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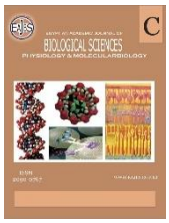

Investigation of Vaginal Microbiota in Infertile Women Compared with Fertile and Evaluation of Correlation Between Elafin As an antimicrobial Peptide with Some Hormones

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ABSTRACT

Background: This study included the collection of 90 blood samples and 90 vaginal swabs, in order to isolate the microbial samples. **Method**: The samples were diagnosed based on routine methods.**Result**: The results of culture and pre-diagnosis showed that 86 swabs gave a positive result for bacterial and yeast growth on the media used. The results showed that *Candida albicans* was the most common pathogen among infertile women, *Staphylococcus aureus* and *Escherichia coli*. While in the control group, *Lactobacillus* was the most common, and then *E.coli*. Results showed a significant increase at the level (P≤0.05) in the concentrations existence of negative correlation between Elafin and (LH and FSH) and positive correlation between Elafin and (GNRH, β-Lipotropin and subfatin).

INTRODUCTION

Infertility or subfertility is the failure to obtain pregnancy after one year of regular sexual intercourse and without use contraceptives (Farquha *et al*., 2019). Almost a third of infertility cases that affect couples are primarily attributed to the woman, a third to the man, and a third to the interaction, between the two, 20% of cases are unexplained infertility (Peterson *et al*., 2007), and approximately 15% of couples are infertile (Huhtaniemi *et al*., 2018). One of the causes of infertility is bacterial vaginosis (BV), which negatively affects the genitals through inflammatory changes, and therefore the pathological conditions that occur due to its lead to a complete loss of fertility (Sleha *et al*., 2013). Also bacterial infection spreads from the vagina to the cervix and then to the upper reproductive system, thus causing infertility (Tsevat *et al*., 2017).

Bacterial vaginosis is a common infection affecting approximately 29% of women of reproductive and is associated with disturbances in the population of vaginal microorganism. BV increases the risk of infertility, pelvic inflammatatory, and endometriosis (Khleifat *et al*., 2022; Nijris *et al*., 2020), and lower reproductive tract infections such as bacterial infection are higher in infected women with infertility (Park, 2017).

It has been shown that there are some pathogens that infect the genitourinary system and cause infections in both men and women, such as *E. coli*, *Enterococcus faecalis*, *Ureaplasma urealyticum*, and *Candida spp* (Ruggeri *et al*., 2016). Elafin is a defense protein produced by epithelial cells and keratinocytes in response to anti-inflammatory cytokines, such as interleukin-1 and tumor necrosis factor-alpha (TNF-α). Elafin is produced as part of the body's immunity, as it is found in skin, lungs, upper gastrointestinal tract, and female reproductive system, it's responsible for regulating proinflammatory paracrine reactions necessary to maintain pregnancy and reduce uterine inflammation. The other functions of Elafin include essential pathways to protect mucous membranes from uterine infections and regulate the menstrual cycle through protein breakdown mechanisms and participation in tissue repair (Shaw and Wiedow, 2011).

The aim of this study is to clarify the relationship between antimicrobial peptides Elafin and some hormone causes of infertility and bacterial and fungal infections.

MATERIALS AND METHODS

Sample Collection and Culture:

90 samples were collected from the Medical City Hospital Baghdad city and some outpatient clinics for the period between 10/23/2022 and 12/23/2022. 60 infertile women and 30 healthy women, by taking cotton swabs (containing a carrier medium) from the vagina. Women diagnosed by specialist doctors as infertile and healthy women of different ages, ranging from 20-39 years, were transferred to external laboratories within a period not exceeding 5 hours, as the samples were placed in sterile tubes containing a carrier medium for the purpose of culturing them immediately upon their arrival on the nutrient medium.

