Bull. Pharm. Sci., Assiut University, Vol. 48, Issue 1, 2025, pp. 727-740

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/

BULL. PHARM. SCI. Assiut Univ.

ALLOPURINOL ALLEVIATES ESTROGEN AND PROGESTERONE-INDUCED MAMMARY GLAND HYPERPLASIA THROUGH TARGETING PI3K/AKT/MTOR AND Ki-67 PATHWAYS IN FEMALE RATS

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***Background and Purpose**: Despite being widely used, there is no study on the effects of allopurinol on mammary gland hyperplasia (MGH). In this work, we elucidate the efficacy of allopurinol in **alleviating** MGH induced in female rats **by** estrogen and progesterone and exploring the underlying mechanisms. **Experimental approach**: Female Wistar rats were allocated into three groups (n=10). Control group administered carboxymethyl cellulose (CMC) 1% orally. Rats in both MGH and MGH+allopurinol groups injected with 0.5 mg/kg/day estrogen (IM) for 25 days followed by 0.5 mg/kg/day progesterone (IM) for 5 days. On the 31st day of the experiment, only MGH+allopurinol group was treated daily with allopurinol 50 mg/kg orally for 30 days. Oxidative stress, inflammatory parameters and PI3K/AKT/mTOR genes expression were measured. Histological and immunohistochemical analysis were performed. **Key Results**: In MGH group, there was significant decrease in tissue Nrf2, SOD and CAT levels and significant increase in tissue MDA and NO levels. Moreover, tissue NF-κB, TNF-α, IL-6 levels were significantly elevated, while tissue IL-10 level was significantly reduced. Mammary PI3K, AKT and mTOR genes were positively regulated. Histologically and immunohistochemically there were proliferative changes including hyperplasia in most lobules, increased number of ducts, acini, and multilayered epithelial lining, and increased expression of Ki-67. Whereas treatment with allopurinol significantly reversed the abnormal biochemical and histological abnormalities. Moreover, restored the expression of PI3K/AKT/mTOR pathway and decreased the expression of Ki-67. **Conclusion & Implication**: Therefore, results demonstrated that allopurinol could alleviate estrogen and progesterone-induced mammary gland hyperplasia through targeting PI3K/AKT/mTOR and Ki-67 pathways.*

***Keywords:** Allopurinol, mammary gland hyperplasia, PI3K/AKT/mTOR, Ki-67, proliferation, oxidative stress*

INTRODUCTION

Mammary gland hyperplasia (MGH) has a major impact on both patient's physical and emotional well-being[1]. Its main pathogenesis is disturbance of estrogen and progesterone secretions causing proliferation of mammary gland lobules[2]. MGH patients' morbidity is rapidly growing, with a significantly higher chance of producing mammary carcinoma[3]. Hormone or endocrine therapy is one of the most often used treatments for MGH clinical symptoms. Nonetheless, adverse effects reduce the quality of life for those receiving long-term treatment [4].

Received : 15/10/2024 & Accepted : 22/12/2024

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Phosphatidylinositol-3-kinase (PI3K), protein kinase B (AKT) and mammalian target of rapamycin (mTOR) signaling pathway is essential for numerous facets of cell growth and survival both in physiological and pathological circumstances. Moreover, activation of PI3K/AKT/mTOR pathway brings about great disturbance in control of cell growth and survival [5]. The proliferative attribute of estrogen results from the activation of PI3K that stimulates the phosphorylation of AKT. The phosphorylation of AKT promotes cell growth and protein synthesis by activating mTOR that has a critical role in **cell proliferation and cancer** development [6]. The normal mammary epithelium, in most mammals, expresses variety of receptors including the cell cycle-associated antigen ki-67. It expressed in cells undergoing mitosis, so it considered as a reliable marker for cell proliferation [7].

Xanthine oxidase (XO) enzyme, which is known to stimulate the production of uric acid, it is considered as one of the two primary enzymes in the body that generates reactive oxygen species (ROS), together with NADPH oxidase[8]. The resulting ROS causes damage to DNA or a protein cell cycle and may lead to uncontrolled division and growth of cells [9]. Increased oxidative stress also, induced pro-inflammatory transcription factors as nuclear factor-κB (NF-κB). Moreover, it is incriminated as a mediator of cell proliferation and neoplastic transformation [10]. XO induces the metabolic activation of carcinogenic compounds and behaves as a tumorigenic agent by creating reactive oxygen and nitrogen species[11]. The expression of XO was reported in the epithelial cells of the mammary glands [12]. Nevertheless, its role in the pathogenesis of MGH has not been previously examined.

Allopurinol, a commonly used anti-gout therapy, is considered a powerful antioxidant through its inhibition of XO [13]. Besides, allopurinol has an anti-inflammatory effect and is used in the treatment of many types of inflammatory diseases[14]. Moreover, Yasuda et al.[15] reported that by inhibiting XO, allopurinol could induce prostate cancer cell apoptosis. In addition, its long-term treatment in gout patients may be associated with a lower incidence of prostate cancer[16]. To our knowledge, no study examines the effect of XO inhibitor, allopurinol on MGH. Therefore, the present study aimed to explore the role of allopurinol in **alleviating** MGH induced in female rats **and** investigate the possible mechanisms of action.

