# The effect of Norethisterone Acetate (NETA) on the ovarian follicles of albino rats: Histological Study.

Raghda I. Abdullah [1], Mahmoud Abd-Elkareem [2], Ahmed Abou-Elmagd Ahmed [2], and Sotohy, A. Sotohy [3]

[1]Department of Anatomy and Histology, faculty of Veterinary medicine, New Valley University, kharga, Egypt.
[2]Department of Cell and Tissues, Faculty of Veterinary Medicine, Assuit University, Assuit, 71526, Egypt
[3]Department of Animal and environmental Hygiene, Faculty of Veterinary medicine, Assiut University.
*Corresponding author: raghdaismaily@gmail.com
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**ABSTRACT:**
***Background:*** *Norethisterone, also known as Norethisterone Acetate (NETA), or Norethindrone, is a commonly used progestogen in various European countries.*
***Aim of the Work:*** *Previous studies have shown that introducing synthetic hormones can alter the effects of sex hormones on the ovarian cycle and lead to negative outcomes for the ovaries. There is a lack of histological reports regarding the effects of NETA on the ovarian morphology. So, the present study focuses on the ovarian histological sequelae following the exposure to NETA in the albino rats.*
***Materials and Methods****: Fourteen adult female albino rats were investigated. They were randomly divided into two groups: Control group (A) and NETA treated group (B). Each one consisted of 7 rats. Albino rats of control group (A) were administered daily food and distilled water only, while rats of NETA treated group (B) were administered daily 20 µg of NETA dissolved in 2 ml distilled water, food, and water. The experiment was continued for three weeks.*
***Results:*** *The findings indicate that the use of NETA has negative effects on the ovarian follicles, including a reduction in their number and signs of atresia across all stages.*
***Conclusion:*** *This treatise underscored Norethisterone Acetate (NETA) has an adverse effect on the ovarian cycle and follicular development of the albino rat.*

**KEYWORDS:** Norethisterone Acetate (NETA), follicles, Histological study.

## 1. Introduction

Birth control has been used since ancient times, but effective and safe contraception only became available in the 20th century[1] . The most effective (but harmful) birth control methods have been found to be sterilization via vasectomy in males and tubal ligation in females, intrauterine devices, and implantable contraceptives, followed by a number of hormone-based methods including oral pills, patches, vaginal rings, and injections ("Family Planning - A global handbook for providers," n.d.). The pill is the most common method of contraception in the United States [2]. In fact, there are three types of birth control pills: estrogen-progesterone combined (which contains both estrogen and progestin), progesterone-only, and continuous or extended-release birth control pills. Progesterone is the hormone that prevents pregnancy, and the estrogen component controls menstrual bleeding [3]. NETA is a common synthetic progestogen hormone used with oral contraceptives [4]. NETA is a synthetic, orally active progestogen used for contraception in premenopausal women for the treatment of secondary amenorrhea, endometriosis, and abnormal uterine bleeding [5]. Research has indicated that while oral contraceptives offer various health advantages, they can also have negative impacts on users, society, and overall well-being. These effects may even alter the anatomical structure of the ovary in women [6]. It is also observed that after using contraceptives with a low dose of progestin, in several cases, the ovaries are enlarged, some microcysts and corpora lutea appear, the ovarian connective tissue increases frequently, and follicular growth is stopped [7]. However, the histomorphological effect of NETA on follicular development in the ovary of the albino rat is scanty. So, this study explored the histological changes during the ovarian follicular development as sequelae of the oral administration of NETA in the albino rat.

## 2. Materials and Methods

### 2.1. Source of the animals

Female albino rats were obtained from the animal house of the Faculty of Veterinary Medicine at Assiut University. The rats were fed twice daily and put under controlled and stable conditions of light (daylight and 7 hours of electric light), temperature (22 - 25 oc), ventilation, and humidity. The experiment lasted for three weeks. The experimental protocol was approved by the Local Ethical Committee and by the Institutional Review Board of the Faculty of Medicine, Assiut University (Approval Number: 04-2023-200250) and was carried out in accordance with relevant guidelines and regulations. This research was done in compliance with the ARRIVE guidelines and regulations (https://arriveguidelines.org)

**Drug:** NETA was obtained from CID Company for Medicines.

### 2.2. Experimental design

A total of 14 non-pregnant female albino mature rats (average body weight of 150–180 g and an average age of 2–3 months) were randomly assigned into two groups, each consisting of seven animals

*A-Control group*

n this group, mature rats were administered daily normal saline and supplied with water and a commercially pelleted diet for three weeks.