The samples were grown on nutrient agar medium using a planning method and placed in the incubator for 24 hours. After that, they were grown on Blood agar to grow most bacterial species and MacConkey agar to grow gram-negative bacteria and differentiate them from gram-positive bacteria. They were also grown on mannitol agar medium. Mannitol salt agar to confirm the growth of Staphylococcus aureus bacteria, and the medium was Sabouraud Dextrase Agar to grow the fungi. The sample was spread using a loop and the planning method near a Bunsen flame, in addition to sterilizing the area in which the cultivation took place. The dishes were then incubated in an incubator at 37°C for 24 hours. Waiting to grow (Tille *et al*., 2017). It was examined microscopically to confirm whether it was positive or negative for Gram stain. As for blood samples, 5 ml obtained from a vein from each woman, using a disposable syringe, and the blood was placed in plain plastic tubes devoid of anticoagulants until hormonal tests were performed, and the blood was separated after coagulation using a centrifuge. Centrifuge at a speed of 4000 rpm for 10 minutes, then the clear blood serum was withdrawn using a micropipette. Each form was numbered, the patient's name was written. The date of the sample was drawn for each patient. The serum was then stored in Eppendorf tubes at -20°C until the required tests were performed.

Diagnosis of Bacterial Isolate:

After culture bacteria on blood medium, MacConkey agar, mannilol salt agar and Sabouraud Dextrase Agar the diagnosis was made as following:

Form: Colonies on these media, colonies under a light microscope using Gram stain, perform biochemical test such as oxidase test, catalase test, Coagulase test, Gelatin test, Imvic test and Germ tube test.

Blood Collection:

Blood samples were obtained from a vein of 5 ml from each woman, using a Disposable Syringe, and placing the blood in plastic tubes free of anticoagulant, until conducting hormonal tests, and separating the blood after coagulation using a centrifuge at a speed of 4000 cycles/min for 10 minutes, then the clear blood serum was withdrawn by means of a micropipette. Each form was

numbered, and the patient's name and the date of drawing the sample were written for each patient. Then the serum was stored in Eppendorf tubes at a temperature of -20 °C until the required examinations were performed.

Hormone Test:

The concentrations of Elafin, β-Lipotropin, GNRH, FSH, LH, and Subfatin were measured via enzyme linked immune sorbent assay (ELISA) by using the commercial kits (ELISA kit, Human, MyBioSource, USA) and procedures followed as given in the kits catalogs.

Statistical Analysis:

All values expressed as mean± S.D data analyzed by analysis of variance (T-test).

RESULTS AND DISCUSSION

90 vaginal swabs were taken, 60 of which were for infertile women visiting the Medical City Hospital in Baghdad and some outpatient clinics. 30 swabs were taken as a control group from non-infertile women within a period of time from 10/23/2022 until 12/23/2022. The results shown in Table 1. 86 swabs gave a positive result for bacterial and yeast growth on the media used, at a rate of 95.5%, as 59 samples (65.5%) and 27 samples (30%) gave positive growth for the sterile women and the control group, respectively, while 4 samples (4.5%) gave negative growth. The reason for the lack of growth in these samples may be due to the use of antibiotics before taking the sample, which reduces the rate of bacterial growth, or the pathogen may be viral (Khleifat *et al*., 2022). These results are consistent with the study of Razzak *et al*., (2011), Which was conducted in Babylon and showed that 95.5% of the samples gave a positive result. It also agrees with the results of Khleifat *et al*., (2022) which was conducted in Samarra and obtained a 95% positive growth rate, while it does not agree with the results of Jarjees (2006) which he conducted in Erbil .Also the results of Al-Muk and Hasony (2001), which was conducted in Basra, and the positive growth rates were 68.3% and 67.6%, respectively.

Table 1: Sample distribution table according to the presence of bacterial growth.

Groups		The presence of bacterial growth		No bacterial growth The ratio
	The number	**The ratio**	**The number**	
Infertile women	59	%65.5	1	%1.1
The control	27	%30	3	%3.3
The total	86	%95.5	4	%4.5

Table 2 shows the numbers and percentages of the group of infertile women by age group, as the ages of the women ranged between 20-39 years. As for infertile women, the highest incidence rate was for the age group of 20-29 years, with 54 samples, or 90%. The lowest incidence rate was for the age group 30-39 years, with 6 samples, at a rate of 10%, as for the control group, it ranged between the age group of 20-29 years, at a rate of 100%. The result of the age group 20-29 years was more affected by infertility. This result is consistent with (Kumari *et al.*, 2016). She indicated that most women suffering from infertility are within the age group 26-35 years.