MATERIALS AND METHODS

Drugs and chemicals

Allopurinol was procured from AK Scientific, Inc. (USA). Estrogen and progesterone ampules were obtained from Misr Co. for Pharma. Ind., Egypt. Carboxymethyl cellulose 1% (CMC) was purchased from Biodiagnostic Company for pharmaceuticals and chemical industries, Egypt. superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and nitric oxide (NO) assay kits were bought from Biodiagnostic Company Pharmaceutical Industries, Egypt. Ki-67 Monoclonal Antibody catalog # 25-5698-82 were bought from Thermo Fisher Scientific Company.

Animals and Administration

The study was conducted in strict accordance with the European Union Guidelines for the Care and Use of Laboratory Animals (European Union Directive 2010/63/EU), which was approved by Sohag University, Faculty of Medicine, Sohag, Egypt Institutional Animal Care and Use Committee (Approval No. Sohag 5-5-4/2024-01).

Adult female albino Wistar rats weighing 190±10 g were brought from the animal house, Faculty of Medicine, Sohag University, Egypt. The animals were fed a commercial pellet diet, placed on a normal light/dark cycle, temperature 20-24 °C, 40-60 % humidity and provided unlimited food and water. Three groups of rats, 10 at each, were distributed at random. Rats of the **control group** received CMC 1% orally. Rats in **MGH and MGH+Allopurinol groups** injected with 0.5 mg/kg/day estrogen IM for 25 days followed by 0.5 mg/kg/day progesterone IM for 5 days[17] to induce experimental MGH model. On the 31st day of the experiment, only **the MGH+Allopurinol group** was treated daily with allopurinol 50 mg/kg [18] orally by gastric gavage for 30 days. Both control and MGH groups were treated with an equal volume of CMC 1% orally.

Twenty-four hours after the last treatment dose, rats from all groups were euthanized under the influence of isoflurane anesthesia. Following skin shaving, mammary gland tissues were gathered and weighed then divided into three parts. The first part was centrifuged at 4000 rpm for 15 min at 4ºC after being homogenized in sodium phosphate buffer (pH 7.4). The supernatant was then separated for biochemical analysis. The second part was swiftly frozen in liquid nitrogen, then kept at -80 ºC, and used for gene expression analysis. The last part was fixed in 10% formalin to be used in histological and immunohistochemical (IHC) analysis.

Biochemical analysis

Measurement of antioxidant and oxidant parameters

Enzyme-linked immunosorbent assay (ELISA) was used to measure the tissue level of nuclear factor erythroid 2-related factor 2 (Nrf2) in accordance with the manufacturer's instructions. Nrf2 showed in ng/mg tissue. It was obtained from Cusabio Technology LLC (USA).

SOD and CAT levels were investigated according to Nishikimi et al. [19] and Aebi [20] respectively and a colorimetric technique was employed. Tissue SOD absorbance change was detected at 550 nm and expressed as U/ mg tissue. As regarded CAT, its absorbance change was detected at 510 nm and expressed as U/mg tissue.

MDA and NO levels were detected relying on Ohkawa et al. [21] and Montgomery and Dymock [22] respectively. Their absorbance change were detected at 534 nm and 540 nm. MDA in the sample stated in nmol/mg tissue. While, nitrite in the sample presented in μmol/mg tissue. For both of them, the technique of colorimetric analysis was utilized.

Measurement of NF-κB, TNF-α, IL-6 and IL-10 level

Tissue levels of nuclear factor-κB (NF-κB), tumor necrosis factor alpha (TNFα), interleukin 6 (IL6) and IL10 were measured by ELISA specific kit. Cusabio., USA provided kits for both NF-κB and IL-10**.** Besides**,** TNF-α and IL-6 gotten from Wuhan EIAab Science Co. Ltd., China, Elabscience., USA respectively. They were expressed in the sample as pg/ mg tissue.

Analysis of PI3K, AKT and mTOR genes expression with quantitative real-time PCR

The TRI reagent (Bioshop Co, Canada) was utilized to isolate the entire RNA from the mammary gland tissue samples. Template cDNA was created using GoTaq 1-Step RT-qPCR kit, Promega institution, USA. The amplification was carried out on an Applied Biosystems 7500 device (USA). The procedure of PCR amplification consisted of 10-minute initial denaturation at 95°C. Next, 40 cycles of denaturation at 95°C for 10 seconds. Annealing at 60 °C for 30 seconds, lastly, extension at 72°C for a further 30 seconds. The $^{2-\Delta\Delta CT}$ comparative method [23] was used to quantify the relative level of gene expression for each sample, standardized to the housekeeping gene β-actin. The primers' nucleotide sequences were as follow **(Table 1)**:

Table 1: Primers sequences for the studied genes.

Gene	Primer sequence: 5′–3′
PI3K	Forward: CTC TCC TGT GCT GGC TAC TGT Reverse: GCT CTC GGT TGA TTC CAA ACT
AKT	Forward: ATC CCC TCA ACA ACT TCT CAG T Reverse: CTT CCG TCC ACT CTT CTC TTT C
mTOR	Forward: TGC CTT CAC AGA TAC CCA GTA C Reverse: AGG TAG ACC TTA AAC TCG GAC C
β-actin	Forward: GGC ACC ACA CCT TCT ACA ATG Reverse: GGG GTG TTG AAG GTC TCA AAC

Phosphatidylinositol-3-kinase (PI3K), protein kinase B (AKT) and mammalian target of rapamycin (mTOR).

