal (alkaline phosphatase, myeloperoxidase, Trimethylamine N-oxide, and free fatty acid receptor 2 gene expression), ovarian tissue (Silent Information Regulator 1 (SIRT1), reduced glutathione, and gene expression of mitogen activated protein kinase (AMPK $\alpha$ ) and SIRT1) were determined. In addition, vaginal swaps, ovarian histopathology, and immunohistochemical staining of BCL2 and Bax were done. **Results:** MSG-induced insulin resistance, dyslipidemia, intestinal dysbiosis, ovarian oxidative stress, and inflammation through modulation of the AMPK $\alpha$ /SIRT1 pathway. On the other hand, flaxseed significantly prevented these findings. **Conclusion:** MSG-induced gut dysbiosis that predisposes to PCOS and ingestion of flaxseed can be considered a prospective protective agent against PCOS development.

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### Graphical abstract

Rats received MSG in a dose of 1200mg/kg orally for 28 days. MSG induced intestinal gut dysbiosis with elevation of LPS & TMAO which induce disruption of intestinal barrier and leaky gut. The systemic endotoxemia developed by dysbiosis triggers activation of immune cells with promoting synthesis of inflammatory mediators (TNF $\alpha$ , & IL-6). Systemic inflammatory state disrupts insulin signaling and initiates state of IR that has its impact on various tissues including ovaries through impairing signaling of AMPK $\alpha$ /SIRT1 pathway which predispose to development of PCO characteristic features. Flaxseed administration before MSG prevents intestinal dysbiosis with its subsequent deleterious effects.

## 1. Introduction

The widespread use of mono sodium glutamate (MSG) with the trade name "China salt" in food, mainly processed one like noodles, soup, and sauces, has increased its consumption without explicit awareness of its acceptable limits [1,2]. This has triggered concerns because of the detected negative health impacts of MSG, such as obesity, metabolic illnesses, and detrimental effects on reproductive organs [3].

Around 1-2 million eggs at birth are found in ovaries, but only 300 of such eggs will mature and be released for fertilization [4].

Polycystic ovarian syndrome (PCOS), a chronic endocrine-metabolic illness affecting women of reproductive age [5], is distinguished by hyperandrogenism, ovulatory dysfunction, and infertility, frequently associated with obesity, insulin resistance (IR), and dyslipidemia [6].

PCOS can be diagnosed by at least two of the following three characteristics: ultrasound imaging of oligo-anovulation, polycystic ovaries, and hyperandrogenism. The PCOS primary cause is uncertain, but obesity and IR appear to play a pivotal role in its etiology [7]. The role of gut microbiota in PCOS is of interest due to their involvement in immune and metabolic systems [8].

Energy balance disruption and increased inflammation associated with metabolic diseases have been related to gut microbial dysbiosis, which has been found in PCOS women [9]. Illness, inheritance, sex hormones, immunity, birth procedures, food, and the location in the gastrointestinal tract all appear to impact the diversity of the gut microbiota [10].

Based on the findings of a previous study by Zhao [11] who demonstrated the importance of

microbiota in the pathogenesis of MSG-induced obesity, this study investigated the role of microbiota in MSG-induced PCOS.

Mitogen-activated protein kinase alpha (AMPK $\alpha$ ) and sirtuins (Silent Information Regulator1 (SIRT1) which is a nicotinamide adenine dinucleotide NAD<sup>+</sup>- dependent histone deacetylase) are found in all eukaryotic cells and may exist together during ovulation [12].

AMPK $\alpha$ /SIRT1 pathway is related to IR, and blood sugar is reduced with the protection of microvascular endothelium from glucose toxicity when the expression of AMPK and SIRT1 is increased [13,14]. Since PCOS is characterized by an IR state so AMPK $\alpha$ /SIRT1 pathway could play a role in the pathophysiology of PCOS.

Over the past few years, interest regarding the use of medicinal plants has been glowed up as an additional treatment strategy for PCOS, along with lifestyle modifications such as diet and exercise [15]. Flaxseed gained interest for its potential efficacy in managing PCOS due to its exceptionally high level of physiologically active components as  $\alpha$ -Linolenic acid, phytosterogenic lignans, and dietary fibers [7]. Flaxseed contains bioactive phenolics, which have antioxidant and free radical scavenging capabilities [16]. It is the richest dietary source of lignin that may influence levels of testosterone by increasing sex hormone-binding globulin (SHBG) and decreasing the amount of circulating free testosterone [17]. Lignin is metabolized by gut microbes to yield enterodiols and enterolactone, which have similarities to estradiol, so flaxseed can alter testosterone levels and potentially act as estrogenic/antiestrogenic agents [18].

Based on the previous observation, we attempted in this research to elucidate the probable mechanism by which MSG could develop PCOS, and if this is due to gut microbial dysbiosis in addition to assessing the role of AMPK $\alpha$ /SIRT1 with investigating the impact of concomitant flaxseed intake as a protective agent.

## Materials and methods

### Chemicals

**MSG:** The white powder of MSG was purchased from (Sigma-Aldrich- PHR2634, No. 1907/2006). 120 gm of MSG was dissolved in 120 ml distilled water (DW), then 1 ml of the aqueous solution (1000 mg/ml) was administered to rats by oral tube in a daily dose equivalent to 1200 mg/kg [19].

**Flaxseed extract:** A packet of 750 grams of organic brown flaxseeds labeled Tassyam (Part number: 13317001000079) was commercially accessible in local stores and was purchased from the Nine Life Company in Egypt.

**Flaxseed extract preparation:** A homogenizer was used to ground flaxseed into a fine powder before being extracted with 70% ethanol as previously reported [20]. A rotary evaporator was used to concentrate the extract at low pressure to obtain a dry semi-solid mass. After that, a fine powder was produced by lyophilizing the semi-solid mass. Following that, it was kept at 4°C in an airtight container. 500 mg of the powder was dissolved in 5 ml of DW, then 1 ml of the aqueous solution (100 mg/ml) was administered to rats by oral tube in a daily dose equivalent to 500 mg/kg [21].

### Experimental Animals

Twenty-four albino local strain female rats aged 8-10 weeks and weighing 150-170g were

used and kept in sanitary, well-ventilated cages with four rats per cage under conventional laboratory settings (12h cycles of light and dark, ambient temperature of 25 $\pm$  2°C, and unrestricted access to food and water). Animals were acclimatized for two weeks before the start of the study. The Faculty of Medicine, Tanta's Animal Care Review Committee examined and approved the experimental procedure (Approval code no. 36260/12/22).

### Experimental protocol

After acclimatization rats were randomly divided into three groups of animals (eight rats in each group) as follows:

Group 1: Control group (n=8) rats: Rats received an equivalent volume (1 ml) of distilled water.

Group 2: MSG group (n=8) rats: Rats received MSG dissolved in water at a dose of 1200 mg/kg daily by oral gavage [19].

Group 3: flaxseed group + MSG (n=8) rats: Rats received flaxseed aqueous extract by oral route in a dose of (500 mg /kg/day) one hour before MSG administration that was given by the same dose of MSG group [21,22].

The treatment procedures continued for 28 days in all experimental groups.

### Vaginal Smear Collection

By grasping the tail of motionless animals, the swab-smear technique was performed. A cotton bud swab measuring 5 mm by 12 mm and dipped in NaCl 0.9% (normal saline) was inserted to a depth of about 1 cm in the vagina. The cells from the vaginal lumen and walls were gently removed by rotating the swab in the same direction. On clean glass slides, the swab tip was gently rolled into a thin coating (smear), which

was then left to air dry. A new swab stick and glass slide were used for each animal. After that, the slides were stained using Wright's Giemsa Stain (G1020, Solar bio, Beijing, China) solution, as instructed by the protocol of the manufacturer. Finally, using a light microscope, the estrous cycle and alterations in vaginal epithelial cells were assessed based on the predominant cell type [23,24].

*For all animals:* rats were weighed individually at the end, just before collecting samples. Under the guidance of a daily vaginal swab (predominance of non-nucleated cornified cells and a minimal presence of leucocytes and epithelial cells) animals in the estrous phase were scheduled for scarification.

#### **Blood sample collection**

After scheduling for scarification, animals were starved overnight, and then rats were given 0.1 ml of 1% sodium barbiturate intraperitoneally for anesthesia[25]. Blood samples were collected by cardiac puncture. To extract the plasma, citrated blood was centrifuged at 3000 cycles per minute. The plasma was then divided into aliquots and kept in a freezer at -80°C for later use.

#### **Tissue collection & preparation of tissue homogenates:**

After blood sampling, an incision in the midline was done on the abdominal wall anterior aspect, intestinal tissues, and ovaries were excised, then the ovaries were weighed. To remove any blood contamination intestinal tissues and ovaries were rinsed with saline and then blotted with filter paper to dry. After that, the intestinal tissue was immediately frozen at -80°C for biochemical analysis, and the ovaries were divided into two parts; one part for histopathological and

immunohistochemical examination, which was preserved in 10% phosphate-buffered formalin solution, while the other part was for biochemical and molecular analysis so it was immediately frozen at -80°C.

For tissue homogenate preparation, 5 volumes of 50mM phosphate buffer (pH 7.4) were used to homogenize intestinal and ovarian tissue, followed by 15 minutes of 11,000xg centrifugation at 4°C. Until the time of analysis, the supernatants were stored in a freezer at -80°C.