Table 2: Sample distribution table according to age

Age	Infertile		Control	
	Number	%	Number	%
20-29	54	90%	30	100%
30-39	6	10%	0	0%
The total	60	100%	30	100%

As for *C.albicans*, they were initially identified based on the external morphology of the colonies and microscopic characteristics. The colonies appeared in colors varying between white and cream, with smooth edges and smooth, shiny surfaces. This result agree with the result of Othman *et al.*, (2018) and Abdulla and Mustafa (2020) in terms of the phenotypic characteristics of the colonies

All isolates also gave positive results for reaction with Gram stain, as the cells appeared oval to spherical, oval to elongated, or cylindrical in shape. This result was consistent with Singh *et al*.,(2013) and that the appearance of Candida cells stained blue in color is a result of the Peptidoglycan layer in the cell wall retaining this dye (Sudbery *et al*., 2004).

The test results showed that all isolates belonging to the type *C.albicans* formed germ tubes when incubated at a temperature of 37°C for 2-3 hours in 0.5 ml of human blood serum. The results of the study agree with Akortha *et al*., (2009) whose study results showed that *C.albicans*. albicans has the ability to form germ tubes, which is a diagnostic characteristic for this species.This result is also consistent with the results of Hashim and Zghair (2023) and Matare *et al*., (2017) who stated that *C.albicans* has the ability to form germ tube and that the germ tube plays a role . It is important for the bacillus to penetrate the layer of epithelial cells lining the body and tissues which reach the blood stream. Additionally, it is believed to be necessary for feeding yeast (Sudbery, 2004).

As for the genus *Lactobacillus spp*, it was diagnosed based on microscopic, cultural, and biochemical characteristics. The results of the study showed that the colonies of the genus *Lactobacillus* were white or cream in color, large, smooth, and round on MRS medium. Positive for the bacillus Gram stain, producing acids and negative for the catalase test. It does not produce the gelatinase enzyme, and it varied in its growth at temperatures of 10°C and 45°C (Imran and Ali, 2013).

Table 3, shows the numbers and percentages of bacteria and yeasts under study isolated from the vaginas of infertile women and the control group. The results showed that *C.albicans* was the most common pathogen among infertile women. 25 isolates were isolated, representing 42.37%, followed by *C.albicans*, *staph.aureus* at 22 (37.28%), then *E.coli* at 12 (20.33%). While in the control group, *Lactobacillus* was the most common, with 23 (85.18%) isolated, followed by E.coli 4 (14.81%). This result agree with the findings of Al-Obady (2012), as *C.albicans* was the most common in vaginal infections. Although the adult female vagina has a microbiota, *Lactobacillus* is responsible for maintaining vaginal pH and preventing overgrowth of potent pathogens. Treatment reduces the frequency of infection, but antibiotics such as broad-spectrum antibiotics can kill or suppress beneficial bacteria in the vagina. The reproductive system, allow resistant organisms to grow (Faruqui, 2018). A wide range of bacteria, viruses, protozoa, and fungi are typically present in the reproductive tract of infertile women and can use a large number of virulence factors to interfere with the normal reproductive process (Vander and Prabha, 2019). Colonizing microorganisms may play an important role in determining many aspects of reproductive health and are capable of causing various gynecological complications (García-Velasco *et al*., 2017). The negative impact on reproduction by microorganisms is through direct damage to the mucous membrane in the reproductive system and the generation of inflammatory responses from the host, or indirectly through influencing the functions of reproductive system organs (Sleha *et al*., 2013).