Histological and Immunohistochemical analysis

The following stains were used to evaluate the normal histological structure of rat's mammary glands and the pathological changes among experimental groups:

- Hematoxylene and Eosin (H&E) stain.
- Immunohistochemical staining using the proliferation marker Ki-67 antibody.

Histopathological evaluation

Routine histological processing was done. Specimens were fixed in 10% neutral buffered formalin for 48 h, dehydrated in increasing concentrations of ethanol, and then embedded in paraffin. Five microns (µm) tissue sections were prepared and mounted on slides. Sections were deparaffinized in hot xylene for 10 minutes and rehydrated using descending grades of alcohol for two minutes each. Finally, sections were stained with H&E to be examined using a light microscope (Olympus Corp., Tokyo, Japan) to evaluate the histopathological findings.

Immunohistochemistry study

Paraffin tissue sections (4µm) were mounted on positively charged slides, deparaffinized in xylene, rehydrated in descending grades of alcohol, and then incubated in 3% H_2O_2 for 20 minutes. Antigen retrieval was done by heating the sections twice in 0.01 M citrate buffer (pH 6.0) in a microwave for 5 min, followed by incubation with a rabbit monoclonal antibody against Ki-67 at 1:100 (Clone SP6, Abcam, USA) overnight at 4ºC. The sections were incubated with a biotinylated goat secondary antibody for 20 minutes. Then, incubated with Streptavidin-biotin peroxidase for 10 minutes at room temperature, with washing in phosphate buffer saline (PBS) after each step. After staining with diaminobenzidine (DAB) chromogen, the tissue slides were counterstained with hematoxylin, dehydrated, and mounted to each slide using DPX.

Evaluation of immunostaining

Ki-67 protein expression appeared as diffuse or granular brown nuclear staining. First, immunostaining was evaluated at low power to select areas with maximal nuclear staining within the epithelial cells. Then, counting of positive cells was done at high power (400X); Ki-67 positivity expressed as the percentage of Ki-67-stained nuclei per 500 cells. The surrounding stroma, vascular component, inflammatory cells, and necrotic areas were excluded from scoring[24].

Statistical analysis

The SPSS software (version 26) was utilized for all statistical analyses. The data were analyzed statistically using one-way analysis of variance (ANOVA), and expressed as the Mean ± SE. The Tukey post hoc test was carried out to evaluate the differences between groups. The difference became significant when $P<0.05$.

RESULTS AND DISCUSSION

Results

Effect of allopurinol on antioxidant and oxidant parameters

Results showed that, Nrf2**,** SOD and CAT levels were significantly decreased in the MGH group compared to the control group. Administration of allopurinol significantly raised tissue levels of Nrf2**,** SOD and CAT matched to the MGH group **(Table 2)**. Significant increase in MDA and NO in the MGH group were present in contrast to the control group. Furthermore, the MGH +allopurinol group displayed significant decrease in tissue levels of MDA and NO in comparison with MGH group **(Table 2)**.

Effect of allopurinol on inflammatory biomarkers

The data of the present study revealed significant elevation in the tissue levels of NFκB, TNFα, IL6 and a significant reduction in tissue IL10 level in MGH group opposed to the control group. Allopurinol administration significantly decreases NF-κB, TNF-α, IL-6 levels and increases the tissue IL-10 level contrary to MGH group **(Fig. 1)**.

Table 2: Effect of allopurinol on antioxidant and oxidant parameters in estrogen and progesterone-induced MGH in female rats.

Parameters	Control	MGH	MGH+Allopurinol
Nrf2(ng/mg tissue)	60.08±3.27	31.25±2.87*	53.72±3.48$^{\#}$
SOD (U/mg tissue)	388.17±15.28	178.83±8.19*	340.33±26.84$^{\#}$
CAT(U/ mg tissue)	541.67±25.48	232.67±22.70*	515.00±19.45$^{\#}$
MDA (nmol/mg tissue)	45.50±4.39	100.82±7.01*	60.72±4.63$^{\#}$
NO(µmol/ mg tissue)	12.73±1.20	30.67±1.49*	18.12±1.13$^{\#}$

Data appear for mean±SE (n=10). *Significant opposed to control group (P<0.05). $^{\#}$ Significant opposed to MGH group (P<0.05). MGH; Mammary gland hyperplasia, Nrf2; nuclear factor erythroid 2-related factor 2, SOD; Superoxide dismutase, CAT; Catalase, MDA; Malondialdehyde, NO; Nitric oxide.

