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The Effect of Estrogen on Vaginal Infection with Candidiasis

Anmar Saad Khalaf Hamid Al-Akidi[1], Dr. Hadi Alwan Mohammed Al-Saadi[2]

Abstract

This study was conducted at the University of Kirkuk - College of Education for Pure Sciences - Department of Life Sciences to determine the effect of estrogen hormone on vaginal infection with candidiasis for women in the city of Kirkuk / Iraq for the period from August 2022 to October 2022, where 200 vaginal swabs were collected from women who declined Consultant of the Maternity, Gynecology and Children's Hospital and some private clinics. Women are aged 17-0 5 years for investigate Candida SPP types and prepare a questionnaire list to record patient information. Laboratory tests, culture and biochemical tests were performed to diagnose Candida. The results of culture on the medium of Sabouraud Dextrose Ager with Chloramphenicol showed the presence of 94 positive cultured samples with a percentage of 50% of the total and 610 negative samples for culture and a percentage of 50%. Four types of Candida albicans, Candida tropicalis and Candida glabrata were isolate Candida Lusitaniae by 69%, 22%, 5% and 3% respectively and the diagnosis of fungi was confirmed using polymerase chain reaction technique.

Theresults showed a significant difference between the category of infected women and non-infected women compared to the control group, where the significance value was equal to (0.046), which is less than (0.05), while the highest incidence of diabeteswas within the age group 17-28 years. As for academic achievement, it was among non-educated women, where it reached .6% 26 with a significant difference at the level of probability (0.05) and the rate of infection outside the city was higher, reaching 37%, with a significant difference at the level of probability (0.05).

Keywords: *Estrogen, vaginal infection, candidiasis.*

Introduction

This study was conducted at the University of Kirkuk - College of Education for Pure Sciences - Department of Life Sciences for the period from August 2022 until October 2022. Hormones Estrogens are a steroid hormone associated with the female reproductive organs that is responsible for the development of female sexual characteristics (Lobu et al., 2016) There are four forms of endogenous estrogen that are responsible for the effects attributed to this hormone: Estrone (El), Estrone, estradiol (E2), Estradiol and Estriol (E3) Estriol and esterol (E4) Etestrol with estradiol being the most prevalent and effective estrogen before menopause (Cui et al., 2013). These hormones are synthesized using cholesterol as the primary sterol where the aromatase enzyme aromatase converts them into estrogen (Blakemore et al., 2016) In premenopausal females these estrogen are synthesized primarily in the ovaries as well as in the placenta during pregnancy, after menopause, the ovaries produce estrogen at

[1] Department of Life Sciences, College of Education for Pure Sciences, Kirkuk University, anmarsaad2223@gmail.com
[2] Department of Life Sciences, College of Education for Pure Sciences, Kirkuk University, Dr.hadi 1959@uokirkuk.edu.iq

sharply reduced levels and the majority of residual estrogen is synthesized in secondary tissues such as the brain, kidneys, bones, skin and adipose tissue (Cooke et al., 2018). Estrogen enters the systemic circulation as a free or protein-binding hormone to either sex hormone-bound globulin (SHBG) or albumin (Sier et al., 2017). Non-protein-bound estrogen has the property to diffuse freely in cells without regulation The physiological response to estrogen begins in the cellular cytoplasm with estrogen binding to either alpha-estrogen receptors or beta-estrogen receptors beta-estrogen then crosses the estrogen receptor complex and activated estrogen into the nucleus of cells to induce DNA transcription by binding to nucleotide sequences known as estrogen response elements (EREs) to activate the physiological response Kumar et al., 2018) estrogen levels in the body are regulated by the negative retrograde effect of estrogen on the hypothalamus and pituitary gland An example of adverse reactions can be observed during PMS (Hamilton et al., 2018).