Candida albicans is the main fungal pathogen affecting the genitourinary system. It is naturally present in the female reproductive system and has the ability to cause various diseases, from simple forms of vaginitis and cervicitis to more serious form, such as recurrent vulvovaginal candidiasis

(RVVC). Which may change the vaginal mucosa or may affect the reproductive process (Vander and Prabha, 2019). The presence of *C. albicans* in the vagina may also lead to weak sperm motility. Various studies have shown the negative effect of *C. albicans* yeasts on sperm parameters. *C. albicans* products have an inhibitory activity on human sperm motility and deterioration of the structural structure of sperm, which is one of the main vital factors. The active proteins produced by it, such as acid proteins, phosphatidases, and other soluble virulence factors, lead to a significant loss of anterograde motility that coincides with multiple sperm damage due to apoptosis and necrosis, and in sub-lethal doses it can also lead to premature loss of the body. Acrosome and sperm DNA fragmentation (Atlee *et al*., 2013). Data emerging from recent studies provide evidence of evidence supporting vaginal colonization by Staphylococci and infertility, as (Orhue *et al*.,2012) showed that 38.7% of *Staphylococcus aureus* was isolated from the vagina and endocervix of infertile females.(Ghiasi *et al*.,2014) showed that S. *aureus* is the main cause of vaginitis in infertile women, as it was isolated in 57.33%. (Prabha and Kaur 2012) also showed that the cause of infertility in female mice is vaginal colonization by *S. aureus* with weakening of sperm, and it is associated with the development of endometriosis and is considered a risk factor. For infertility in women, this has been proven as cytokines that are released in the vicinity of the uterus in response to the *S.aureus* bacteria can impair the implantation of blood vessels in the placenta, leading to recurrent miscarriage (Toth *et al*., 2010).

Most vaginal infections are through infection from the urinary system, as Devi (2013) stated that the *E.coli* bacteria is considered a cross-contamination as it is transmitted from the intestines to the urethra, through which it is transmitted to the vagina. It is considered one of the main bacteria and is often isolated from cases of bacterial vaginosis. Agrawal *et al* (2013) hypothesized that the possible mechanism of infertility is through LPS, a component of the cell wall of *E.coli* bacteria. They also reported that the presence of LPS changes the levels of the cytokine TNF-α, as well as LH and FSH and that this disturbance in immune and hormonal factors may lead to failed implantation or early pregnancy loss.

The study by (Sukarjati *et al*.,2013) indicated that the negative effect of *E.coli* bacteria on sperm, such as the bacteria directly attaching to male gametes or producing factors that dissolve sperm. Adhesion occurs through bacterial pilis, which attach to the plasma membrane of sperm and leads to their agglutination and loss of vitality. It has been observed that *E.coli* als can interfere with the fusion of egg and sperm.

It was found that intravaginal inoculation of sperm attached to *E.coli* led to infertility in mice (Kaur and Prabha, 2014). The presence of the genera *E.coli* and *S.aureus* in the vagina is considered microbiota and can become pathogenic and cause inflammation when there is a change in the pH, as well as also due to external environmental causes or the entry of other pathogens. That is, the composition of the vaginal ecosystem is changes over time and in response. to internal and external influences (Faruqui, 2018).

Lactobacillus is one of the natural bacteria present in the vagina, which is of great importance in maintaining the balance of normal flora and reduces the risk of infection with the Human Immunodeficiency Virus (HIV). Lactobacillus bacteria contribute to reducing the incidence of infections resulting from the presence of Candida yeasts, as one study showed. That *Lactobacillus* bacteria isolated from the vagina of healthy women have the ability to inhibit two types of yeasts that cause vaginal infections. *Gardnerella vaginalis* and *C. albicans,* by reducing the process of adhesion of yeasts to the surfaces of the epithelial cells lining the vagina and restoring the normal bacterial system (Kaewsrichan *et al*., 2006). In addition, (Zarate and Nader, 2006) indicated that the presence of bacteria in the

vagina has an effect in inhibiting *S.aureus* bacteria, which cause infections in the genitourinary system as a result of preventing pathogenic bacteria from adhering to the walls of the epithelial cells lining the vagina.

Table 3: Numbers and percentages of diagnosed isolates taken from vaginal samples of infertile women and the control group.