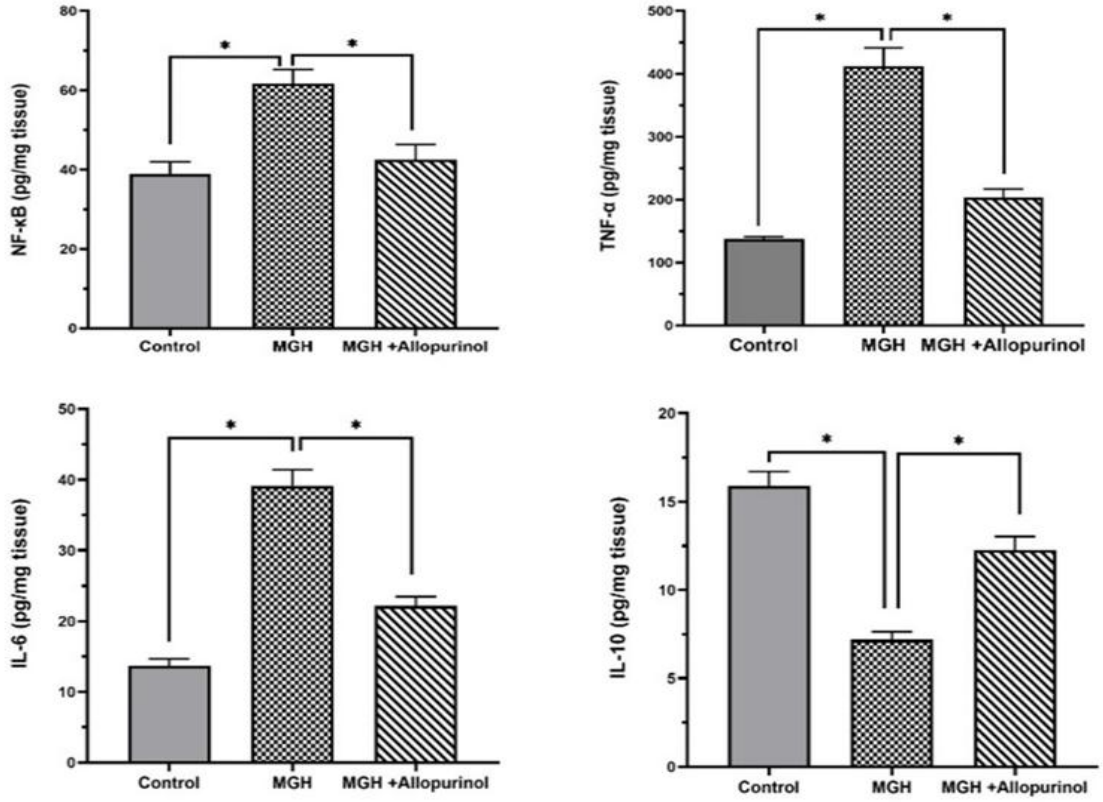

Fig. 1: Effect of allopurinol on inflammatory biomarkers in estrogen and progesterone- induced MGH in female rats.

Results are represented as mean ±SE (n=10). *Significant at P<0.05. MGH; Mammary gland hyperplasia, NF-κB; nuclear factor-kappa B, TNF-α; Tumor necrosis factor alpha. IL-6; Interleukin 6, IL-10; Interleukin 10.

Effect of allopurinol on PI3K, AKT and mTOR gene expression

It can be revealed from **Fig. (2)** that in the MGH group PI3K, AKT and mTOR gene expression in the mammary gland were up regulated compared to the control group. In the MGH +Allopurinol group there was an obvious down regulation in the above-mentioned genes in comparison to the MGH group.

Histological and Immunohistochemical changes

Hematoxylin and eosin stain

Hematoxylin and eosin stained sections of the control group showed the classic histologic appearance of the mammary gland tissue. Scattered lobes of acini and ducts within an abundant adipose tissue stroma. Each was lined by a well-organized single layer of cuboidal epithelial cells surrounded by an outer basal layer of flat myoepithelial cells. The lumen of ducts were patent, and all were surrounded by thin connective tissue (CT) sheets with a scattered few undeveloped acini were also seen in some sections **(Fig. 3A&B)**. H&E stained sections of the rats of the MGH group exhibited an obvious increase in the lobulo-alveolar epithelial component on expense of surrounding fibrofatty CT stroma. Increased and dilated acini and ducts with hyperplasia of the lining epithelial cells. Some ducts were cystically dilated. The ducts were lined by more than two layers of epithelial cells. Some lining epithelial cells revealed mild cellular pleomorphism. Some revealed discontinuity of their lining epithelium with the detachment of some epithelial cells to the lumen. Disturbed fatty cellular eosinophilic stroma with excessive collagen fibers, thickened CT sheaths and dilated congested blood vessels **(Fig. 3C&D)**. However, sections of the MGH+Allopurinol group revealed marked improvement in the hyperplastic changes of mammary epithelial cells. Most ducts and acini lined by well-organized layers of epithelial cells with prominent nuclei. Necrotic eosinophilic materials occasionally present in their lumens. Some ducts were surrounded by mild cellular eosinophilic stroma with thin CT sheaths. (**Fig. 3E&F).**