Vaginitis is a common clinical gynecological disease caused by vaginal bacteria, vaginal fungi,Trichomonas vaginalis and other pathogens (Sparks et al. 1991) including vulvar candidiasis. which accounts for 20-45% of all cases of vaginitis and is the second most common vaginitis only after bacterial vaginosis and is mainly vaginosis B vulvar leukemia which accounts for 85-90% resulting in itching in the vulva or vagina before menstruation and other systems (Rajalakshmi et al., 2016).

Vaginal candidiasis is the most common fungal infection, affecting three out of four women in their lifetime (Bas-Neves et al., 2008), with 75% of adult women developing at least one episode of candidiasis by candidiasis during their lifetime. About 40-50% of women who have had their first attack can recur their infection and 5% of women have suffered from infection. One of the "recurrent seizures" characterized by the appearance of three or more episodes of infection annually (Willems et al., 2020) is that the formation of vaginal microbes is affected by the presence of estrogen, as recent studies have shown that estrogen stimulates the deposition of glycogen in the epithelial tissues of the vagina, where glycogen is metabolized into glucose inside the vaginal epithelium and then converted to lactic acid by cellular metabolism, where symptoms of the disease appear in the form of red or yellow ulcers accompanied by vaginal discharge with itching and burning Painful and discomfort and the incidence increases in pregnant women, those who use antibiotics excessively and randomly and those who use estrogen-containing pills (Nghamish et al., 2010).

Theobjective of this study is to:

1 Isolation and diagnosis of candida types associated with the vagina of women innately, biochemically and confirmed by PCR technology.

2- Measuring the level of estrogen and its relationship with infection with some vaginal fungi.

3 Generator and working methods

Materials and Methods

Sample collection

200 vaginal swab samples were collected from women visiting the consultant of the Maternity and Gynecology Hospital, the Children, Azadi Teaching Hospital and some private clinics in the city of Kirkuk - the center of Kirkuk province.The samples included non-pregnant ladies for the period from August 2022 to October202, the age of the women ranged between 16-50 years The swabs were placed in sterile tubes containing the Physiological Saline Solution.

Direct microscopic examination

The eyes were examined by placing a drop of the sample taken with swabs after shaking it in the Physiological Saline Solution well on a glass slide and covered with the lid of the slide and passed on the flame two or three times while avoiding boiling because it leads to the crystallization of potassium hydroxide and examined under a light microscope with a strength of 400X and 100X to observe yeasts and false fungal filaments (Kwon-Chung and Bennett) 1992,).

Transplant and biochemical tests to diagnose candida types. Candida spp.

Chlamydospores formation with tween 80 test

This test was done where the center of corn meal agar was planned with three parallel lines of length 10 mm at an angle of 45 degrees, this test is one of the diagnostic characteristics characteristic of the types of candida, then inoculated with yeast to be diagnosed and placed on it a sterile slide cover on the surface of the middle and incubated dishes at a temperature of 37° C for 48 hours and then examined by microscope to observe chlamydospore spores as well as false fungal filaments(Konemam et al., 1979).

Surface Growth test

This test was performed by inoculating the test tubes containing liquid sucrose sucrose medium (SSB), part of the yeast colony, and then incubating the dishes at a temperature of 25-30 °C for 24 hours and using this test to observe surface growth (Van Der watt, 1970).

Grow test on Chrome Candida Differential Agar

This test was performed by taking a small part of the colony fungal growing on the medium of the sabouraud's dextrose Agar (SDA) for 24 hours , and planted on the medium of Chrome Agarand incubated for 24-48 hours at a temperature of 37 °C.

Ability to ferment sugar test Fermentation Sugar

This experiment was conducted according to the method of Lodder (1974), by adding 2 ml of the fermentation medium of sugars to the test tubes containing a Dirham tube, by placing an inverted position and adding 2 ml of sugar storage solution for sugars (glucose, sucrose, lactose, maltose and calctose) and then adding drops of phenol red. Phenol Red When the color of the medium A changed to red, then the tubes were inoculated with yeast stuck and then incubated at a temperature of 30 °C, the results were followed up daily for 10 days to observe the change in the color of the medium from red to yellow and the formation of gas in the AED tubes.