#### **Biochemical assays**

The following parameters were assessed:

**HOMA-IR** was calculated according to the formula: fasting insulin (microU/L) x fasting glucose (mmol/L)/22.5 [26]. This is carried out by using a Glucose Colorimetric Detection Kit (Life Technologies Frederick, USA, Catalog number: EIAGLUC). Fasting Insulin by ELISA kit (MyBioSource, Inc. San Diego, USA, Catalog Number: MBS724709).

#### **Assessment of plasma lipid profile, & hormonal profile**

The enzymatic colorimetric method was used to assess plasma *triglycerides*, *LDL cholesterol*, and *HDL cholesterol* by using Kits obtained from (Biodiagnostic, Dokki, Giza, Egypt) (Cat. No. TR 20 30, CH 12 31, CH 12 30 respectively).

ELISA technique was used to assess plasma levels of *testosterone*, *Luteinizing Hormone (LH)*, and *Follicle-Stimulating Hormone (FSH)* by using kits supplied by (MyBioSource, Inc. San Diego, USA) (Cat. No. MBS4502799, MBS4500760, MBS2021901 respectively). Also, the calculation of the LH/FSH ratio was done.

### Assessment of intestinal dysbiosis

*Plasma lipopolysaccharide (LPS)* level was assessed by using ELISA Rat LPS kits (MyBioSource, Inc. San Diego, USA, Catalog No. MBS268498). *Intestinal Myeloperoxidase (MPO)* activity was determined using the colorimetric method by kits supplied by (Abcam Company Cambridge, UK Cat. No. ab105136). *Intestinal alkaline phosphatase (IAP)* activity by using an ELISA Intestinal kit (ALPI) supplied by (MyBioSource, Inc. San Diego, USA, Cat No: MBS732772). *Intestinal tissue Trimethylamine N-oxide (TMAO)* level which is a microbiota-derived metabolite was assessed by ELISA Kit obtained from (MyBioSource, Inc. San Diego, USA, Cat. No: MBS8807602).

### RNA Extraction and Quantitative RT-PCR Detection of Colonic Free Fatty Acid Receptor 2 (FFAR-2)

Total RNA was extracted from intestinal tissue homogenates using the Gene JET RNA Purification Kit (Cat. No. K0731, Thermo Scientific, Waltham, USA) according to manufacturer's instructions. 5  $\mu$ g of total RNA

was reverse transcribed using Thermo Scientific's RevertAid H Minus Reverse Transcriptase (Cat. No. EP0451, Thermo Scientific, Waltham, USA) to produce cDNA. Reactions of PCR were performed by usage of Power SYBR Green PCR Master Mix (Cat. No. 4367659, Life Technologies LTD, Warrington, UK), and the used primers for PCR reaction were mentioned in (Table 1).

The cycling pattern was as follows: one 10-minute cycle at 95°C, after that 40 amplification cycles at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 1 minute. After the cycle threshold (Ct) values for target genes and the housekeeping gene were determined, the  $2^{-\Delta\Delta Ct}$  technique was used to detect relative gene expression [27].

### RNA Extraction and Quantitative RT-PCR Detection of Ovarian AMPK $\alpha$ and SIRT1

Total RNA was extracted from ovarian tissue homogenates by the method described above in Quantitative RT-PCR detection of colonic FFAR-2 and the used primers for PCR reaction were mentioned in (Table 1).

**Table 1: Primers for the PCR reaction.**

Primer	Sequence	GenBank Accession No
Rat FFAR-2	FOR: 5' CTACGAGA ACTTCACCCAAGAG 3' REV: 5' GAAGCGCCAATAACAGAAGATG 3'	XM_039104536.1
AMPK $\alpha$	FOR: 5'-TAAACC CACAGAAATCCAAACACC-3' REV: 5'-ACAACCTTCCATTCATAGTCCAAC-3'	XM_054352910.1
SIRT1	FOR: 5'-AACCACCAAAGCGGAAAAAAGAA-3' REV: 5'-CCACAGCAAGGCGAGCATAAATA-3'	NM_001314049.2
Rat GAPDH	FOR: 5' CAACTCCCTCAAGATTGTCAGCAA 3' REV: 5' GGCATGGACTGTGGTCATGA 3'	NM_001394060.2

FOR: forward primer, REV: reverse primer, FFAR-2: Free fatty acid receptor 2, AMPK $\alpha$ : Mitogen-activated protein kinase alpha, SIRT1: Silent Information Regulator1, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

### Assessment of inflammatory and oxidative stress biomarkers

*Plasma TNF- $\alpha$*  was assessed by using a Rat (TNF- $\alpha$ ) ELISA kit (RayBio Inc. Norcross,

Georgia, USA, Catalog #: ELR-TNF $\alpha$ ). *Plasma IL-6* was assessed by Rat IL-6 ELISA kit (Cat No. CSB-E04640r, CUSABIO, Houston, USA). *Plasma adiponectin* was assessed by using an

ELISA kit (RayBio Inc. Norcross, Georgia, USA, Catalog #: ELR-Adiponectin). *Ovarian tissue Silent Information Regulator 1 (SIRT1)* level was assessed by ELISA kit (MyBioSource, Inc. San Diego, USA Cat. No. MBS705558). *Ovarian tissue reduced glutathione (GSH)* level was assessed by the colorimetric method by kits (Biodiagnostic, Dokki, Giza, Egypt Cat. No. GR 25 11).

### **Histological and Immunohistochemical Examination of the ovarian tissues:**

#### **Histological examination of the ovarian tissue:**

Paraffin blocks were obtained by embedding formalin-fixed ovarian samples in paraffin wax. Hematoxylin and eosin were used to stain sections of 5–6  $\mu\text{m}$  thickness for routine histological analysis [28] and examined by Olympus light microscope model BX43F. Using image analysis tools, a morphometric study was conducted by counting the number of various types of ovarian follicles and measuring the thickness of the granular and theca layers. Magnification 400 was used to count primordial follicles, whereas magnification 100 was used to count other types of follicles. Ten non-overlapping readings were obtained for each slide in all groups (8 slides for each group), and the mean values were obtained.

#### **Immunohistochemical staining of the ovarian tissue Bcl2, & Bax**

Using immunohistochemical methods, the expression of Bcl2, and Bax in ovarian tissue was evaluated by dewaxation, rehydration, and incubation of paraffin-embedded sections with rabbit monoclonal antibodies against Bcl2 and Bax, using Bcl2 (Cat. no. M0887, clone 124, Dako, Carpinteria, CA, USA) and Bax (Cat. no. 18–0218, clone 2D2, Zymed Laboratories Inc., South San

Francisco, CA, USA). Using image analysis tools, the quantification of immunohistochemical images was done. (Image J, 1.46a, NIH, Bethesda, MD, USA) [29]. The mean color intensity of the immunohistochemical reaction of both Bcl2 and Bax was measured in ten non-overlapping fields (400) on each slide (8 slides for each group).

#### **Statistical analysis:**

For data expression, the mean  $\pm$  SD was used. Tukey test which follows One-way analysis of variance (ANOVA), was used for several groups' multiple comparisons. Pearson correlation analysis was used to investigate the relationship between two variables.  $p$  values  $<0.05$  were considered statistically significant.

SPSS (IBM SPSS Statistics for Windows, IBM Corp, Version 23.0. Armonk, NY, USA) is the program that was used for statistics.

### **Results**

#### **Effect of MSG and flaxseed on body weight, ovarian weight, HOMA-IR, TG, HDL-C, and LDL-C.**

As shown in (Table 2), compared to group 1, MSG in group 2 and flaxseed administration before MSG in group 3 didn't significantly modify body weight, but MSG significantly increased ovarian weight compared to group 1, but flaxseed significantly decreased ovarian weight compared to group 2. Moreover, MSG induced IR state by significant elevation of HOMA-IR in addition to the dyslipidemic state by significant elevation of plasma levels of TG, and LDL-C and significant lowering of plasma levels of HDL-C compared to control. However, flaxseed extract administration before MSG prevented insulin resistance and dyslipidemic state by significant lowering of TG, and LDL-C and significant elevation of plasma levels of HDL-C compared to group 2 restoring the

previous parameter to their normal level with non-significant difference when compared to group 1 except for the plasma level of TG which was significantly higher than in group 1.

#### Effect of MSG and flaxseed on plasma testosterone, LH, FSH, and LH/FSH ratio.

As shown in (Fig. 1a-d), MSG administration significantly increased the plasma levels of testosterone, LH, and FSH as well as the LH/FSH ratio in group 2 compared to group 1. On the other hand, these hormones were significantly decreased by flaxseed extract administration before MSG in group 3 compared to group 2 and their levels were non-significant compared to group 1 except for testosterone which was significantly higher when compared to group 1.

#### Effect of MSG and flaxseed on markers of intestinal dysbiosis.

As shown in (Table 3), it was evident that MSG elicited an intestinal inflammatory state by significant elevation of intestinal MPO together with intestinal dysbiosis by significant elevation of plasma LPS, intestinal TMAO, together with a significant reduction in both IAP activity and intestinal relative mRNA expression of FFAR-2 in group 2 in comparison to group 1. These

parameters were significantly reversed by the intake of flaxseed extract before MSG administration in group 3 when compared to group 2.