Groupe	Number	%	Bacterial isolation	Number	Ratio
Infertile women	59	68.61%	*C. albicans*	25	%42.37
			S.aureus	22	%37.28
			E.coli	12	%20.33
Control	27	31.39%	*Lactobacillus spp*	23	%85.18
			E.coli	4	%14.81
The total	86	100%			

Correlation Between Elafin As an antimicrobialpeptied And Bacteria:

Elevated Elafin concentrations have been observed in cases of infection by opportunistic microorganisms, invasion by pathogens, excessive activity of the immune system, and mucosal dysfunction. The mechanisms that normally control the inflammatory response to the mucous layer have been found to be abnormal, causing severe damage to the mucosal tissue. Among the endogenous mechanisms that have been described to control mucosal immunity to prevent excessive tissue damage, different classes of proteins have been studied, the most important of which is Elafin, which is able to simultaneously control excessive tissue protein degradation, inflammatory cell infiltration and activation, and microbial invasion (Deraison *et al*., 2023).

Elafin protein had activity against Gram-positive and Gram-negative bacteria, such as *S.aureus*, *Pseudomonas aeruginosa, E.coli, Klebsiella pneumoniae*, *Mycobacteriu tuberculosis, Aspergillus fumigatus*, and *C.albicans* (Wilkinson *et al*., 2009; Baranger *et al*., 2008). Since Elafin and its precursor trappin2 have antibacterial activity in mucosal defense through homology to what is known about the mechanism of action of other antimicrobial peptides (Alexander *et al*., 2021). Elafin is increased in response to infection and failure to stimulate the trappin2 response/ Elafin induces bacterial vaginosis, and Elafin is directly induced in response to local infection, causing disruption of bacterial membranes and inhibition of viral replication or attachment to epithelial cells (Drannik *et al*., 2013; Scott *et al*., 2011). A study by Abbott *et al.* (2014) added that Elafin was elevated in response to infection/inflammation and processes leading to spontaneous preterm birth (PTB). Another study also indicated that failure to stimulate an appropriate trappin2/elafin response in cervicovaginal fluid (CVF) leads to bacterial vaginosis. (Stock *et al*., 2009). A recent study conducted by Matsuda *et al.* (2023) demonstrated that Elafin is elevated in the cervical region containing Gardnerella vaginalis (GV) and controlled by normal flora. Inflammatory cytokines, chemokines and other inflammatory substances are secreted from epithelial cells exposed to GV components, specially IL-8 is released at the highest concentration, which enhance neutrophil migration and activation. Activated neutrophils release elastase, which causes damage to epithelial cells. Therefore, epithelial cells exposed to GV components release Elafin. The mechanisms of its action lie in its being an effective antimicrobial against GV first, and its being an anti-elastase to prevent tissue damage second.

Uterine mucosa is an important tissue barrier whose main function is to provide protection against pathogens and toxic factor while maintaining a symbiotic relationship with commensal microbes. All mucosal tissues share these characteristics and the uterine mucosa is unique because it changes periodically during the menstrual cycle as

well as during pregnancy (Zhou *et al*., 2018).

In addition to its role in the defense process, the immune system plays an important role in reproduction because it ensures local immune tolerance to fetal antigens and paternal antigens, trophoblast invasion, and vascular remodeling. The human endometrium contains a conspicuous population of immune cells, especially natural killer cells (NK Natural Killers), which are phenotypically different from cytotoxic peripheral NK (McGuckin *et al*., 2011). The uterine lining also contains a small number of lymphocytes, which include B cells and CD8+ T cells. The number and pattern of these cells change during the menstrual cycle. Recently, the uterus has been considered a non-sterile compartment as it appears to contain its own microbiota and for the homeostatic relationship between host and microbes to resist pathogen colonization, there has been increasing interest in characterizing the nature of intrauterine microbial colonization and its apparent impact on fertility and pregnancy (Belkaid and Harrison, 2017). Elafin is an antiproteinase inhibitor and an antimicrobial molecule that is expressed at epithelial sites, such as the cervix. The study by King *et al*(2003), showed details of the expression and regulation of Elafin in the endometrium by examining Elafin mRNA and protein expression in the endometrium throughout the menstrual cycle, peaking during menstruation. Elafin protein is localized in white blood cells circulating in the endometrial stroma during the secretory and late menstrual phases. Elafin was expressed by endometrial neutrophils around the time of menstruation. A combination of proinflammatory mediators, IL-1β and TNFα, increased Elafin mRNA levels by 4.6-fold. The results showed that the endometrium expresses Elafin in a menstruation-dependent manner, attributable to The presence of leukocyte infiltration and increased inflammatory signals, as Elafin regulates proteolytic enzymes during menstruation and will contribute to innate immune self-defense against uterine infection (Neto *et al*., 2014).

