

## The Reproductive Effect of Terbinafine in Ewe: Effects on Estrous Cycle and Ovarian Follicles

Muhannad A. A. AlBayaty<sup>\*</sup>, Eman Rasol AlShaty<sup>\*</sup> and Alaa Karim Jassim<sup>\*\*</sup>

<sup>\*</sup> Department of Physiology and pharmacology, College of Veterinary Medicine, University of Baghdad,

<sup>\*\*</sup> Ministry of Higher Education and Scientific Research, Research and development

### Summary

Terbinafine is a fungicide given orally with the dose of 100 mg/kg body weight per day for each ewe for 60 days. The vaginal smears of ewe, body and ovarian weight were daily administered at slaughter time; ewes were slaughtered at 60<sup>th</sup> day. Estrous cycle was affected by showing a significant reduce in the estrous cycle length of each phases of estrous cycle with associated significant increase in the diestrus phase in terbinafine treatment as compared with control group (olive oil treatment as adjuvant) of ewes. There was a significant reduce in the number of follicles and a significant raise in the number of atretic follicles in treated group as compared with control group as well as upsurge the progesterone/estrogen ratio. The body and ovarian weight were significant diminished in terbinafine treatment. These observed effects of terbinafine on the ovarian activity may be due to a direct effect as antiproliferative agent or the hypothalamus - hypophysial - ovarian axis causing hormonal inequality.

### التأثير التناسلي للترينافين في النعاج: التأثير في دورة الشبق والجريبات المبيضية

مهند عبد الستار البياتي\* وإيمان رسول الشاطي\* وعلاء كريم جاسم\*\*

\* كلية الطب البيطري- جامعة بغداد \*\*وزارة التعليم العالي والبحث العلمي , البحث والتطوير

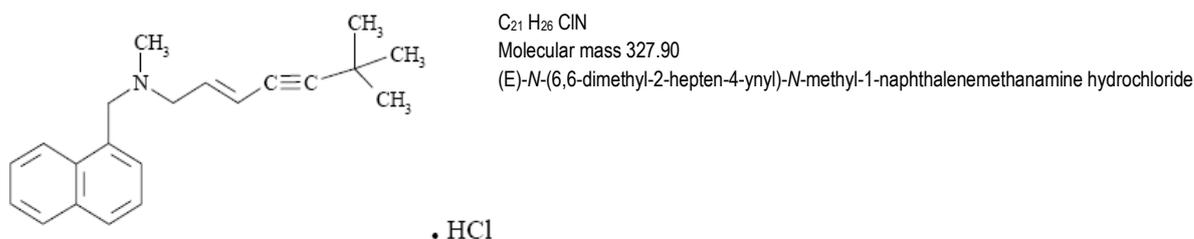
#### الخلاصة

عد التيرينافين مبيد فطري جرع فمويًا بجرعة 100 ملغ/كغم من وزن الجسم يوميًا لنعاج لمدة 60 يوم. وثقت المسحات المهبلية يوميًا ووزن الجسم والمبايض في وقت الذبح ومن ثم ذبحت نعاج التجربة المعاملة بالتيرينافين والسيطرة في اليوم 60. أظهرت مجموعة المعاملة بالتيرينافين اختزالًا في عدد وطول دورة الشبق وأطوارها مصحوبة بزيادة معنوية بطور الأصفرى مقارنة بالنعاج لمجموعة السيطرة المجرعة بزيت الزيتون كمادة حاملة. فضلًا عن ذلك انخفض عدد الجريبات وزاد عدد الجريبات المرتقة معنويًا لمجموعة معاملة التيرينافين مقارنة بمجموعة السيطرة فضلًا عن ارتفاع نسبة البروجستيرون إلى الاستروجين. إن المعاملة بالتيرينافين خفضت من وزن الجسم ووزن المبيض معنويًا. إن من الملاحظ في تأثير التيرينافين في دورة الشبق والجريبات المبيضية قد يعود إلى تأثير المبيض المباشر كمادة مضادة لانقسام الخلايا أو الاختلال الهرموني المتأني من تأثير المحور تحت المهاد - الغدة النخامية - المبيض.