#### Effect of MSG and flaxseed on inflammatory and oxidative stress biomarkers.

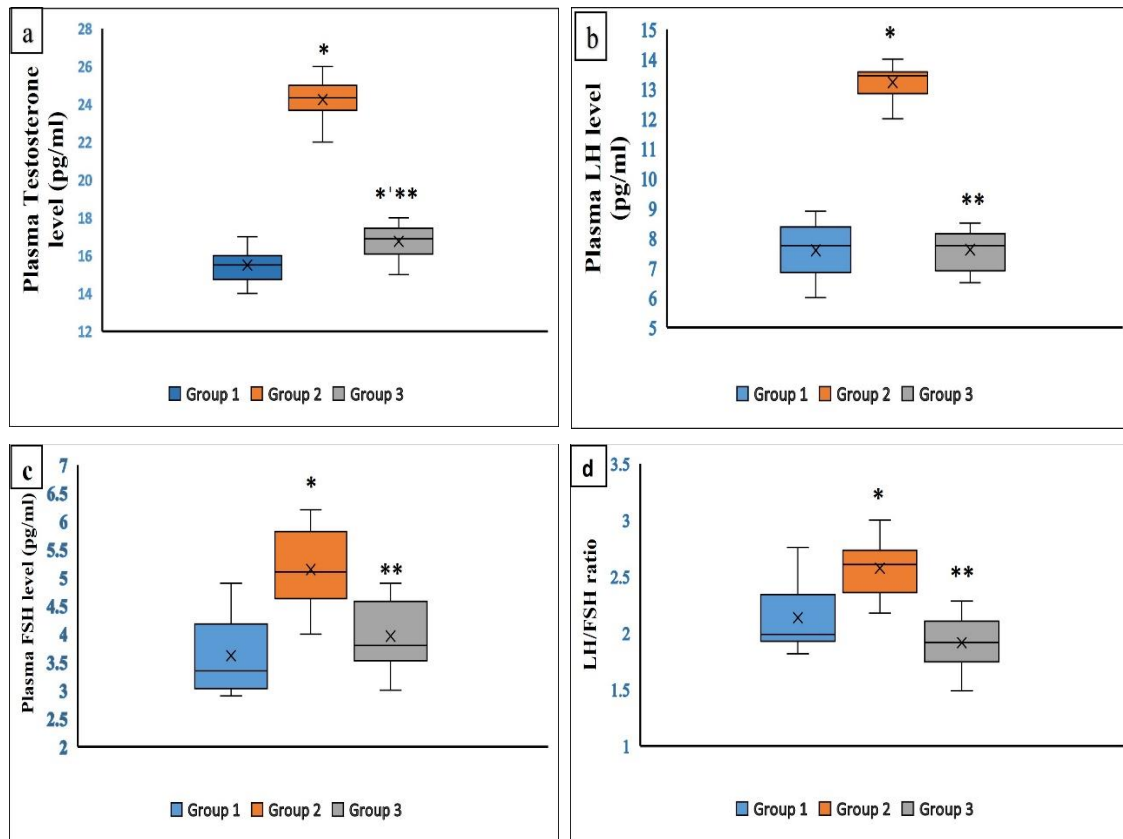
As shown in (Table 4), MSG induced systemic inflammation by the significant raising of plasma levels of TNF- $\alpha$ , & IL-6 and lowering of plasma adiponectin in group 2 compared to group 1. In addition, MSG triggered ovarian oxidative stress and inflammation by significant decrement of ovarian SIRT1, GSH levels, and relative mRNA expression of both AMPK $\alpha$  and SIRT1 in group 2 compared to group 1. Flaxseed administration before MSG in group 3 counteracted the effect of MSG either systemically by a significant lowering of plasma level of TNF- $\alpha$ , & IL-6 with a significant raising of plasma adiponectin or at the ovarian level by a significant increase in ovarian SIRT1, GSH levels, relative mRNA expression of both AMPK $\alpha$  and SIRT1 when compared to group 2, in group 3 the change in these parameters were non-significant in comparison to group 1 except for plasma adiponectin level which was significantly lower than group 1.

**Table 2:** Effect of MSG and flaxseed on body weight, Ovarian weight, HOMA-IR, TG, HDL-C, and LDL-C among all studied groups.

Groups /Parameters	Group 1 N=8	Group 2 N=8	Group 3 N=8
Body weight (g)	180.13 $\pm$ 4.26	182.50 $\pm$ 4.44	179.12 $\pm$ 4.32
Ovarian weight (mg)	0.028 $\pm$ 0.005	0.049 $\pm$ 0.006*	0.026 $\pm$ 0.005**
HOMA-IR	1.63 $\pm$ 0.20	3.81 $\pm$ 0.40*	1.81 $\pm$ 0.22**
Plasma TG (mg/dl)	134.69 $\pm$ 1.89	156.20 $\pm$ 1.41*	138.81 $\pm$ 1.51***
Plasma LDL-C (mg/dl)	78.13 $\pm$ 2.42	106.31 $\pm$ 4.30*	81.06 $\pm$ 2.57**
Plasma HDL-C (mg/dl)	44.88 $\pm$ 2.47	22.25 $\pm$ 1.83*	42.38 $\pm$ 2.13**

Data are expressed as mean $\pm$  SD. Superscript \*, and \*\*denote a statistically significant difference at (P < 0.05). \*: p<0.05 Vs. Group 1, \*\*: p<0.05 Vs. Group 2 using one way ANOVA with Tukey post hoc test. N: number of rats, HOMA-IR: Homeostatic Model Assessment for Insulin Resistance, TG: Triglycerides, LDL-C: Low-density lipoprotein cholesterol, HDL-C: High-density lipoprotein cholesterol.





**Fig. 1** Effect of MSG and flaxseed on plasma testosterone, LH, FSH, and LH/FSH ratio among all studied groups. Data are expressed as median and interquartile range. Superscript \*, and \*\* denote a statistically significant difference at ( $P < 0.05$ ). \*:  $p < 0.05$  Vs. Group 1, \*\*:  $p < 0.05$  Vs. Group 2 using one-way ANOVA with Tukey post hoc test. LH: luteinizing hormone, FSH: follicular stimulating hormone.

**Table 3:** Effect of MSG and flaxseed on markers of intestinal dysbiosis among all studied groups.

Groups/ Parameters	Group 1 N=8	Group 2 N=8	Group 3 N=8
Plasma LPS level (EU/ml)	0.24±0.024	0.48±0.018*	0.26±0.019**
IAP activity (µg/mg of tissue)	105.13±3.31	62.88±1.81*	107.50±2.62**
Intestinal MPO activity (µg/mg of tissue)	11.8750±1.46	19.75±1.04*	11.00±1.20**
Intestinal (TMAO) level (pmol/mg protein)	22.99±1.88	75.88±2.36*	30.13±1.46***
Relative mRNA expression of intestinal (FFAR-2) gene	1.00±0.116	0.50±0.018*	0.97±0.010**

Data are expressed as mean± SD. Superscript \*, and \*\* denote a statistically significant difference at ( $P < 0.05$ ). \*:  $p < 0.05$  Vs. Group 1, \*\*:  $p < 0.05$  Vs. Group 2 using one way ANOVA with Tukey post hoc test. N: number of rats, LPS: lipopolysaccharides, IAP: Intestinal alkaline phosphatase, MPO: myeloperoxidase, TMAO: Trimethylamine N-oxide, FFAR-2: Free fatty acid receptor 2.