*B-Norethisterone Acetate (NETA) treated group*

In this NETA treated group, each rat was orally administered daily 20 µg of NETA dissolved in 2 ml distilled water and supplied with water and a commercially pelleted diet for three weeks.

### 2.3. Sample collection

At the end of the experiment, rats were euthanized by cervical dislocation and ovaries were dissected and fixed in 10% neutral buffered formalin.

### 2.4. Histological preparation

The fixed materials were dehydrated in ascending grades of ethanol, cleared in methyl benzoate, and then embedded in paraffin wax. Transverse and longitudinal serial paraffin sections at 3-7 µm in thickness were cut and stained with Haematoxylin and Eosin for general histological examination (Harris, 1900).

### 2.5. Morphometrical analysis

The number of different types of the ovarian follicles was counted in both experimental groups.

### 2.6. Statistical analysis

The data were expressed as mean values ± S.E. The data were subjected to statistical analysis using independent samples T test (IBM SPSS statistics 22 Software). The significance value was set at ($P< 0.05$).

## 3. Results

**Table 1:** Showing the effect of NETA on the number of different follicles in rat ovary.

| Type of follicles | Control group | NETA treated group |
|---|---|---|
| Primordial follicles | 9.00 ± 1.39 [a] | 4.50 ± 1.06 [b] |
| Primary follicles | 2.33 ± 0.33 [a] | 1.50 ± 0.34 [a] |
| Growing follicles | 5.83 ± 0.95 [a] | 3.83 ± 0.65 [a] |
| Small antral follicles | 4.67 ± 0.80 [a] | 1.17 ± 0.40 [b] |
| Large antral follicles | 1.67 ± 0.42 [a] | 0.17 ± 0.17 [b] |
| Atretic follicles | 4.00 ± 0.73 [a] | 10.17 ± 1.05 [b] |

Values (Means ± SE) with different letters (a and b) in the same raw are significantly different ($P< 0.05$) between control and NETA treated groups.

The microscopical examinations revealed that the outer ovarian surface epithelium of the control and the NETA treated group consisted of a single layer of squamous cells or cuboidal cells in certain areas while in other regions, it was multi-layered (Fig. 1 A & B). In the control group, the primordial follicles were found beneath the tunica albuginea. These follicles were formed of an oocyte surrounded by a single layer of flattened follicular cells. Furthermore, it was noted that the control group had a higher number of primordial follicles (Fig. 1 C)) compared to the NETA treated group (Fig. 1 D). Both the control group and the NETA treated group showed that the