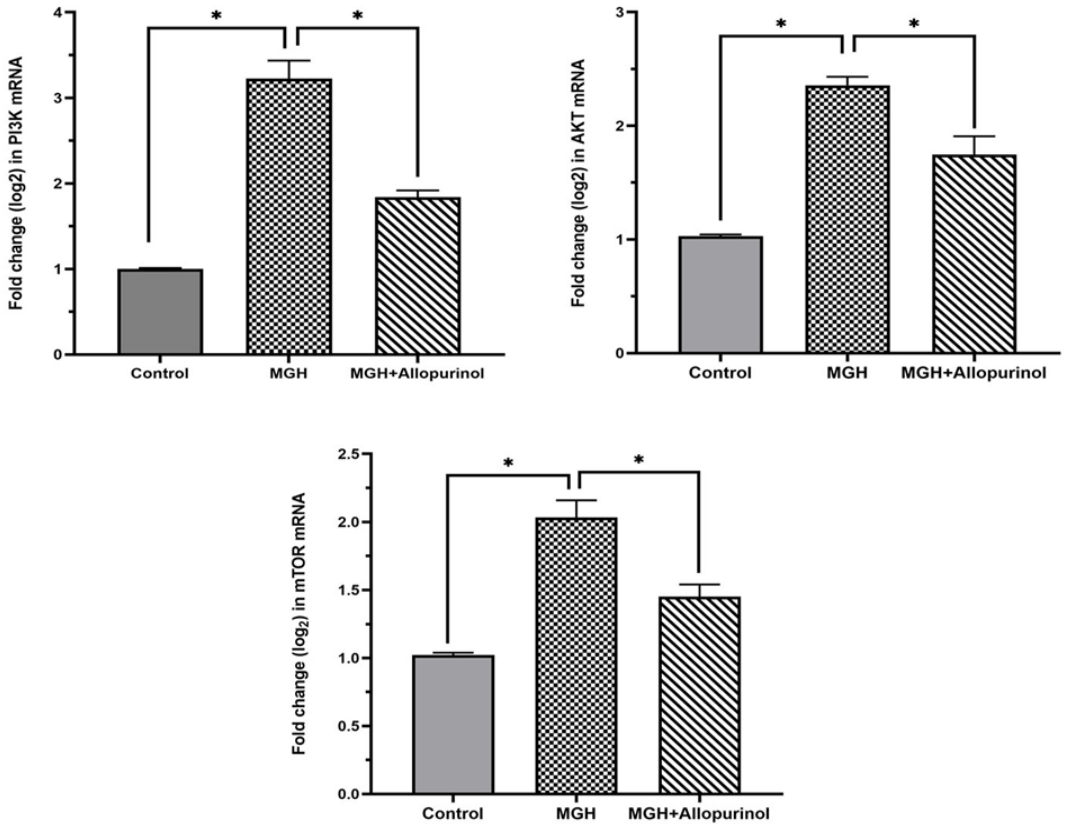

Fig. 2: Effect of allopurinol on PI3K, AKT and mTOR mRNA expression in estrogen and progesterone induced MGH in female rats.

Results are represented as mean ±SE (n=10). *Significant at P<0.05. MGH; Mammary gland hyperplasia, PI3K; Phosphatidylinositol-3-kinase, AKT; protein kinase B, mTOR; mammalian target of rapamycin.

Immunohistochemical studies (Ki-67)

Mammary glandtissue sections of the control groupshowed few sporadic positive immunoreactivity of scattered epithelial cells were detected across the epithelial component of the mammary tissue appearing as light brown nuclear staining. While, the stroma showed negative reactivity (**Fig.4A**). As regard MGH group, strong nuclear brown positive immunostaining of epithelial cells of the ducts and acini with immunopositive secretions in their lumens. The positivity involved more proportion of the epithelial cells compared with the control group. Few immunostaining of the surrounding stroma (**Fig.4B**). In contrast, the mammary gland tissue of MGH+Allopurinol group showed light brown nuclear positive immunestaining of scattered epithelial cells which was nearly comparable to the control group with few immunopositive cells of the surrounding connective tissue (**Fig.4C&D**).

Percentage of Ki-67 positive cells

In **Fig. (4E)**, it was observed that percentage of Ki-67 positive cells was significantly higher ($P< 0.05$) in MGH group in comparison with control group. However, percentage of Ki-67 positive cells significantly lower in MGH +Allopurinol group in contrast to MGH group.