**Table 4:** Effect of MSG and flaxseed on inflammatory and oxidative stress biomarkers among all studied groups.

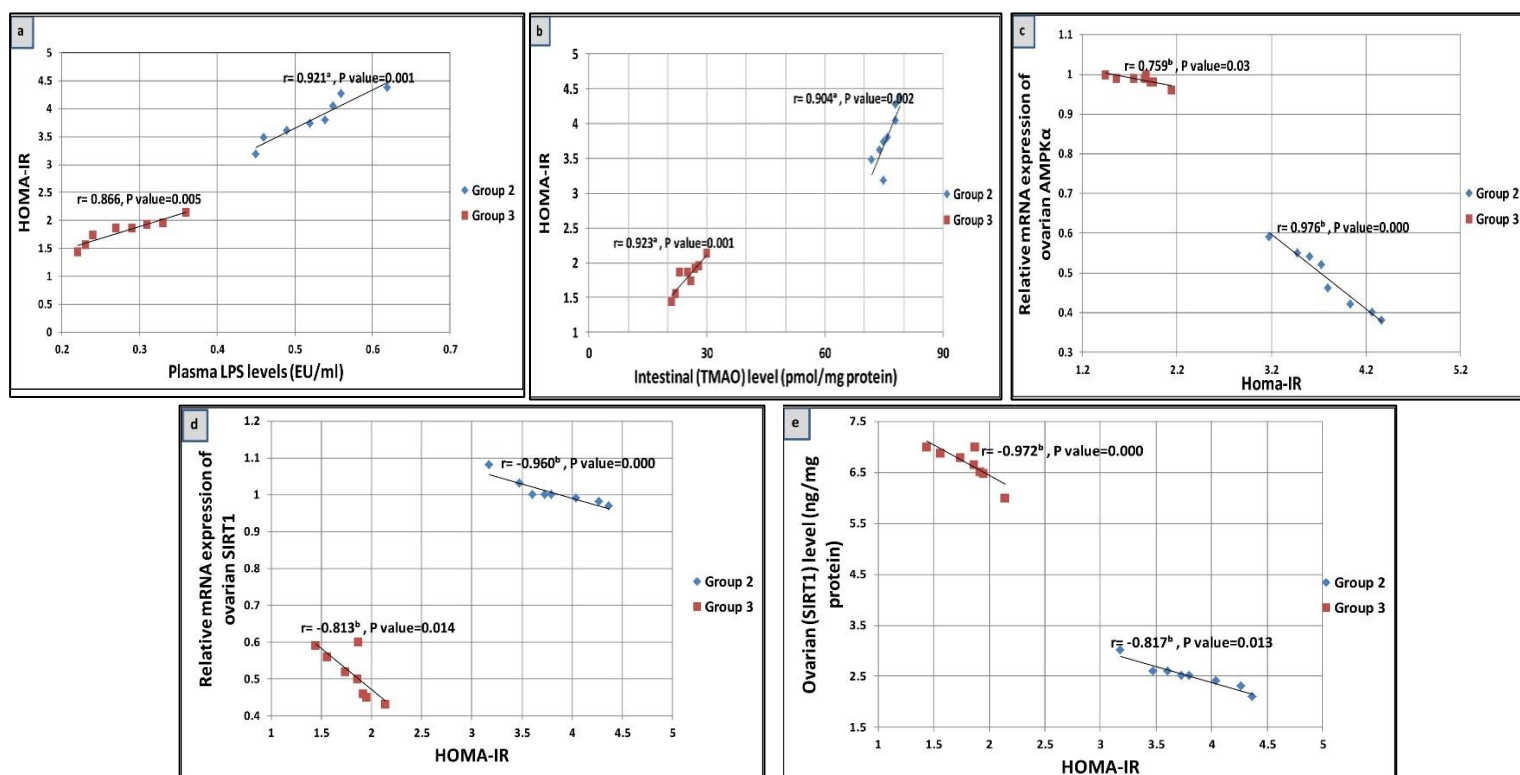
Groups/ Parameters	Group 1 N=8	Group 2 N=8	Group 3 N=8
Plasma adiponectin level (ng/ml)	3.64±0.42	2.09±0.09*	2.64±0.35***
Plasma (TNF-α)(pg/ml)	171.13±4.58	229.50±13.03*	175.38±2.62**
Plasma (IL-6)(pg/ml)	19.60±1.48	31.01±1.05*	20.01±1.98**
Ovarian (SIRT1) level (ng/mg protein)	6.48±0.35	2.50±0.26*	6.67±0.33**
Ovarian (GSH) activity (ng /mg protein)	40.13±1.48	22.80±1.64*	38.31±1.49***
Relative mRNA expression of ovarian AMPKα	1.00±0.04	0.48±0.08*	0.99±0.01**
Relative mRNA expression of ovarian SIRT1	1.00±0.03	0.51±0.07*	0.97±0.03**

Data are expressed as mean  $\pm$  SD. Superscript \*, and \*\* denote a statistically significant difference at ( $P < 0.05$ ). \*:  $p < 0.05$  Vs. Group 1, \*\*:  $p < 0.05$  Vs. Group 2 using one way ANOVA with Tukey post hoc test. N: number of rats, TNF- $\alpha$ : Tumornecrosing factor, SIRT1: silent information regulator 1, GSH: Reduced Glutathione.

### Correlation analysis:

Correlation analysis was carried out to determine the relation between gut dysbiosis and HOMA-IR with subsequent changes in the ovarian AMPK $\alpha$ /SIRT1 pathway. As shown in (Fig. 2a-e) in group 2&3 there was a positive correlation

between Plasma levels of LPS and intestinal TMAO levels and HOMA-IR and a negative correlation between HOMA-IR and ovarian SIRT1, Relative mRNA expression of ovarian AMPK $\alpha$  and Relative mRNA expression of ovarian SIRT1



**Fig. 2 (a-e)** Correlation analysis in groups 2 and 3. Superscripts a and b denote a statistically significant difference at ( $P < 0.05$ ). a denotes statistical significance at  $P < 0.05$  (positive correlation). b denotes statistical significance at  $P < 0.05$  (negative correlation). LPS: lipopolysaccharide, TMAO: trimethylamine N-oxide, SIRT1: Silent Information Regulator 1, AMPK $\alpha$ : Mitogen-activated protein kinase alpha)

### Effect of MSG and Flaxseed on Histological Morphology

#### Hematoxylin and Eosin staining

Group 1 ovarian tissue examination revealed normal histological architecture. Ovaries are formed of two regions: one region is covered with germinal epithelium, which is the outer cortex and the other region is formed of stromal cells and blood vessels in loose connective tissue which is the inner medulla. Follicles at various

developmental stages (primordial, primary, growing, and mature Graafian follicles), as well as many corpora lutea, were detected in the ovarian cortex as in (Fig. 3b-d). In the primordial follicle, the oocyte is surrounded by a single layer of squamous epithelium. While in the primary follicle, the oocyte is surrounded by the cuboidal epithelium of one layer of granulosa cells. Moreover, in the secondary follicle, the oocyte and zonapellucida were present and surrounded by a

varying number of concentric layers, two or more cuboidal granulosa cells, and without an antrum, as shown in (Fig. 3e). The antral cavity of the Graafian follicles was clearly developed, with an isolated oocyte surrounded by zonapellucida that is well-defined, a corona radiata with numerous granulosa cells layers, and an outer fusiform theca cellas shown in (Fig. 3f).

Examination of the ovarian tissue after MSG administration in group 2 revealed that there were several cystic follicles in the ovarian cortex and these several dilated follicular cysts with large antrum and thinner layers of granulosa cells. In addition, the granulosa cells got thinner as compared to group 1. It was made up of 2-3 layers, with flattened granulosa cells adjacent to the antrum. Furthermore, the cystic follicles had no oocytes and corona radiata and had a theca cell layer that was well-developed. Moreover, there was an apparent proliferative ovarian stroma with some growing Graafian follicles and corpora lutea embedded within. There were many atretic follicles found in addition to highly vascular medullary stroma detected as shown in (Fig. 4a-d).

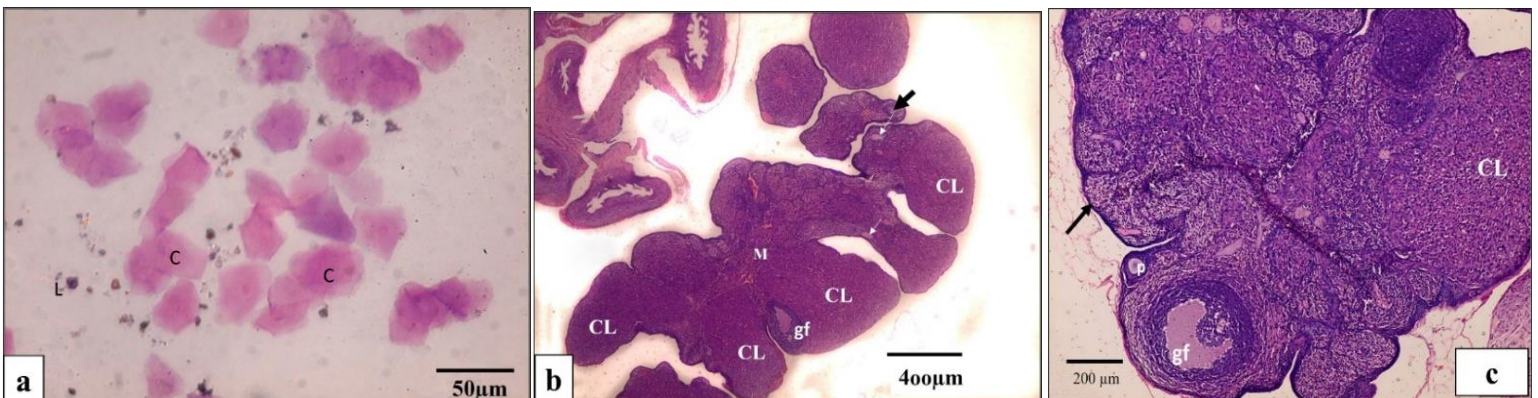
Examination of the ovarian tissue of group 3 showed that flaxseed extract intake before MSG administration resulted in a nearly typical ovarian structure with different stages of follicular development in the cortex, numerous growing and Graafian follicles, and some corpora lutea. Additionally, only a few atretic follicles and follicular cysts were found compared to group 2.