### Introduction

Antifungal vary from any other chemical substances because they are knowingly used for long duration, therefore, a great part of the living animal population might be exposed in either the abuse or accidentally treatment, in other wise several attempting to use a new generation of antifungal to avoid resistance and adverse effects. The use of antifungal, takes place mostly through topical or systemic route (1 and 2). The really exposure determines the detrimental consequence that this exposure could had deleterious effects on reproductive performance. In animals, if primordial follicles are shattered widely, they could not be renewed; which can cause premature ovarian failure and early aged (3).

The terbinafine HCl (allylamine derivative) displayed in figure 1, which has a broad-spectrum antifungal activity, is a synthetic form, which exerts its' antifungal effect by inhibiting squalling epoxidase, a key enzyme in sterol biosynthesis in fungi further terbinafine inhibits the production of ergosterol in such a way as to lead to the buildup of the ergosterol precursor known as squalene. Having the wrong structural components disrupts the function of the cell wall as a barrier. This action results in a deficiency in ergosterol and a corresponding accumulation of sequalene within the fungal cell and causes fungal cell bereavement (4 and 5). It is chiefly used against most strain of the following organism dermatophyte mycetoma, ringworm and in particular, Aspergillus species (6). Yeganeh and McLachlan (7) reported that the terbinafine dwindled sexual function and ended testicular risk in rats (8).



**Figure 1: The molecular structure of terbinafine (4).**

The terbinafine fungicide also has been lately report in the initiation of steroidal hormone disruption to woman-dosed contraceptive after chronic exposure to terbinafine and interacting with contraceptive (9, 10 and 11). In view of the above findings, the present study had been undertaken to be acquainted with the effect of a terbinafine fungicide, on estrous cycle and folliculogenesis in ewe.

### Materials and Methods

Terbinafine sample was purchased from Dr. Reddy's Laboratories (UK) Ltd, 6 Riverview Road, Beverley, HU17 0LD, UK, Terbinafine dissolved in olive oil as a vehicle for oral administration. Twenty ewes aged one year (Local bread), weighing between 30–40 kg, screening regular estrous cycle, which were divided in to two equal groups (control and terbinafine treated group). Ewes groups were housed in to semi-closed arena (private Ownership: AlWahda) and had free access to concentrate pellets diet and water *ad libitum* all through the experiment. The lighting schedule was ~12:12 h light and dark cycle, the protocol of experimental design done at 10<sup>th</sup> December to 20<sup>th</sup> February. Terbinafine treated ewes group dosed 100 mg/kg per day orally for 60 days. The control group received an equal volume of olive oil. Vaginal smear and body weight recorded daily during the study.

The phases of estrous cycle were determined by observing the vaginal smear in the morning (10 to 11 hour). Experimental ewes in both control and terbinafine treated group were slaughtered at end 60<sup>th</sup> days of experiment time (12).

**Diestrous index** was obtained after loading terbinafine duration of treatment as a relationship between diestrous period to treatment period (13), which calculated as follows:

$$\text{Diestrus index} = \frac{\text{Number of days with clear diestrous smear}}{\text{Total duration of treatment}}$$

Blood samples were collected at 60<sup>th</sup> on the day of estrous phase. Samples were immediately placed on ice and centrifuged within 1 h. Plasma was recovered and stored frozen until analyzed by radioimmunoassay (Baghdad hospital Lab.) for both estrogen and progesterone.

Morphometric analysis of follicular development :- Ovaries of five animals randomly selected in both groups were taken for follicular development studies. The estrous cycle phases of the five examined ovaries exhibited were in diestrus phase. The weight of ovaries of the ewes nearest to the mean weight of the ovaries of respective group was selected. The ovaries were put in Bouin's fixative fluid, sectioned at 5- $\mu$ m thickness, and stained with hematoxylin and eosin. Every one of successive sections of the ovary was counted for assorted stages of development of follicles as describe by (14). Follicles were classified according to (14, 15 and 19) into small, medium, and large follicles. Healthy and atretic follicles were classified as defined by (16). In this study, three classes of ovarian follicles were categorized using the comparative cross sectional diameter of the follicle as measured from the outer margins of the granulosa cell layers. These quantitative criteria represent a substantial generalization of an

elaborated grading system proposed by Pedersen and Peters (14) and Lundy, *et al.* (15), with eight stages and several sub-stages to differentiate between primordial oocytes (Type 1) to antral follicle (Type 8).