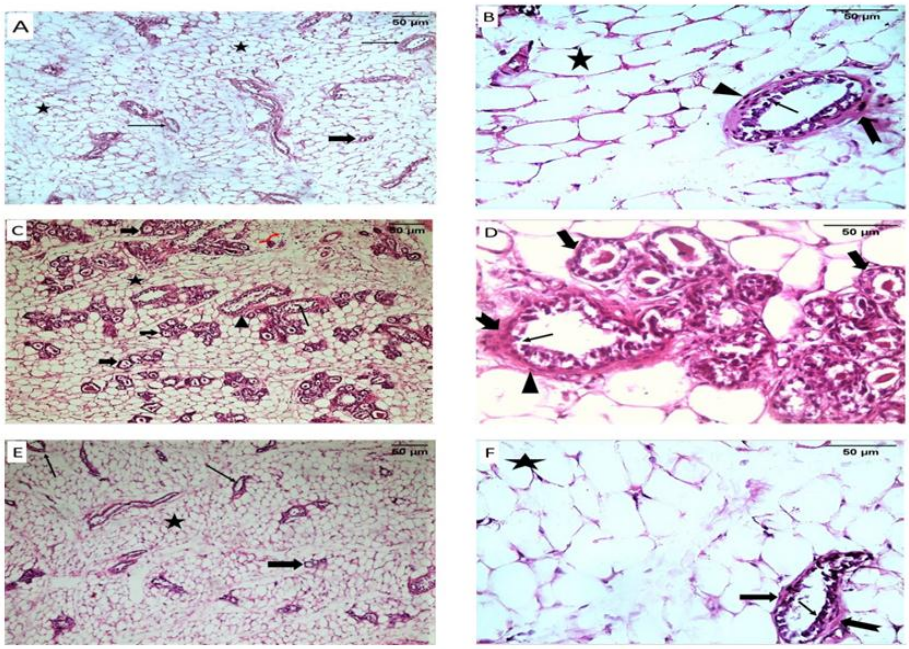

Fig. 3A:a photomicrograph of H&E stained section of mammary gland (control group)showing scattered ducts (thin arrow)within abundant fat cells (star) , few under developed alveoli (thick arrow) (H&E X 100). **B:** a magnification of previous section showing that the duct lined by one layer of cuboidal cells (thin arrow) with outer layer of flat myoepithelial cells (arrow head) surrounded by thin connective tissue sheath (indented arrow) within abundant fat cells (star) (H&E X 400). **C:a photomicrograph of H&E stained section of mammary gland (MGH group)**showing dilated duct with detached epithelial cells in the lumen (thin arrow), surrounded by thick connective tissue sheath (arrow head) with increased and dilated alveoli (thick arrow). Some inflammatory cellular infiltrations noted (curved red arrow) within less fatty stroma (star) (H&E X 100). **D:a photomicrograph of H&E stained section of mammary gland (MGH group)** show dilated duct lined by more than one layer of disarranged epithelial cells (thin arrow), and outer layer of flat myoepithelial cells (arrow head). Which is surrounded by thick connective tissue sheath (indented arrow) with increased and dilated alveoli with secretion in its lumen (thick arrow) (H&E X 400). **E:a photomicrograph of H&E stained section of mammary gland (MGH +Allopurinol)** showingscattered ducts (thin arrow) within abundant fat cells (star) and few alveoli (thick arrow) (H&E X 100). **F:** a magnification of previous section showing that the duct lined by one layer of cuboidal cells (thin arrow) with outer layer of flat myoepithelial cells (thick arrow) and surrounded by thinconnective tissue sheath (indented arrow) within abundant fat cells (star) (H&E X 400).