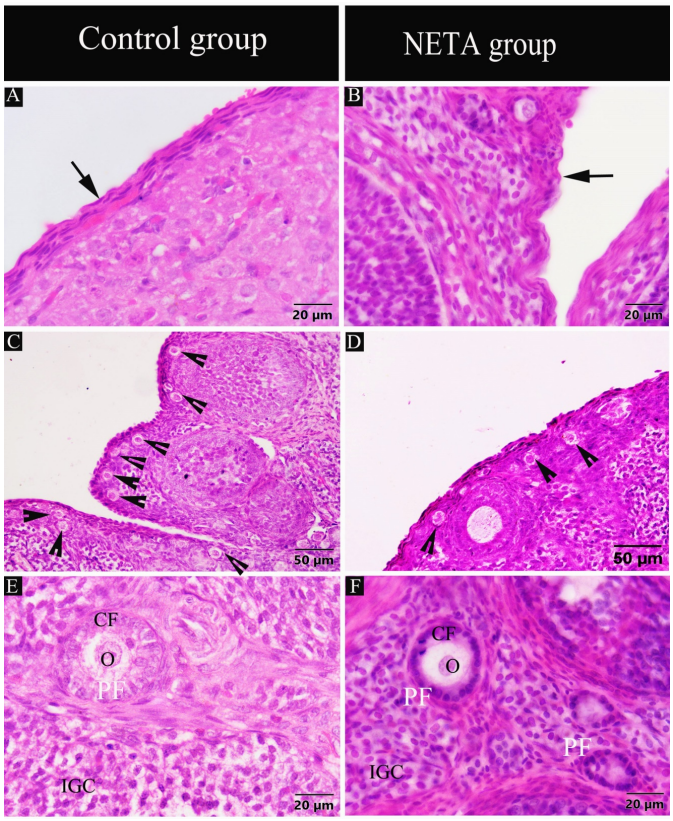


**Figure 1:** Photomicrograph of paraffin section stained with Hx and E of the ovary of Albino rat showing the effect of NETA on follicular development. Simple squamous epithelium (arrow) covers outer surface of the ovary in the control group (A) and in the NETA treated group (B). The primordial follicle (arrowhead) appeared degenerated, and the number of primordial follicles (arrowhead) in the control group (C) is remarkably larger than that in the NETA-treated group (D). Control group (E) and NETA treated group (F) showing primary follicle (PF) formed of an oocyte (O) surrounded by cuboidal follicular cells (CF). Note Interstitial gland cells (IGC).

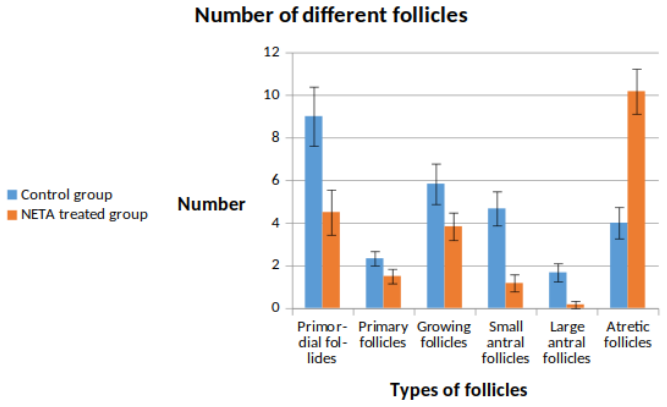


**Figure 2:** Showing the effect of NETA on the number of different follicles in rat ovary

primary follicle consisted of an oocyte that was encircled by a layer of either cuboidal or columnar follicular cells (Fig. 1 E & F) In the control group, the growing follicles were formed by an oocyte surrounded by multiple layers of granulosa cells. A distinct theca folliculi surrounds the growing follicles externally. The theca follicular cells display two well-defined layers, theca interna and theca external (Fig. 3 A). In contrast, the NETA treated group displayed signs of atresia in the growing follicles. The granulosa layer became loose, and there were some deeply stained pyknotic nuclei. Furthermore, apoptotic bodies were observed in the granulosa cell layer in the treated group (Fig. 3 B). The control group explored that the small antral follicles contained small cavities filled with an acidophilic fluid. The zona pellucida surrounding the oocyte in the small antral follicle was a homogenous, acidophilic membrane, as seen in the Haematoxylin and Eosin paraffin section (Fig. 3 C). While the treated group showed signs of atresia in their small antral follicles. There was an amount of apoptosis of granulosa cells, dead cells, and apoptotic bodies found in the follicular fluid (Fig. 3 D) Our results revealed that the mature Graafian follicle of the control group was distinguished by the presence of a single large follicular cavity. It was large in size and was formed of the following layers from inside to outside as follows: The oocyte was surrounded by thick acidophilic zona pellucida. The corona radiata was formed of a layer of columnar follicular cells arranged radially around the zona pellucida. The lining of the follicular cavity was made up of stratified epithelium, which formed the mural granulosa cells. Cumulus oophorus cells connected the ovum and its surrounding cells with mural granulosa cells. The granulosa cells are separated from the theca follicle by a basement membrane layer. A properly arranged theca folliculi surrounded the large antral follicles from the outside. The theca folliculi were divided into two parts: the theca interna and the theca externa. The theca interna was more vascular and cellular; polyhedral or elongated cells, and less fibrous. The theca externa, on the other hand, was more fibrous, less vascular, and less cellular (Fig. 4

A, C & E). We observed that in the NETA treated group, the mature Graafian follicle displayed signs of atresia; the granulosa cell layer appeared to be loosening. Additionally, several deeply stained pyknotic nuclei were observed in this layer, along with apoptotic bodies. Furthermore, the zona pellucida had thinned and some cavities had formed in the oocyte, corona radiate and cumulus oophorus had been disorganized (Fig. 3 B, D & F). Our observations