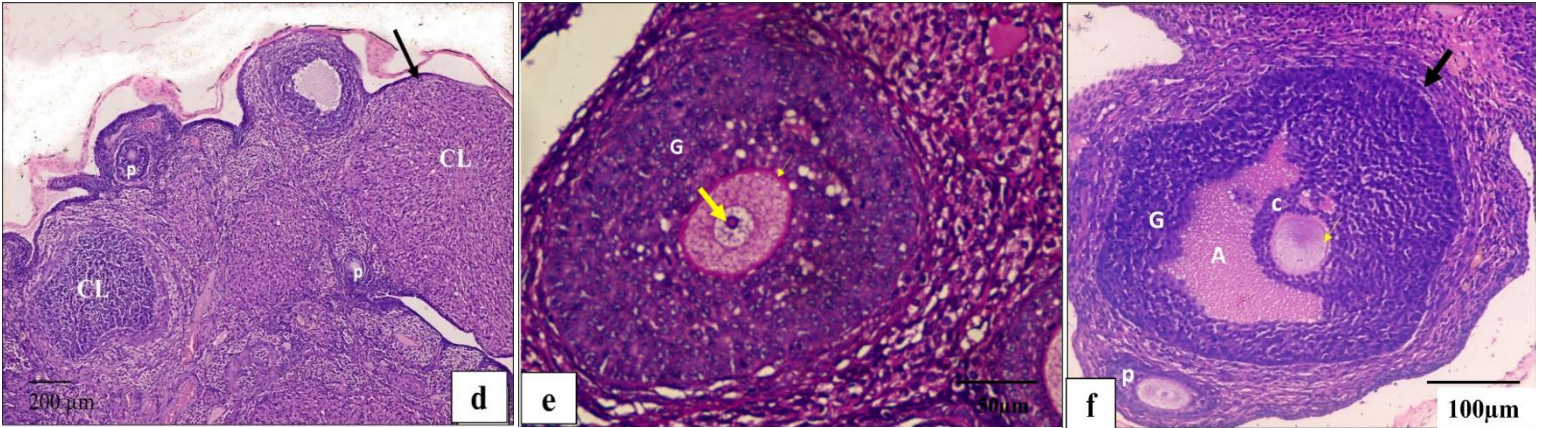
Furthermore, a highly vascular medulla was noted as shown in (Fig. 4 e, f).

### Morphometric analysis

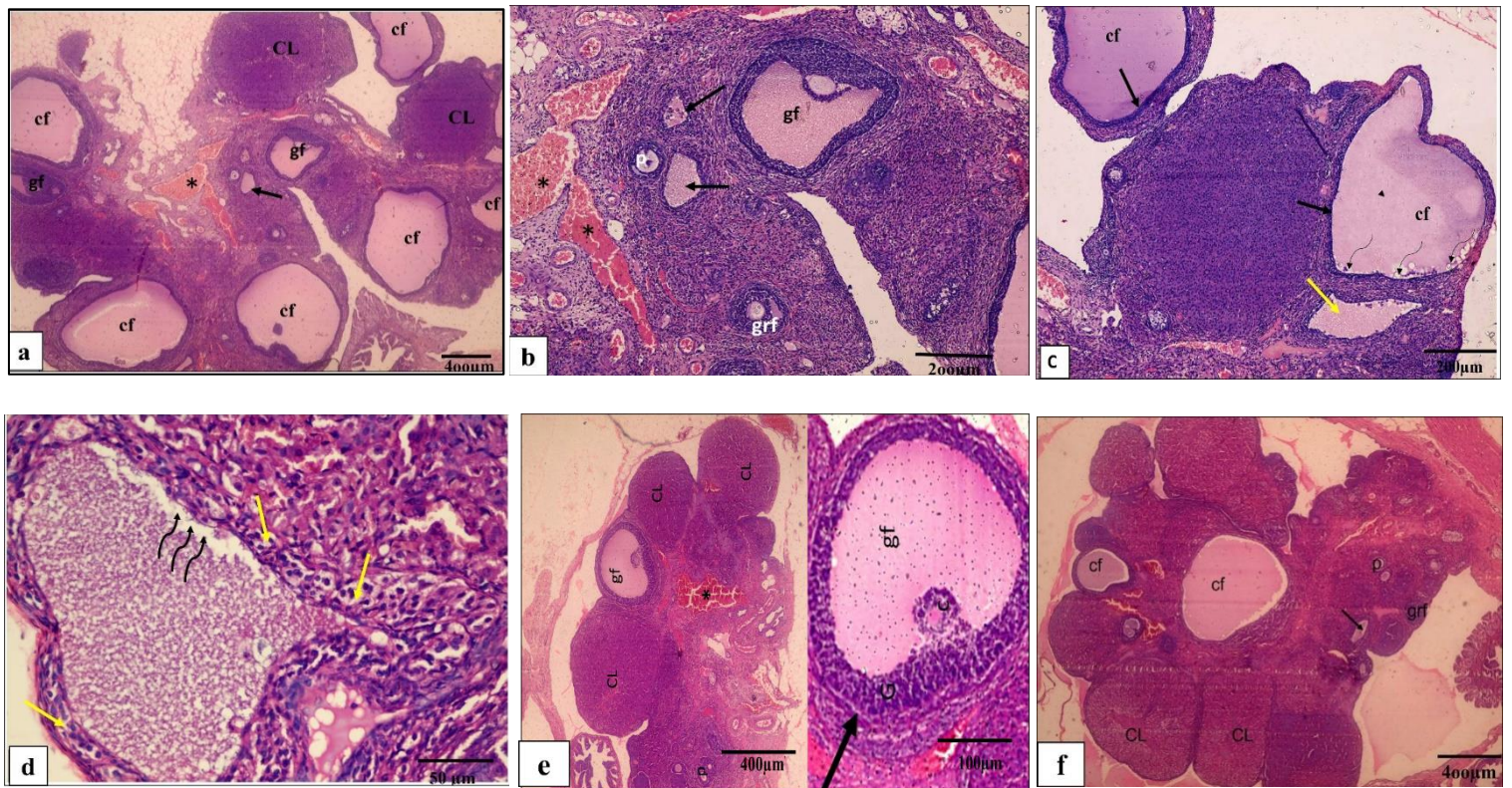
As shown in (Fig. 4 g & h) MSG administration in group 2 resulted in a significant decrease in the number of primordial, primary, mature Graafian follicles and corpus luteum with a significant increase in the number of cystic follicles as compared to group 1. On the other hand, flaxseed extract intake before MSG administration in group 3 resulted in a significant increase in the number of primordial, primary, and Graafian follicles and a significant decrease in the number of cystic follicles when compared to group 2 with no significant change when compared to group 1.

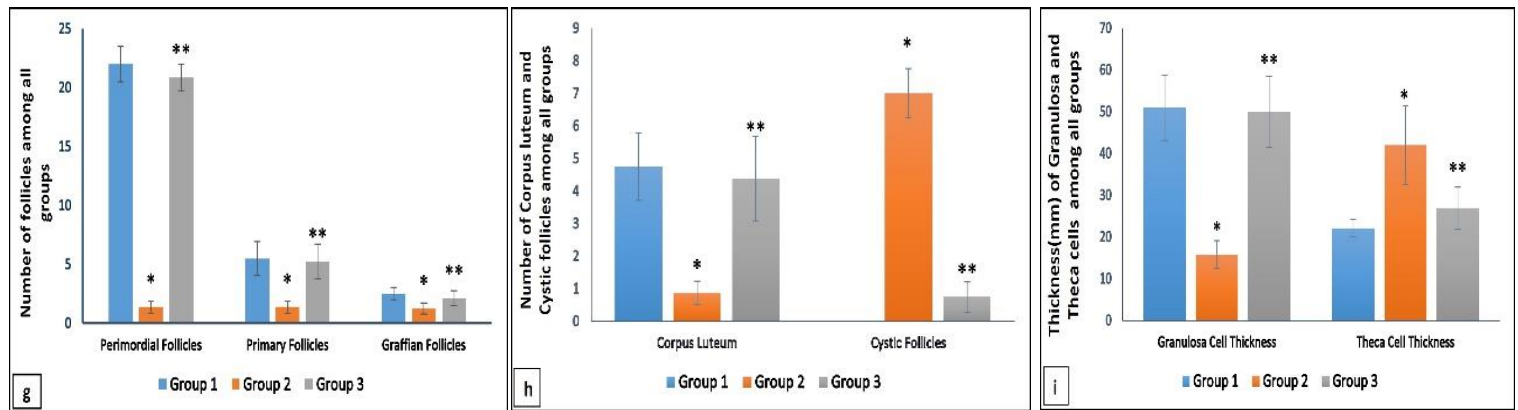
Furthermore, there was a significant decrease in the morphometric analysis of granular layer thickness coupled with a significant increase in theca layer thickness in group 2 when compared to group 1. On the other hand, flaxseed extract intake before MSG administration in group 3 showed a significant increase in the morphometric analysis of granular layer thickness coupled with a significant decrease in theca layer thickness when compared to group 2 with no significant difference in both parameters between group 3 and group 1 (Fig. 4 i).