Elafin topical suppressant of vaginal secretions and is responsible for regulating the paracrine inflammatory reactions necessary to maintain pregnancy and reduce uterine infections. Other functions of Elafin include intrinsic pathways for protection in mucous membranes against uterine infections, regulation of the menstrual cycle through antiproteolytic mechanisms, and participation in tissue repair (Zakizadeh *et al*., 2020). The uterine mucosal immune system is quite unique compared to other mucosal surfaces because it must adapt to menstruation in response to hormonal stimuli. Low levels of LH and progesterone are responsible for the menstrual phase, which begins with long-lasting and intermittent vasoconstriction, with subsequent necrosis of the vessel walls as well as In endometrial tissue of the functional layer (Zhou *et al*., 2018). Antimicrobial molecules, which are produced in the mucous membrane of the female reproductive tract, are greatly influenced by estrogen, which acts differently in the upper and lower reproductive tract. Elevated levels of estrogen, a characteristic of the pre-ovulatory period, increase the production of certain antimicrobial peptides, e.g. Peptidase inhibitor secretory leukocyte peptidase inhibitor, and Elafin secreted from endometrial epithelial cells (Wira *et al*., 2014) endometrial epithelium.

On the other hand, estrogen suppresses the secretion of pro-inflammatory cytokines, including TNF-α, macrophage proteins, IL-1β, IL-6, and IL-8 from uterine epithelial cells.Immunodeficiency in the lower reproductive tract may contribute to enhanced survival. Sperm in the pre-ovulatory period. In addition, the rise in estrogen in the pre-ovulatory period increases the levels of antimicrobial peptides and reduces pro-inflammatory cytokines in the upper genital tract, which prevents ascending infection by vaginal pathogens and provides favorable conditions for the crossing of animals. Sperm and embryos (Trifonova *et al*., 2014). Leukocytes are distributed in the female reproductive system either in aggregates or dispersed in the epithelial layer,

lamina propria, stroma, and despite their differential distribution in each organ of the female reproductive system, the predominant immune cells are T cells, macrophages/ dendritic cells. macrophages/dendritic cells, NK cells, neutrophils, and mast cells. (Lee *et al*., 2015). Sex steroid hormones greatly affect these cells even though they lack estrogen and progesterone receptors. As in peripheral blood, leukocyte counts in the female reproductive tract fluctuate periodically during the menstrual cycle, perhaps in indirect response to estrogen and progesterone (Lee *et al*., 2010). Recntly there are negative correlation between Elafin and (LH and FSH) and positive correlation between Elafin and (GNRH, β-Lipotropin and subfatin) Table 4.

Table 4: Correlation between Elafin and hormone

Pearson Correlation	Elafin
B-lipo	0.168997
FSH	-0.3227
LH	-0.08675
GNRH	0.08973

Conclusion

From these results, we conclude that one of the causes of infertility (unexplained infertility), is bacterial infection and that at the beginning of the infection or the entry of the foreign microscopic organism. It leads to the stimulation of the production of the Elafin protein in the lining of the vagina and uterus, which leads to the inhibition of the bacterial wall or any foreign microbe, and thus its elimination. Thus, the higher the protein level. The higher the resistance, the greater the inhibition of pathogenic bacteria

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