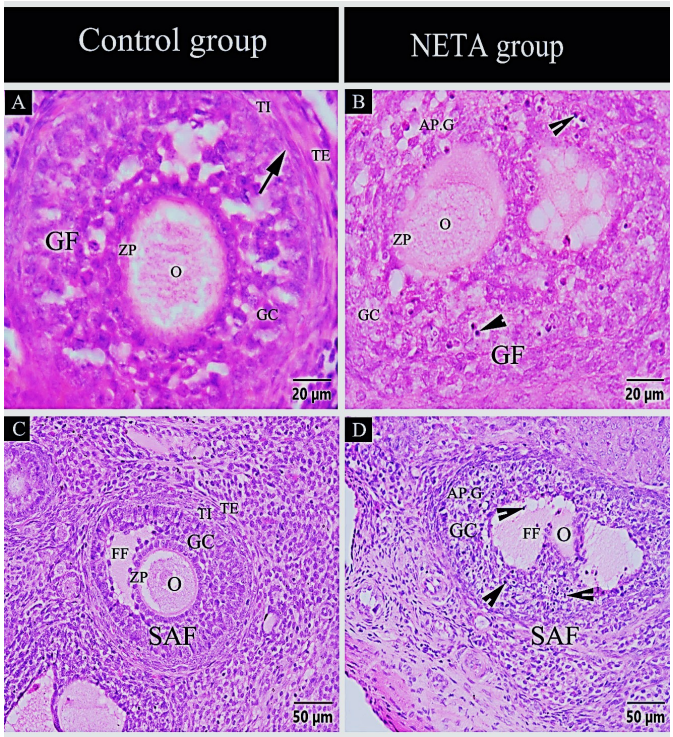


**Figure 3:** Photomicrograph of paraffin section stained with Hx and E in the ovary of Albino rat showing the effect of NETA on follicular development. A: Normal growing follicle (GF) in the control group formed of an oocyte (O) surrounded by zona pellucida (ZP), granulosa cells (GC), theca interna (TI), theca externa (TE). B: Ovary of NETA treated group showing atretic growing follicle (GF); loosing granulosa cell layer (GC), and apoptotic bodies in the granulosa layer (arrowhead). C: Small antral follicle (SAF) in the ovary of a control group formed of an oocyte (O), zona pellucida (ZP), theca interna (TI), theca externa (TE), and follicular fluid (FF). D: In the ovary of NETA treated group, an atretic small antral follicle (SAF) showed loosing granulosa layer (GC), apoptotic bodies (arrowheads) in the granulosa layer, and follicular fluid (FF).

revealed that follicular atresia was occurred in all stages of the follicular development. The condition known as atresia of the antral follicles was marked by several specific signs. These included the loss of the mural granulosa, fragmentation of the follicular cells within the follicular cavity, the appearance of apoptotic bodies in both the granulosa layer and the follicular cavity, detachment of the oocyte, and thickening of the theca interna layer (Fig. 5 A). During the early stage of atresia, the large antral follicle displayed several characteristics. These included apoptosis, irregular arrangement of the mural granulosa cells, and the presence of macrophages and degraded granulosa cells in the large follicular cavity. Additionally, the theca interna underwent thickening due to hypertrophy and proliferation of its cells. The mural granulosa cell layer varied in thickness, and in some areas, the continuity of the granulosa cells was lost. The cumulus oophorus was disorganized, and the oocyte was loosely attached to the mural granulosa cell layer. The corona radiata was disorganized, and the oocyte was observed freely in the follicular cavity. Finally, the thick zona pellucida became disorganized and degenerated (Fig. 5 A).

In the advanced atresia of the large antral follicles, the mural granulosa disappears completely, the follicular fluid becomes more condensed, and the theca interna thickened (Fig. 5 B & C). Sometimes, the atresia progresses to a point where the only remnants of the follicles were small ova residues and folded hyalinized basement membranes. The surrounding theca interna cells transform into interstitial gland cells, giving rise to structures known as corpora atretica (Fig. 5 D).

Our results revealed that the number of primordial, small antral, large antral and atretic follicles was significantly decreased in NETA treated group compared to the control one. Whereas the number of primary and growing follicles was non-significantly decreased in NETA treated group compared to the control one (Table 1and Fig. 2)

## Discussion

The results of this study showed certain histological changes in the ovaries of the Albino rats under oral administration of Norethisterone acetate in doses equivalent to human therapeutic doses for three weeks. We have shown through our research that the use of Norethisterone acetate results in a lower count of primordial follicles compared to the control groups. This finding is consistent with the