**Fig. 3** (a) Photomicrograph of vaginal smear from estrus phase shows non-nucleated cornified cells (C) and few leucocytic infiltrations (L). **Fig. 3 (b, c, d, e & f)** Photomicrographs of H&E stained sections from rat ovarian tissue of group 1. **(b, c & d)** Show ovarian tissue sections determine cortex (thin black arrow) formed of ovarian follicles at various stages of maturation; primary (p), growing follicle (short thick black arrow), and mature Graafian follicles (gf). corpora lutea (cl) are noticed. Medulla (M) with stromal cells and blood vessels in loose connective tissue is observed. Few atretic follicles were observed (white arrow). **(e)** Shows the oocyte (thick yellow arrow) and zona pellucida (thin yellow arrow) in the secondary follicles are surrounded by a variable number of concentric layers, two or more cuboidal granulosa cells (G) without an antrum. **(f)** Shows the primary follicle is composed of an oocyte surrounded by cuboidal epithelia of one layer of granulosa cells (p), mature Graafian follicle shows a clearly formed antral cavity (A) with an isolated oocyte surrounded by a well-defined zona pellucida (thin yellow arrow), a corona radiata (c) with multiple layers of granulosa cells (G), and an outer fusiform theca cell (black arrow). (H&E  $\times$  40, scale bar = 400  $\mu$ m, H&E  $\times$  100, scale bar = 200  $\mu$ m, H&E  $\times$  200, scale bar = 100  $\mu$ m & H&E  $\times$  400, scale bar = 50  $\mu$ m )





**Fig. 4 (a-d)** Photomicrograph of H&E-stained sections from rat ovarian tissue of group 2. **(a&b)** Show many cystic follicles (cf) with an inner granulosa layer and an enlarged antral cavity and many atretic follicles (black arrow) are detected. Highly vascular medullary stroma is seen (asterisk). Additionally, there were primary follicles (P), growing follicles (gr), Graafian follicles (gf), and corpora lutea (CL), embedded within an apparently proliferated ovarian stroma. **(c)** Shows large cystic follicle (cf) filled with homogenous pink exudates with attenuated thin granulosa cell layer (black arrow), where the inner cells are flat and epithelioid (curved arrows). **(d)** Shows atretic follicles with a dilated antral cavity, thin granulosa cell layers with many vacuolations (yellow arrows), and some granulosa cells are detached into the antral cavity (curved arrow). **Fig. 4 (e&f)** Photomicrograph of H&E stained sections from rat ovarian tissue of group 3. **(e& f)** Show multiple follicles at different stages of development primary follicle (p), the growing follicle (grf), mature Graafian follicle (gf), and corpora lutea (CL) are seen. Few cystic follicles (cf), the atretic follicle (black arrow), and highly vascular medulla are noticed. At higher magnification, the mature Graafian follicle (gf) shows a clearly formed antral cavity with an isolated oocyte surrounded by a well-defined zona pellucida, a corona radiata (c) with multiple layers of granulosa cells (G), and an outer fusiform theca cell (black arrow). (H&E  $\times 40$ , scale bar = 400  $\mu\text{m}$  and H&E  $\times 100$ , scale bar = 200  $\mu\text{m}$ , H&E  $\times 200$ , scale bar = 100  $\mu\text{m}$ ). **Fig. 4 (g, h, &i)** Morphometric analysis of primordial, primary, mature Graafian follicles and corpus luteum, cystic follicles, and thickness (mm) of granular and theca layer among all studied groups. **(g)** Showed the number of follicles among all studied groups. **(h)** Showed the number of Corpus luteum, and cystic follicles among all studied groups. **(i)** Showed thickness (mm) of granular and theca layer among all studied groups. Data are expressed as mean  $\pm$  SD. Superscript \*, and \*\* denote a statistically significant difference at ( $P < 0.05$ ). \*:  $p < 0.05$  Vs. Group 1, \*\*:  $p < 0.05$  Vs. Group 2 using one-way ANOVA with Tukey post hoc test.

#### Immunohistochemical staining of ovarian tissue Bcl2, & Bax with morphometric analysis of their immunostaining

As regards Bcl2, immunostaining sections from rats' ovarian tissue from group 1 showed a strong positive immunoreaction of the cytoplasmic Bcl2 in the form of a brownish coloration in granulosa cells, with no immunoreaction for Bcl2 was detected in thecal cells (Fig. 5a,&b). Meanwhile, MSG administration in group 2 showed that granulosa cells of cystic follicles had a negative reaction (Fig. 5c,&d). Moreover, flaxseed extract intake before MSG in group 3 showed that there was moderate to strong reactivity in the granulosa cells of primary, secondary, and Graafian follicles (Fig. 5e,&f).

On the other hand, Bax immunostaining sections from rats' ovarian tissue from group 1

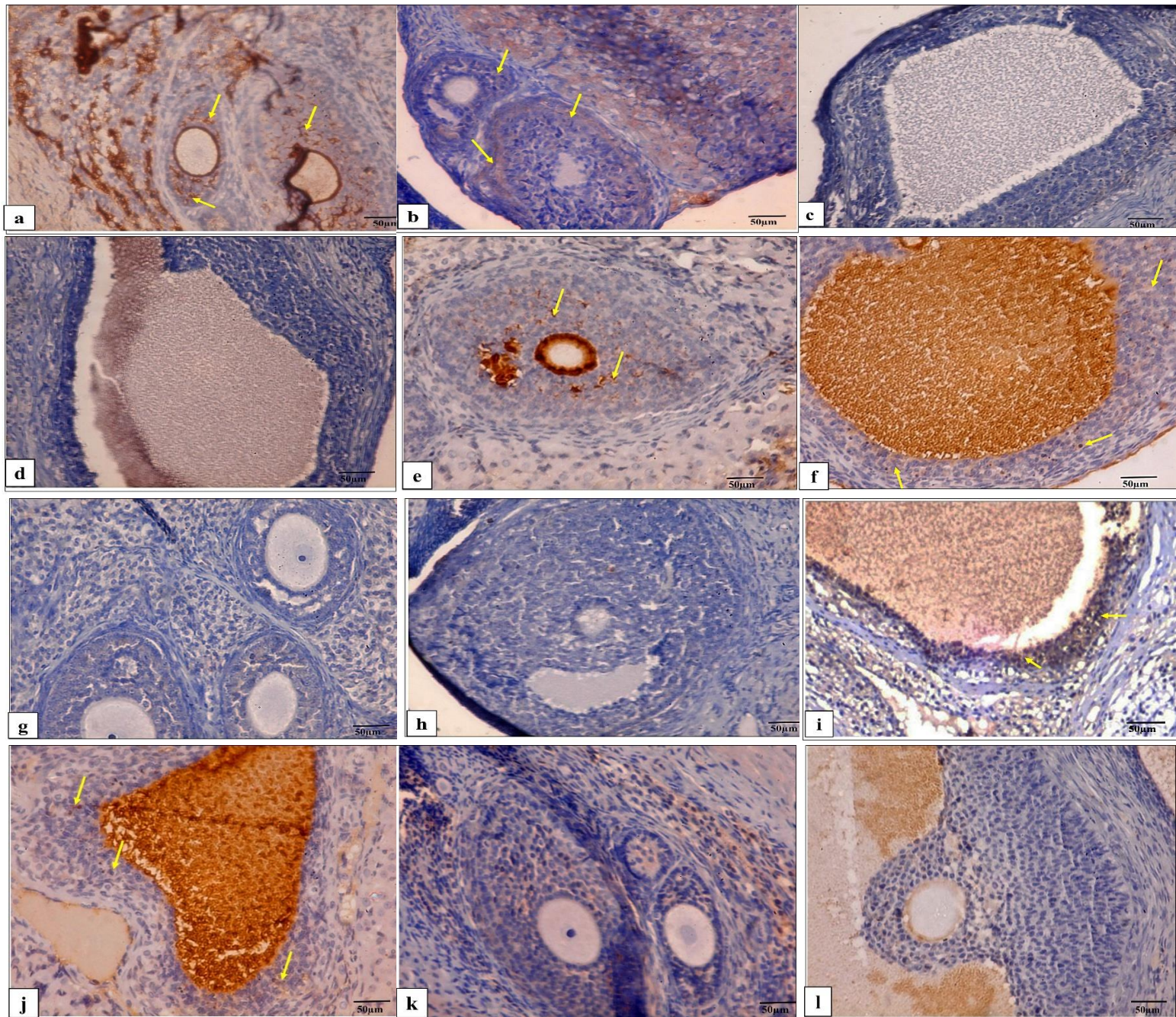
showed negative immunoreactions (Fig. 5g&h). Meanwhile, MSG administration in group 2 revealed that sections showed a strong positive reaction (Fig. 5i&j). On the other hand, flaxseed extract administration before MSG in group 3 showed that sections had weak immunoreactivity of Bax in the cytoplasm of some granulosa cells of primary and secondary follicles in the form of a very faint brown color in the cytoplasm (Fig. 5k) while granulosa cells of Graafian follicles showed negative reactions (Fig. 5l).

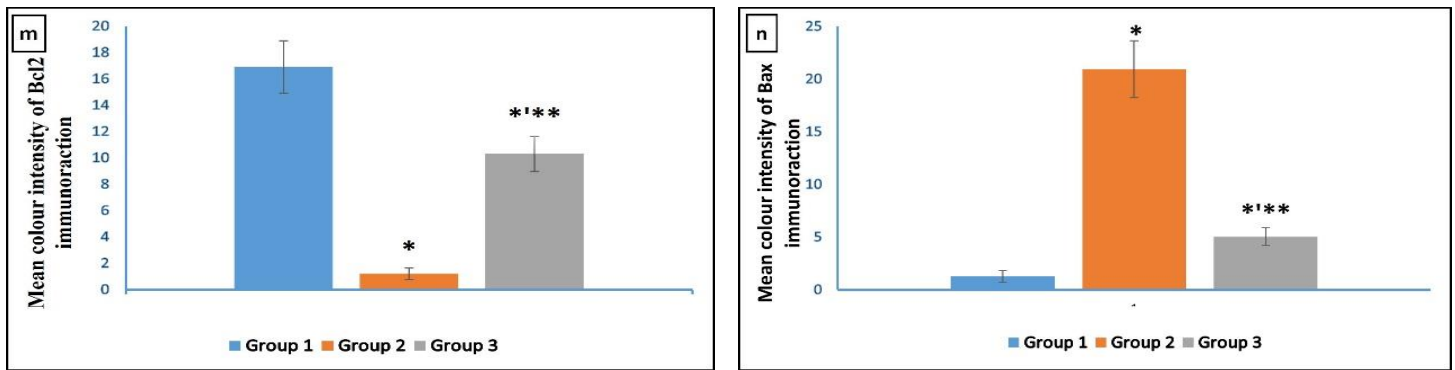
The morphometric analysis of the mean color intensity of Bcl2 positive immunoreaction showed a significant decrease in group 2 when compared to group 1. While in group 3 resulted in a significant increase in ovarian Bcl2 immunoreactivity when compared to group 2,

but it was still significantly lower than group 1 as shown in (Fig. 5m).