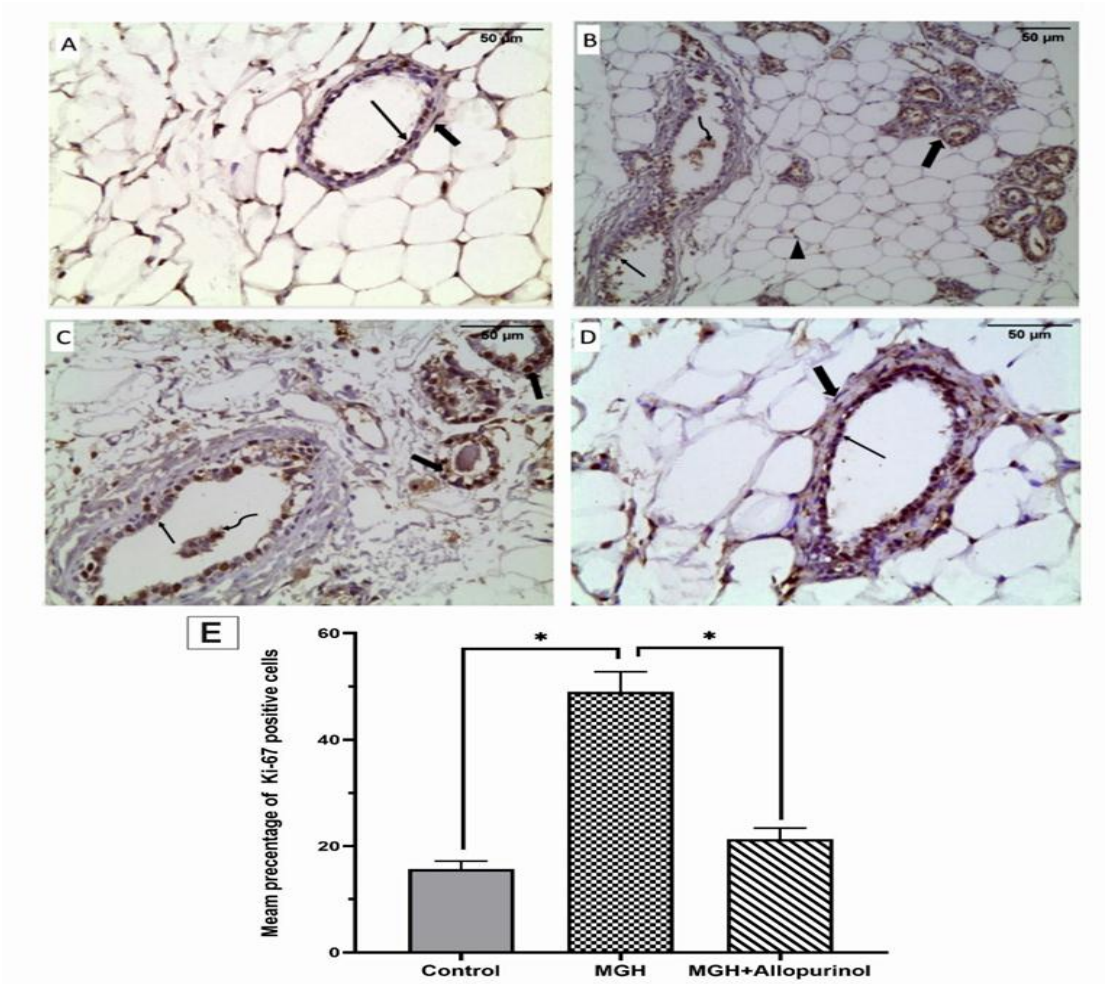

Fig. 4 A:a photomicrograph of section of mammary gland (control group) showingfew positive Ki-67 immunostained cells in the ductal epithelium (thin arrow) with minimal reactivity in the myoepithelial cells (thick arrow) (X 400). .**B:a photomicrograph of section of mammary gland (MGH group)** showing multiple positive Ki-67 immunostained ductal epithelial cells (thin arrow) with detached immunopositive cells in its lumen (curved arrow) and alveolar cells with immunopositive secretions in their lumens (thick arrow) . Few immunostaining of the surrounding stroma (arrow head) (X 200). **C: a photomicrograph of section of mammary gland (MGH group)** showing multiple positive Ki-67 immunostained ductal epithelial cells (thin arrow) with detached immunopositive cells in its lumen (curved arrow) and alveolar cells with immunopositive secretions in their lumens (thick arrow) (X 400). **D:a photomicrograph of section of mammary gland (MGH +Allopurinol)** showing light brown nuclear positive immunostaining of some epithelial cells (thin arrow) with few immunopositive cells of the surrounding connective tissue (thick arrow) (X 400). **E**:**Graphical presentation of the mean percentage of Ki-67 positive cells in the different groups.**

Discussion

One common benign breast condition that frequently acts as a precursor to other breast diseases is mammary gland hyperplasia[25]. Women face a serious public health risk due to MGH, a disease that is common in the middle age and has a strong cancerous tendency. MGH pertains to endocrine disorders, the majority of which caused by an estrogen and progesterone imbalance[26,27]. Consequently, this study investigates the efficacy of allopurinol in **alleviating** mammary gland hyperplasia induced in female rats **by** estrogen and progesterone, as a new indication of this drug.

In MGH group, histological results demonstrated evident proliferative changes including hyperplasia in most lobules, increased number of ducts, acini, and multilayered epithelial lining. These findings **are compatible with** Liu et al., Li et al. and Zhang et al. [17, 28, 29]. In addition, the cell proliferation marker Ki 67 expression was increased in mammary gland tissue. The relation between breast cell proliferation and

the breast cancer development is well established. In addition, it had been shown that the risk of breast cancer was reduced if breast proliferation was reduced [30]. In the current study, administration of allopurinol significantly suppressed these changes. The proliferative degree of mammary lobules and number of acini and ducts markedly decrease. **Allopurinol inhibits XO enzyme, which has gained recognition for its proliferative ability and for mediating neoplastic transformation and metastasis aggressiveness in cancer models**[31]**.**

Estrogen contributed to the growth and proliferation of the mammary gland by promoting the growth and proliferation of mammary cells [32]. MGH and oxidative stress, which resulted from a hormonal imbalance, are closely related. Excess estrogen can produce significant amounts of ROS and increase oxidative stress from its intermediate metabolites, 4-hydroxyestradiol semiquinone **and quinone** [33]. Besides, oxidative stress modified the immune system and has a major effect in estrogen-induced MGH [34]. In the present study, the oxidant antioxidant imbalance was proved by the significant decrease in the tissue level of antioxidant parameters **(**Nrf2, **SOD and CAT)** in MGH rats and an obvious increase in the tissue level of MDA and NO which indicates increased oxidative stress in the mammary gland. These results are in harmony with Chen et al.[3]. Nrf2 is responsible for the expression of heme oxygenase-1 (HO-1), SOD and glutathione S-transferases and functions as a master regulator of redox homeostasis[35]. Deficit in Nrf2 lowers ROS scavenging by reducing the expression of antioxidant genes and causing damage to DNA. Allopurinol successfully reduced ROS content[36]. Interestingly, in the present study, allopurinol increases the level of Nrf2 in mammary gland tissue and concurrently increases the tissue levels of SOD and CAT. Moreover, it reduces MDA and NO tissue levels in MGH+allopurinol treated group, thereby correcting abnormalities in oxidative stress parameters. These results agree with Gokcen et al. [8]. The antioxidant effect of allopurinol is due to inhibition of XO enzyme, which can produce reactive oxygen species and therefore causes inflammation and tissue damage [37]. Moreover, Hosseinzadeh et al.[18] mentioned that allopurinol antioxidant effect may be mediated by its uric acid-lowering effects which constrain the harmful overproduction of ROS.

In addition, the current study showed that in **the** MGH group there was an increase in the tissue NF-κB, TNF-α and IL-6 levels along with decreased tissue IL-10 level. One important modulator of inflammation is the pro-inflammatory transcription factor NF-κB that **regulates inflammatory responses.** NF-κB can be activated by numerous external stimuli; oxidative stress is a major factor in NF-κB activation [38] and it stimulates multiple inflammatory cytokines like TNF-α and IL-6. According to Brantley et al. [39]. NF-κB is crucial for controlling the growth, branching, and preservation of the normal epithelial structure of mammary epithelium. M**ammary gland** hyperplasia causes intricate inflammatory diseases, which elevated the lesion risk for malignancy. In addition, **increasing the activity of NF-κB led to** MGH [29]. According to results of the present study, NF-κB was triggered, leading to elevated levels of proinflammatory cytokines regulated by this factor, including TNF-α, and IL-6. Moreover, there was a decrease in IL-10 level, which has immunomodulatory and anti-inflammatory properties and this in agreement with Tilg et al.[40]. Allopurinol's anti-inflammatory effect in the present study is related to the reduction in the tissue level of NF-κB, TNF-α and IL-6 and elevation in IL-10. This result is in harmony with Yang et al. [41]. Besides, Li et al. [29] reported that, blocking NF-κB activation within the mammary glands led to decrease lobulo-alveolar proliferation, whereas increasing NF-κB activity through estradiol administration caused the mammary gland to hyperplasia.