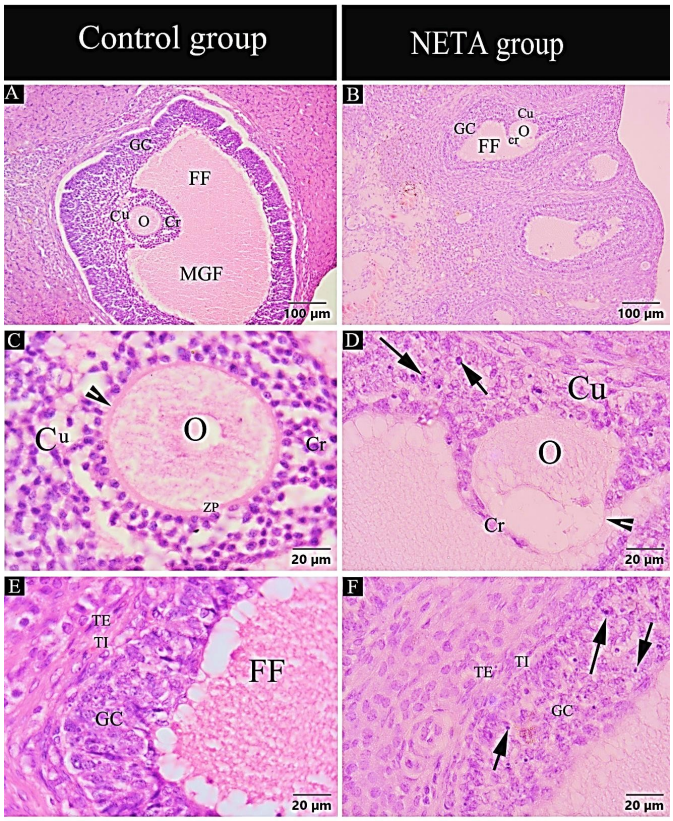


**Figure 4:** Photomicrograph of paraffin section stained with Hx and E in the ovary of Albino rat showing the effect of NETA on follicular development. A, C, and E: A mature Graafian follicle (MGF) in ovary of control group formed of the oocyte (O) surrounded by zona pellucida (ZP) covered by radially arranged cells of corona radiate (Cr), cumulus oophorous (Cu) connected the ovum and its surrounding cells with the mural granulosa cells (GC), theca interna (TI), theca externa (TE), and follicular fluid (FF). B, D, and F: An atretic mature Graafian follicle (MGF) in NETA treated group showing thinning of zona pellucida (arrowhead) and some cavities formed in the oocyte (O), apoptotic bodies are observed in disorganized granulosa cell layer (GC), disorganized corona radiate (Cr) and cumulus oophorous (Cu) cells.

study conducted by [8], who indicated that the administration of oral progestin effectively inhibits the onset of follicular activity in the ovary of felines. The current study highlighted that Norethisterone acetate was responsible for reducing the count of the growing and the small antral follicles and exhibited indications of degeneration of the growing and the small antral follicles. The granulosa layer became loose and disorganized with some deeply stained pyknotic nuclei. Furthermore, apoptotic bodies were observed in the granulosa layer and the antrum. These results coincided with a report conducted by [9] where he showed a decrease in the numbers of follicles, and cystic change following the administration of the progestin-only pills. Similarly [10] found that administering Depto Provera (100 mg depot medroxyprogesterone acetate) and mesgyna (progestin plus estrogen) through injections as contraceptives in albino rats can lead to atresia and degeneration of growing follicles, with little corpus luteum and some cystic enlargements. Additionally, our study found that daily use of Norethisterone acetate resulted in the absence of pre-ovulatory follicles and indications of atresia in the granulosa cells of large antral follicles. A similar finding was also shown in a study conducted by [11] who observed that rats treated with 200 mg of NET-EN