The morphometric analysis of the mean color intensity of Bax-positive immunoreaction showed a significant increase in group 2 when compared to group 1. While in group 3 resulted in

a significant decrease in ovarian Bax immunoreactivity when compared to group 2, but it was still significantly higher than group 1 as shown in (Fig. 5n).





**Fig. 5 (a-n)** Photomicrograph of Bcl2 and Bax immunohistochemical stained sections from rat ovarian tissue of all studied groups with morphometric analysis. **Fig. 4 (a, b, c, d, e & f)** Photomicrograph of Bcl2 immunohistochemical stained sections from rat ovarian tissue in all studied groups (**a, & b**) Show strong positive Bcl2 immunoreaction in the form of a brownish coloration of cytoplasm in granulosa cells of ovarian follicles (yellow arrows) in group 1. (**c, & d**) Show a negative reaction in group 2 when compared with control rats (arrows). (**e, & f**) Showed moderate to strong reactivity of Bcl2 in the cytoplasm of granulosa cells (yellow arrows) in group 3. **Fig. 5 (g, h i, j, k, & l)** Photomicrograph of Bax immunohistochemical stained sections from rat ovarian tissue. (**g & h**) Show negative Bax immunoreactions in the cytoplasm in group 1. (**i, & j**) Showed a moderate to strong positive reaction (yellow arrows) in group 2. (**k & l**) Show weak immunoreactivity of Bax in the cytoplasm of 1ry and 2ry follicles but a negative reaction in Graafian follicles in group 3 (Bcl2 and Bax immunostaining:  $\times 400$ , scale bar = 50  $\mu\text{m}$ ). **Fig. 5 (m & n)** Morphometric analysis of the mean color intensity of Bcl2 and Bax immunoreaction among all studied groups. (**m**) Morphometric analysis of the mean color intensity of Bcl2 immunoreaction among all studied groups (**n**) Morphometric analysis of the mean color intensity of Bax immunoreaction among all studied groups. Data are expressed as mean  $\pm$  SD. Superscript \*, and \*\* denote a statistically significant difference at ( $P < 0.05$ ). \*:  $p < 0.05$  Vs. Group 1, \*\*:  $p < 0.05$  Vs. Group 2 using one-way ANOVA with Tukey post hoc test.

## Discussion

It was proposed by *Mondal et al.* [30] that MSG inhibits the reproductive function by its nature as an excitatory transmitter on brain glutamatergic neurons that enhances the activity of the hypothalamic-pituitary-gonadal (ovarian) axis. Through a positive feedback loop, the hypothalamic glutamatergic neurons enhance the LHRH and FSHRH producing neurons, which in turn stimulates the production of pituitary LH and FSH.

However, considerable gut flora imbalance was detected in PCOS patients [31]. Given this, we postulated that the gut microbiota could assume a role in the pathogenesis of MSG-induced PCOS by causing systemic low-grade inflammation and IR, as well as promoting changes in the sex hormones, the gut-brain axis, and other pathogenic pathways.

In the present study, MSG induced dysbiosis was detected by elevation of plasma LPS, intestinal myeloperoxidase, and TMAO along with lowering of IAP and FFAR-2 gene. These results are also recorded by [32,33].

In women with PCOS, *Liu et al.* [34] reported an increase in gram negative bacteria (*Bacteroides* and *Desulfovibrio*). So, the elevation of LPS (a product of gram-negative bacteria) after MSG administration indicates intestinal dysbiosis. LPS triggers GIT inflammation as the first organ by affecting gut microbiome and gut-associated lymphoid tissue [35]. This study recorded elevation of intestinal myeloperoxidase which is an indicator of GIT inflammation and leucocytic infiltration. This aligns with the findings of *Bertani et al.* [36], who associated elevated LPS with disruption of the intestinal barrier, inflammation (leaky gut), and intestinal dysbiosis.

Plasma LPS contributes to the PCOS' pathophysiology by triggering "endotoxemia," and systemic inflammation [37]. So, in the current study the leaky gut by high LPS could be the cause of elevation of plasma  $\text{TNF-}\alpha$  and IL-6 in group 2. As it is through the CD14 toll-like receptor complex (TRL-4), LPS binding protein binds to the cell surface of the innate immune cells, triggering their signalling cascade resulting in

endotoxin-induced macrophage activation. The enhanced immune system activity (systemic inflammation) hinders the function of insulin receptors, causing IR [38,39].

The considerable reduction in IAP in group 2 confirms the increase in LPS. IAP enzyme at the brush border of the duodenum is a detoxifying agent for LPS by dephosphorylation of the lipid A region (toxic region). The reduction in IAP activity may precede or follow inflammation, and it contributes to the development or maintenance of gut inflammation and high plasma LPS levels [40].

Furthermore, gut dysbiosis by MSG was confirmed by escalation of TMAO levels, as TMAO is a hazardous metabolite generated by bacterial activity on quaternary amines such as choline found in common dietary constituents. Flavin mono-oxygenase enzyme in the liver converts choline to TMAO [41]. TMAO remains relatively stable in plasma, and can have detrimental effects. As a result, its concentration may indicate the degree of disruption in the gut microbiota [42]. TMAO by itself prompts inflammatory markers through NF $\kappa$ B activation [43] which is in harmony with our results.

Short-chain fatty acids (SCFAs): acetate, butyrate, and propionate are three dietary fibers' primary degradation products when dietary fibres are decomposed by the gut microbiota [44]. Butyrate, in particular, has anti-inflammatory properties as well as maintains the intestinal mucosal barrier function [45].

SCFAs activate FFAR-2 which is G-coupled protein receptors [46]. Since colonic inflammation was detected in FFAR-2-deficient mice, the FFAR-2 determines the protective effects

of SCFA in intestinal inflammation [47]. These findings matched our results, which showed that FFAR-2 mRNA gene expression was downregulated after MSG administration, and this further confirms gut inflammation.

The role of gut dysbiosis to trigger IR was confirmed by the positive correlation between plasma LPS and intestinal TMAO with HOMA-IR.

In addition, TNF- $\alpha$  provokes insulin receptor substrate 1 (IRS1) serine phosphorylation with subsequent attenuation of its ability to transduce insulin-mediated cellular events. Moreover, it has an impact on independent IRS insulin signalling [48].

IR impacts ovarian tissue's excitometabolic signal pathway [49]. In the present study, there was a reduction of ovarian relative mRNA expression of both AMPK $\alpha$  and SIRT1 after MSG administration. Reduced AMPK $\alpha$  and SIRT1 could be linked to IR as there was a negative correlation between HOMA-IR and ovarian AMPK $\alpha$ , SIRT1 gene expression, and ovarian tissue SIRT1 after MSG administration.

Reduced AMPK $\alpha$  (the mitochondrial energy deprivation response key regulator) is due to the developed local ovarian IR that inhibits AMPK through its phosphorylation by protein kinase B (AKT). AKT phosphorylates S485 of the AMPK $\alpha$ 1-subunit without phosphorylation of an equivalent site in the AMPK  $\alpha$ 2-subunit (S491), thus blocking upstream kinases from phosphorylating threonine 172 residue on its  $\alpha$  subunit [50].

Reduced SIRT1 in ovarian tissue and its gene expression is linked to the reduced AMPK $\alpha$  as it was reported by *Cantó et al.* [51] that AMPK $\alpha$  simultaneously activates SIRT1 by up-



regulating the SIRT1 co-substrate intracellular levels (NAD<sup>+</sup> or nicotinamide activity). Also, AMPK could be activated by SIRT1 via deacetylating the liver kinase B1, which then translocate from the nucleus to the cytosol, where it is activated with subsequent phosphorylation and activation of AMPK.

Pancreatic cell secretion of insulin is positively regulated by SIRT1 [52]. Moreover, enhancing SIRT1 expression, improved insulin sensitivity, especially under insulin-resistant conditions [53]. So, the interplay between SIRT1 and IR could explain the disturbed insulin signalling and impaired local ovarian response to it with the deleterious effects of IR on ovarian tissue.

In addition SIRT1 can regulate the hypothalamic-pituitary-ovarian axis, has a role in controlling basic ovarian functions, and promote the estrus cycle, while the deficiency of SIRT1 can disturb the estrus cycle [54].