1. Small follicles—(Pedersen and Peters Types 1-3b) consisted of an isolated oocyte or an oocyte surrounded by a partial or unbroken layer of granulosa cells.

2. Medium/growing follicles—(Pedersen and Peters Types 4-5b) have an oocyte surrounded by multilayered, solid mantle of granulosa cells.

3. Large/antral follicles—(Pedersen and Peters Types 6- 8) were characterized by central oocyte and fluid filled space bordered by number of granulosa cells.

By using these principles, mean diameters of follicles have been measured at approximately < 20 µm for small, 20–70 µm for medium and >70 µm for large follicles in mice. Follicles showing the nucleus of the oocyte were measured by using a calibrated ocular micrometer to avoid repeated counting. The maximum diameter and diameter at the right angle to it were used to find a mean diameter for each follicle. A follicle was considered to be undergoing atresia or to regressing when two or more pyknotic granulosa cells would be establish in a single section or whether the oocyte showed signs of degeneration, such as fragmentation, loss of nuclear membrane, or thinning of cumulus oophorus as proposed by (17).

#### Body and organs weight

The body weight was intended based on the weight taken on the 1<sup>st</sup> day following the oral administration measured as the initial body weight and the weight taken on the end day of experiment before slaughter was measured as the final weight. Ovary, was dissected, freed from adherent tissue and weighed. Ovaries weights were expressed per body weight.

#### Statistics

Statistical analysis of the control and terbinafine treated group data were subjected to analysis of variance (ANOVA) one-way analysis. A probability of  $p < 0.05$  was assumed to denote a significant difference. LSD test was used for group comparison.

## Results

### Estrous cycle studies

The control group showed signs of ordinary estrous cycle and usual period of each phases of estrous cycle. Treatment with 100 mg/kg per day terbinafine caused a significant ( $p < 0.05$ ) delayed in the length of estrous cycle and period of proestrus, estrus and metestrus with associated significant ( $p < 0.05$ ) amplify in the period of diestrus phase.

*Diestrus index* was also exaggerated the index following the oral administration of terbinafine (Table 1). However, the treated ewes were depressed and display lost of their normal activity.

**Table (1) the effect of terbinafine on the estrous cycle phase**

Groups	Total estrous cycle length (days)	Estrous cycle period				Diestrus index
		Proestrus %	Estrus %	Metestrus %	Diestrus %	
Control	21.39±2.80 a	15.36±3.07 a	18.55±1.72 a	22.14±2.60 a	43.93±2.13 a	42.16
Terbinafine treatment	25.51±3.33 b	7.07±0.074 b	10.55±0.019 b	22.49±0.063 a	59.87±0.308 b	74.39

N = 20 ewe

Values presented as mean ± standard error

Letters: ( $P < 0.05$ ) vs. differences between treatment and control group.

### Morphometric analysis of follicular growth studies

The histologic pattern of the control ewe group exhibited number of different developing follicles, Graafian follicles, atretic follicles and Corpora lutea. Treatment with 100 mg/kg per

day terbinafine showed a significant ( $p < 0.05$ ) reduce in the number of healthy follicles with concomitant significant ( $p < 0.05$ ) increase in the number of atretic follicles (Tables 2 and 3) when compared with the control group of ewes.

**Table (2) effect of terbinafine on ovarian follicular numbers**

Groups	Number of follicles according to size classification (diameter)			Total number of follicles
	Small < 20 $\mu\text{m}$	Medium 20-70 $\mu\text{m}$	Large > 70 $\mu\text{m}$	
Control	26.5 $\pm$ 5.42 a	4.29 $\pm$ 0.37a	0.88 $\pm$ 0.06 a	31.67 $\pm$ 0.21a
Terbinafine treatment	17.5 $\pm$ 1.38 b	2.86 $\pm$ 0.24 b	0.42 $\pm$ 0.03 b	20.78 $\pm$ 0.55b

N = 20

Values presented as mean  $\pm$  standard error.