It has been observed that the activation NF-κB and PI3K/AKT are linked leading to an inflammatory response, which is in coordination with the finding of Sun et al. [42]. The current results showed up **regulation of PI3K, AKT** and mTOR **genes in** MGH group compared with control group. **The PI3K/AKT/mTOR pathway regulates** cell proliferation and survival in both healthy and pathological conditions. Therefore, maintaining **PI3K/AKT/mTOR** expression is critical for preserving healthy cell physiology. **Estrogen and many other stimuli can**

promote AKT signaling pathway activation.[29]**.** ROS can activate PI3K/AKT by reversibly inactivating phosphatase and tensin homolog (PTEN) (**tumor suppressor protein**) within the cell. PTEN inhibition is required for ROS-induced tumor growth [43,44]. In the present work, MGH was attenuated by allopurinol administration through restoring the expression of PI3K/Akt/mTOR in the treated group compared to the MGH group. This indicates that allopurinol could be effective in retarding mammary hyperplasia.

Additionally, the expression of the proliferation marker Ki-67 was increased in MGH group as compared to the control group. These finding were in line with previous studies [17,45]. Moreover, Ki-67 expression in allopurinol-treated mammary gland showed decreased nuclear positivity in the epithelium of the ducts and acini. This indicates decreased cell proliferation and this in accordance with Guo et al. [46]. They reported that treatment with allopurinol reduced proliferation of hepatocellular carcinoma. Allopurinol suppressed the histopathological changes in estrogen and progesterone-induced MGH in female rats.

To the best of our knowledge, after searching the literature, no previous study examined the effect of XO inhibitor allopurinol on MGH in female rats; however, some recent articles discussed the effect XO inhibitors in other types of tissues as endometrial hyperplasia and benign prostatic hyperplasia [31,33].

Conclusion

The current study highlights that allopurinol can alleviate estrogen and progesterone-induced mammary gland hyperplasia through antioxidant, anti-inflammatory and anti-proliferative effects. The underlying mechanisms could include up regulating Nrf2 and inhibiting NF-κB and thus production proinflammatory cytokines, its capacity to modulate the expression of PI3K/AKT/mTOR genes and Ki-67 in mammary gland tissue. Therefore, allopurinol's antioxidant, anti-inflammatory and anti-proliferative effects suggests that it may be a potentially effective treatment for patients with MGH. The significance of these findings also emphasizes how our knowledge of even older, commonly used medications is constantly growing and necessitates further research to maximize therapeutic safety.

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Bull. Pharm. Sci., Assiut University, Vol. 48, Issue 1, 2025, pp. 727-740

نشرة العلوم الصيدليـــــة
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ألوبيورينول يعمل على تخفيف تضخم الغدة الثديية الناجم عن هرموني الاستروجين والبروجيستيرون من خلال استهداف مسارات PI3K/AKT/mTOR وKi-67 في إناث الجرذان

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يستخدام الوبيورينول على نطاق واسع، إلا انه لا توجد دراسة حول تأثيرات الوبيورينول على تضخم الغدة الثديية (MGH). العمل الحالى يوضح فعالية الوبيورينول في تخفيف تضخم الغدة الثديية الناجم عن هرمونى الاستروجين والبروجسترون في إناث الجرذان. تم تقسيم إناث جرذان ويستار البيضاء البالغة إلى ثلاث مجموعات (ن = ١٠). تم إعطاء المجموعة الضابطة (1%) CMC عن طريق الفم. تم حقن الجرذان في كل من مجموعتي MGH وMGH + الوبيورينول بجرعة ٠.٥ مجم / كجم / يوم من هرمون الاستروجين (IM) لمدة ٢٥ يومًا تليها جرعة ٠.٥ مجم / كجم / يوم من البروجسترون (IM) لمدة ٥ أيام. بدءاً من اليوم الحادي والثلاثين من التجربة، تم علاج مجموعة MGH + الوبيورينول فقط يوميًا بجرعة ٥٠ مجم / كجم من الوبيورينول عن طريق الفم لمدة ٣٠ يومًا. في مجموعة MGH، كان هناك انخفاض كبير في مستوى كل من Nrf2 وSOD وCAT في الأنسجة وزيادة كبيرة في مستوى كل من MDA وNO في الأنسجة. بالاضافة إلى ذلك، ارتفع مستوى كل من NF-κB وTNF-α وIL-6 في الأنسجة بشكل كبير، بينما انخفض مستوى IL-10 في الأنسجة بشكل كبير، كذلك ايضا تم تسجيل زيادة مستوى جينات PI3K وAKT وmTOR الثديية. من الناحية النسيجية والمناعية النسيجية كانت هناك تغييرات شملت تضخم في معظم الفصيصات وزيادة عدد القنوات وزيادة التعبير عن حين Ki-67. في حين أن العلاج بالوبيورينول عدل بشكل كبير التغيرات الكيميائية والنسيجية. علاوة على ذلك، تم تسجيل تحسن فى مسار جينات PI3K / AKT / mTOR و خفض لمستوى جين Ki-67. لذلك، أظهرت هذه الدراسة أن عقار الوبيورينول يمكن أن يخفف من تضخم الغدة الثديية الناجم عن هرمونى الاستروجين والبروجيستيرون من خلال استهداف المسار الجينى لكل من جينات PI3K / AKT / mTOR و Ki-6.