The developed hyperandrogenic state in the form of elevated testosterone, LH, FSH, and LH/FSH ratio after MSG administration could be due to decrease ovarian SIRT1 as SIRT1 regulates steroid hormone synthesis in granulosa cells by deacetylation and upregulation of aromatase enzyme so granulosa cells could utilize androgens for the synthesis of estradiol. Dysregulation of stereognosis and granulosa cell function reduces inhibin and disturbs the negative feedback mechanism of the hypothalamic-pituitary-ovarian axis with an elevation of FSH, and finally, this hyperandrogenic state promotes excessive follicular growth [55]. This was evident in the present study by histomorphological changes with an increase in ovarian weight and cystic follicles after MSG administration.

IR can excite insulin receptors of the pituitary gland, causing the release of LH, and that promotes ovarian and adrenal gland secretion of androgen [56].

On the other hand, the developed hyperandrogenic state after MSG administration could be also similar to IR linked to high inflammatory cytokines (TNF- $\alpha$ , and IL-6). TNF- $\alpha$  is the major player in PCOS as it promotes androgen secretion by upregulating the expression of CYP17A1 (steroid 17-monooxygenase, 17-hydroxylase, 17,20-lyase) and CYP19A1, two key enzymes involved in androgen synthesis and conversion [57, 58].

Hyperandrogenic condition disturbs the hypothalamic-pituitary-ovarian axis' delicate balance by diminishing the negative feedback tone. This frequent stimulation of hypothalamic neurons led to the frequent pulsatile release of gonadotrophin-releasing hormone and an increase in the LH/FSH ratio [59]. This is in agreement with the results of the present study.

Reduced ovarian antioxidant capacity by reduction of ovarian GSH is linked to reduced ovarian SIRT1 as normally SIRT1 enhances superoxide dismutase, catalase, and GSH by upregulation and deacetylation of the factor which is a regulator of cellular resistance to oxidants (nuclear factor erythroid 2) [60]. Moreover, SIRT1 could protect ovarian mitochondria from damage by regulating ovarian autophagy by activating AMPK $\alpha$  together with inhibition of Phosphatase and tensin homolog induced putative kinase 1 in granulosa cells of PCOS [61].

The ovarian apoptosis after MSG administration in the form of decreased immunohistochemical staining of Bcl2 and

increased immunohistochemical staining of Bax agreed with [62] and could be due to decrease ovarian SIRT1 as it was reported by *Han et al.* [63] that SIRT1 increased granulosa cells' resistance to apoptosis by activating the ERK1/2 signaling pathway and inhibiting the NF $\kappa$ B signaling pathway, which has anti-inflammatory functions.

In addition, hyperandrogenic state exaggerates ovarian oxidative stress and apoptosis as excess androgen synthesis by ovarian theca internal cells under high LH, causes macrophage inflammasome production in granulosa cells, which promotes apoptosis in granulosa cells of ovarian follicles [64].

The Dyslipidemia state induced by MSG could be explained by the resistance to insulin's action on lipoprotein lipase in the peripheral tissues and impairment of proteins associated with lipid metabolism by hyperandrogenemia [65].

IR and hyperandrogenic conditions are associated with low plasma adiponectin levels which serve as an insulin-sensitizing agent by increasing fatty acid oxidation [66]. This could be the cause of the decrease of HDL together with the elevation of triglycerides and LDL in the current study after MSG administration.

In the current study, histological analysis of ovarian tissue after MSG administration revealed several cystic follicles with large antrum and thinner granulosa cell layers compared to the control group. No corona radiata or oocytes were found in these cystic follicles. Atretic follicles and a well-vascularized medullary stroma were also observed. These findings align with previous research by *Eweka et al.* [67], who linked MSG-induced oxidative stress to ovarian cystic

degeneration and atrophic changes in oocytes. Morphometric and statistical analysis further confirmed a significant reduction in the number of different ovarian follicles and a significant increase in the number of cystic follicles due to MSG administration, consistent with findings reported by *Ali et al.* [68].

However, this study demonstrated that flaxseed extracts before MSG administration improved dysbiosis of gut microbiota and the maintained the intestinal barrier by reducing gut permeability by lowering of plasma LPS, intestinal myeloperoxidase, and intestinal TMAO together with the elevation of IAP and mRNA gene expression of FFAR-2. As a result of maintaining gut permeability translocation of LPS from the intestines to the systemic circulation is reduced, thus alleviating peripheral endotoxemia, and ultimately contributing to the suppression of systemic and ovarian inflammation. This was evident in the present study by reduction of plasma level of TNF- $\alpha$  and IL-6. This also was observed by [69].

Moreover, flaxseed's high content of vitamin E, omega-3 fatty acids, flavonoids, and flax lignan complex phenolics, are responsible for direct antioxidant, anti-inflammatory, and anti-apoptotic properties of flaxseed as these content can scavenge superoxide radicals and other ROS with subsequent protection of immune cell polarization and activation [32]. This might explain the reduction of intestinal myeloperoxidase and gut inflammation in group 3.

The decreased systemic inflammation by flaxseed results in decreased IR by lowering of HOMA-IR and this was proved by a positive

correlation between plasma LPS and intestinal TMAO with HOMA-IR in group 3.

In addition, the high lignan content of flaxseed improves peripheral insulin sensitivity, glycogenolysis, and gluconeogenesis through antioxidant properties [70,71].

Furthermore, flaxseed's water-soluble fibres activate the extracellular signal-regulated kinase pathway to modulate the release of a glucagon-like peptide-1, raising insulin secretion, enhancing the action of insulin through increasing glucose transport and correcting hyperglycemia, which is connected to resistance to the action of insulin in PCOS [32].

Co-administration of flaxseed extract before MSG induced the raising of adiponectin; increasing adiponectin prevents the dyslipidemia in group 3 as it could invigorate AMPK in the muscle, eventually improving insulin sensitivity, revealing a decrement in triglycerides and LDL while an increase in HDL [72].

The reduced IR by flaxseed in group 3 had its impact on ovarian tissue and induced elevation of AMPK $\alpha$  and SIRT1 gene expression together with the increase in ovarian SIRT1 as proved by the negative correlation between HOMA-IR and ovarian AMPK $\alpha$  and SIRT1 gene expression together with ovarian SIRT1 in this group.

Increasing ovarian SIRT1 by enhanced insulin sensitivity after flaxseed administration may be the clue for preventing the local ovarian IR deleterious, this agreed with *Wu et al.* [73] who postulated that immunoreactivity of both AMPK $\alpha$  and SIRT1 increases after metformin alone or combined with resveratrol with improvement in PCOS' associated metabolic disorders.

The hyperandrogenic state is prevented by flaxseed administration in group 3 due to enhancing insulin sensitivity with decreased HOMA-IR, and also due to increased ovarian SIRT1 (a regulator for steroidogenesis)[54], both ultimately lead to a lowering of testosterone, LH, FSH, and LH/FSH ratio.

In addition, the lignan content of flaxseed has an anti-androgenic effect, which may increase the production of SHBG which lowers excessive levels of testosterone and androgen [74].

Moreover, the increase in ovarian GSH and increased immunohistochemical staining of Bcl2, and decreased immunohistochemical staining of Bax with flaxseed administration could be due to the antioxidant and antiapoptotic properties of SIRT1 [63]. Also, a decrease in androgen levels with flaxseed administration suppresses androgen-mediated inflammatory changes in the ovaries, blocks the apoptotic pathway, and enhances ovarian folliculogenesis[74].

The biochemical results of group 3 were confirmed by histomorphological ovarian changes with flaxseed administration as there was a reduction in ovarian weight and the number of cystic follicles that was due to a decrease in androgen. This was in harmony with [75].

#### Conclusion

In conclusion, our research provided fresh insight into how MSG-induced dysbiosis contributes to the pathogenesis of PCOS. Such dysbiosis of the gut microbiota damages the intestinal barrier and triggers endotoxemia, and a systemic inflammatory state which increases the danger of developing IR that has a local effect on the ovary through modulating AMPK $\alpha$ /SIRT1

pathway and subsequent decrement in SIRT1 in the PCOS rats' ovarian tissue could be a mediator for the progression of PCOS.

By modulating the hormone-inflammation-gut/microbiota axis, flaxseed extract protects against MSG-induced PCOS, potentially allowing access to a low-cost PCOS protective option in cases of high consumption of food additives.

### Recommendation

Because the human intestine contains roughly  $10^{14}$  bacteria, fecal 16S rDNA sequencing is advised to evaluate gut microbiota species at the phylum and genus levels, and the molecular mechanism by which MSG induces dysbiosis needs further investigation. SIRT1 modulators can be an effective target for the management of PCOS, but this needs further studies to determine their bioavailability and specificity.

### Authors' contributions:

Conceptualization, M.M.A. and R.A.A.E.; methodology, M.M.A, R.A.A.E, S.I; software, B.A.F.H; validation, P.M.H., N.H.M.A. and A.A.; formal analysis, M.M.A.; investigation, N.H.M.A; resources, R.A.A.E.; data curation, S.I.; writing original draft preparation, M.M.A. , R.A.A.E. and S.I.; writing review and editing, R.A.A.E and B.A.F.H; visualization, A.A.; supervision, R.A.A.E;. All authors have read and agreed to the published version of the manuscript. The authors declare that no paper mill was used.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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