Letters: (P < 0.05) vs. differences between treatment and control group.

**Table (3) effect of terbinafine on ovarian atretic follicles number**

Groups	percentage of atretic follicles according to size classification (diameter)		Total number of atretic follicles
	Medium 20 - 70 $\mu\text{m}$	Large > 70 $\mu\text{m}$	
Control	58.4 $\pm$ 2.61 a	94.72 $\pm$ 3.28 a	3.01 $\pm$ 1.12 a
Terbinafine treatment	55.81 $\pm$ 1.99 b	98.24 $\pm$ 6.45 b	2.51 $\pm$ 1.31 b

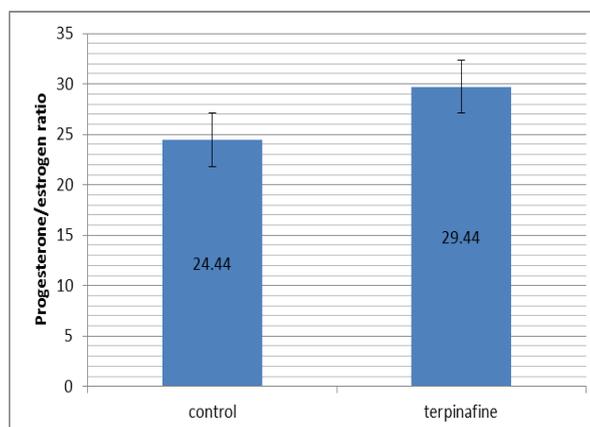
N=20

Values presented as mean  $\pm$  standard error

Letters: (P < 0.05) vs. differences between treatment and control group

**Estrogen/progesterone ratio:**

The figure 2 showed significant ( $p < 0.05$ ) increase in progesterone to estrogen ratio in terbinafine treated group 29.73  $\pm$  1.51 as compared control group 24.44  $\pm$  0.46.



**Figure 2: The effect of terbinafine treatment on progesterone/estrogen ratio in control and terbinafine treated groups.**

N=20

Values presented as mean  $\pm$  standard error

Letters: (P < 0.05) vs. differences between treatment and control group

**Body and organs weight**

The ewe treated with terbinafine for 60 days showed a significant ( $p < 0.05$ ) decrease in the gain of body weight with terbinafine treatment. There was a significant decrease in the weight of the ovary (Table 4).

**Table (4) effect of terbinafine on body weight and ovarian weigh to body weight ratio**

Groups	Body weight kg	Ovarian weight g	Ovarian weight to body weight ratio %
Control	35.62 ± 0.54 a	37.21 ± 0.019 a	0.104 ± 0.031 a
Treatment terbinafine	30.84 ± 0.95 b	28.14 ± 0.026 b	0.091 ± 0.069 b

N=20

Values presented as mean ± standard error

Letters: (P < 0.05) vs. differences between treatment and control group

## Discussion

Cyclic transforms of the vaginal smear see in the estrous cycle offers a sensible index of the ovarian vital activity and its hormonal biosynthesis of estrogen, progesterone, and prostaglandins, the echelons of these hormones are controlled by hypothalamus gonadotropin releasing hormone and pituitary gonadotropin (18). The consequences obtained in the present study indicate that the control ewe exhibited regular ~21.39 days estrous cycle. Ewes treated with 100 mg/kg per day terbinafine produces diminishes in the estrous cycle length and the period of proestrus and estrus with attendant significant amplify in diestrus phases. However, diestrus index was expansion following the treatment of terbinafine as compared with control group.

Comparable outcomes have been indication that the rat extravagances with an allylamine fungicide reason a significant increase in the estrous cycle period associated with of the proestrus, estrus, and metestrus period with a concomitant increase in diestrus phase (11). As if outcomes have also been reports with steroidal hormone interaction, in controversy, demonstrated an ability to persuade the fast migration estrus phase, thus affecting the number sequence of estrous cycle resultant from the hormonal disparity and reduced estrus phase (20). In experiment, it has been revealed that treatment with terbinafine displayed extended diestrus phase and therefore, terbinafine perhaps have interacted with steroidal metabolism and it has been demonstrated in the liver cytochrome inhibitor compounds treated animals. It has been reported that at least seven cytochrome P450 isoenzymes are involved in its metabolism with major contributions from CYP 2C9, CYP 1A2, CYP 3A4, CYP 2C8 and CYP 2C19. Terbinafine down regulated these CYPs, steroidal hormones, on the other hand, is metabolized associated CYPs of terbinafine (21).