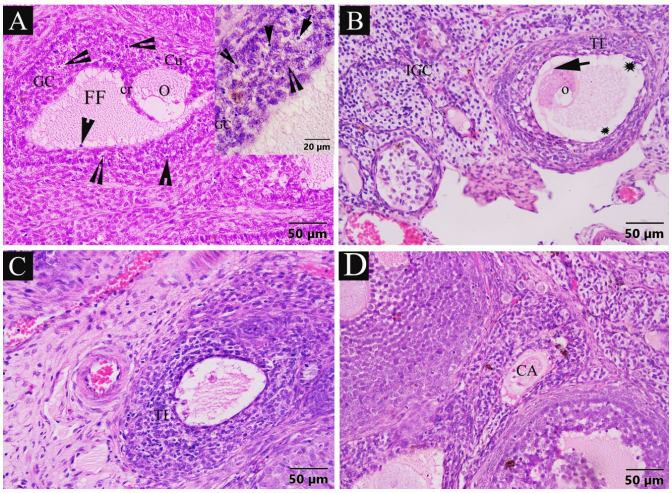


**Figure 5:** Photomicrograph of paraffin section stained with Hx and E in the ovary of Albino rat treated with NETA. A. Early stage of atresia in a large antral follicle showing apoptosis, irregular arrangement of the mural granulosa cells (GC), apoptotic bodies in the granulosa cells and the follicular fluid (arrowhead), disorganized corona radiate (Cr), cumulus oophorous cells (Cu), zona pellucida (ZP), and some cavities appears in the oocyte (O). B & C: Advanced atresia in the large antral follicles showing complete disappearance of mural granulosa (starburst) and thickening in theca interna (TI). The oocyte was observed freely in the follicular cavity (O). D: showing the corpora atretica (CA).

(norethindrone enanthate) showed an absence of mature follicles and recent corpora lutea, indicating ovulation blockade. This was explicated in Massawe's report in 2018 [12] which highlighted that injectable and oral contraceptives cause a negative feedback response on the hypothalamus. This response leads to a decrease in the pulse frequency of the gonadotropin-releasing hormone, which ultimately decreased GNRH distorts the ovarian histoarchitecture by asphyxiating follicular development, through

decreased estradiol levels. Also,[13] stated that development and breakdown of ovarian follicles occur in response to heightened levels of circulating gonadotropins. They also produce peptides involved in ovarian hormone synthesis regulation. Follicle-stimulating hormone (FSH) induces granulosa cells to express luteinizing hormone (LH) receptors on their surfaces; when circulating LH binds to these receptors, ovarian cycle and proliferation stops, and may trigger the follicular atresia. Additionally,[13] proposed that a reduction in the number of growing follicles could be linked to the recruitment of fewer follicles into the growing pool caused by elevated suppression of the hypothalamic-pituitary-ovarian axis with every successive cycle of oral contraceptive administration. The hypothalamic-pituitary system ultimately eliminates ovulation. In a similar manner,[14] discovered that consistent usage of a low-dose oral contraceptive containing Levonorgestrel 90 mcg/Ethyle Estradiol 20 mcg can successfully prevent ovarian activity and ovulation. Also, our results are consistent with [15] who suggested that the use of Dienogest led to histological outcomes that included apoptotic changes and loss of aromatase staining in the granulosa cells of the dominant follicle. Studies indicated that throughout fetal and adult life, ovarian follicles undergo apoptosis [16]. So, According to [17], who conclude that preantral and antral follicular atresia is caused by the activation of different cell-death pathways as antral follicular degeneration is triggered by massive granulosa cell apoptosis, while preantral follicular atresia occurs mainly via enhanced granulosa cell autophagy. Atresia can happen because of various factors. Multiple molecules, including pro-apoptotic molecules such as Fas, caspases, TNF, TVB, Par-4, p53, prohibitin, c-Myc, IFN, and endothelin, are involved in this process pro-survival molecules (such as gonadotrophins, insulin-like growth factor-1, interleukin-1$\beta$, epidermal growth factor, basic fibroblast growth factor, TGF-$\alpha$, bcl-2, bcl-long) and teratogenic factors, and the outcome depends upon an accurate balance between these molecules as mentioned by[18, 16]. In the current study, It was found that NETA led to atresia of the follicles, which included all stages, but it is unclear how NETA interacting with the previous factors which lead to an increase in follicular atresia and the mechanism of programmed cell death in granulosa cells. However, we are hopeful that future studies will shed light on this topic. Studies showed that norethindrone can effectively delay menstruation without causing any spotting and is also well-tolerated. However, it does lead to slight weight gain, unlike contraceptives. On the other hand, norethindrone doesn't affect fertility and allows for a swift return to the normal menstrual cycle after discontinuing its use [19]

## Conclusion

After careful observation, we have concluded that the use of Norethisterone Acetate (NETA) results in a decrease in the number of follicles and an occurrence of atresia in all stages of the follicles. As a result, NETA is an effective contraceptive as it interrupts folliculogenesis and blocks ovulation.

## ETHICAL CONSIDERATION

Ethical clearance was sought from the "Institutional Review Boar" of the Faculty of Medicine at Assiut University, Assiut, Egypt. IRB Iocal approval number: 04-2023-200250.

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