Several possibilities may explain the pharmacological interactions with estrogen. Since terbinafine's protein binding (99%) is stronger than that of steroidal hormones (40-60%), terbinafine might have displaced sexual steroidal hormones from the protein binding sites, increasing its serum level and causing disrupting cyclicity rhythms and period and disturbance of mechanism. It is also possible that terbinafine would have displaced bupropion and/or quetiapine from their binding sites, increasing their levels and causing the described side effects. However, the presence of high estrogenic serum level, with the onset of symptoms, and normalization of its serum level, with the resolution of symptoms, suggests a probable cause (22).

Further, terbinafine and steroid hormone share the metabolic pathway at CYP3A4, CYP450 3A4, isoenzymes. It is possible that terbinafine displaced the steroid at these isoenzyme sites (27), leading to its own metabolism. Further, steroid's potent induction at CYP 3A4, 1A2 could also have caused the metabolism and excretion of terbinafine, while estrogen levels continued to increase. The half-life of estrogen, in most cases, is ~13 hours. However, in

the above case, steroid levels were detectable after 10 days of stopping medications (11 and 20). The long terminal down regulation of metabolic enzyme of terbinafine might explain this.

Terbinafine may be revealed to block the ovulation via restrain the pustule secretion of LH as sequence of sexual hormone disruption (23). Since terbinafine may be probable that it would deleterious effect indirectly on the hypothalamus - pituitary axis with adversely side effects (20), affect the ovary, which in turn affects the estrous cycle and folliculogenesis due to the hormonal imbalance in estrogen - progesterone ratio. That coincided with hormonal result displayed in figure 1 showed comparable between progesterone/estrogen ratio the ewes in terbinafine treated group complain from rising their ratio as compared with control.

Plowchalk, (24) have described that the quantitative appraisal of follicle number is a marker of the usual function in addition to noxious replies in the ovary. Follicles are the principle functional units of the mammalian ovary. The majority vital directors of follicular maturity are follicular stimulating hormone (FSH), Luteinizing hormone (LH) created from the pituitary gland, and the ovarian estrogen formed through granulosa cells. Although all follicles are in fact exposed to the equivalent frequencies in these hormones, several ovulated follicles, and others become atretic, indicating the attendance of intra-gonadal regulatory factors and steroidogenesis with steroidal hormone turnover, which adapt the outcome of these major hormones (25). In this experiment revealed that the number of vary sized follicles and total numbers of healthy follicles were drastically reduced with attendant significant augment in medium, large and total number of atretic follicles in terbinafine treated group of ewes.

On the other hand, recently, we have shown that a number of antifungal agents exert antiproliferative and/or apoptotic activities in various malignant cells *in vitro* and *in vivo*. For instance, our previous studies showed that terbinafine; (allylamines class) induced cell cycle arrest at the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle and the occurrence of apoptosis cells, which highlight the molecular mechanisms of TB-induced antitumoral activity (26). In the present study, the antiproliferative activity of terbinafine may be presumably referred to bad sequence of folliculogenesis and reduced their numbers.

Treatment with terbinafine showed adverse effect in terms of body weight which significant decrease in the body weight in terbinafine treated group, as there may be suppression food and water intake due to anorexia (28); this may presumably one of the motives for low body weight and alteration in the estrous cycle. The ovarian weight was decreased with terbinafine treatment. Similar observations were ended in rats complain from malnutrition that produced decrease in ovaries weight and size due to widespread atretic follicles (29).

Poly-pharmacy carries an inherent risk that needs to be assessed before animal are prescribed multiple medications. In addition, this case may represent an interaction due to abused drug, which may not apply to the majority of the animal for a long time course. However, this report suggests that a probable relationship exists between terbinafine and physiology of estrous cycle which where violates the safeness information through literature notions. Until further research is available, care should be exercised whenever they are prescribed for certain period margin safety.

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