Candida spp spp susceptibility test. In the representation of sugars Candida Susceptibility Test in the Sugar Assimilation

Prepared medium representation of sugars (SAM) and then poured into dishes Petri and after hardening was the work of drilling diameter6 mm in the middle and then add a volume of 1 ml of yeast solution at the age of 48-24 hours by spreading it with a glass rod and adding solutions of sugar stored prepared in the pits and then incubated at a temperature of 30° C for 4-2 days and then the yeast growth or lack of quality is observed in the reserve(Refai et al., 1996)

Polymerase chain reaction (PCR)

Diagnostic method using PCR examination

The application of PCR molecular diagnosis technique based on heterogeneity was carried out in the ITS region (internal transcribed spacer) to confirm the diagnosis of Candida spp fungal isolates. DNA was extracted and the ITS target region amplified using both prefixes

ITS1 '-TCC GTA GGT GAA CCT GCG G-3 '5 و ITS4 '-TCC TCC GCT TAT TGA TAT GC-3'5 (White 1990,).

Schedule(1) Prefixes (ITS4,ITS1) used to confirm the diagnosis of Candida spp.

Initiator Name	**Sequence of nitrogenous bases Secquence Nucluotide**
ITS1	'3 TCCGTAGGTGAACCTGCGG5
ITS4	' TCCTCCGCTTATTGATATGC35

A extract DNA from Candida spp.

TheDNA of the 24-hour candida fungus was synthesized using Chelex®100 BioRad , USA.

1. A small amount of fungal colonies was taken per sample and transferred to a sterile 0.6ml tube containing 200µL of Chelex ®100 and 100 µL TE.

2. Then the pipes were placed in a water bath at 95 degrees Celsius

3. Ten minutes later the samples were removed and ejected at 13,000 rpmfor ten minutes

4. The upper water layer containing DNA was then carefully removed from the samples.

5. Then put in tubes with a volume of 0.6 ml

6. It was stored in the refrigerator at a temperature of -(4) degrees Celsius, a mixture of PCR reaction (25 µL) was prepared according to the company's instructions as in the following table:

Schedule(2)PCR mix sizes

Volume	**PCR master mix**
12.5 µL	Master mix
1 µliter	Forward primer
1 µliter	Reverse primer
5 µL	Free nucleas water
5.5 µL	Genomic DNA
25 µL	Total

Then the components of the PCR reaction mixture mentioned in the table above were placed in special 0.2 ml tubes containing the rest of the PCR reaction components and all the tubes were transferred to the centrifuge vortex exispin at a speed of 3000 rpm for three minutes and then placed in the Thermocycler PCR device to perform DNA amplification according to the ideal conditions of thermal cycles.

Thermocycles conditions for PCR PCR Thermocycler test conditions

The polymerase chain reaction test was performed using a PCR thermocycler device as shown in the following table:

Schedule (3) Thermal cycle conditions for PCR examination

Time	**Temperature**	**Repeat cycle**	**PCR Step**
5min	95 c	1	Initial denaturation
40sec	95 c		Denaturation
1 min	58 c	35	Annealing
40sec	72 c		Extension
7min	72 c	1	Final extension

PCR results analysis

Electrorelay was performed using Agrose gel electrophoresis at a rate of 1.5% and according to the mentioned method to read the result of the PCR product analysis as follows:

Dissolve 1.5 g of Agarose gel in 100 ml of TBE buffer 1 X using a Microwave device for 2 minutes.

Then leave the gel to cool to 50 °C and then add 3 microliters of radioactive DNA dye Ethidium bromide and mix well with the gel.

Pour the Agrose gel electrophoresis into the tray containing a comb to determine the PCR places, then leave the gel to harden at room temperature for 15 minutes, then carefully remove the comb from the gel and transfer to the electric relay basin.

The samples carried the product of the PCR product and placed in the gel pit.

DNA ladder starting from 55-700 bp was used to measure PCR output and put in the first pits.

After the loading process, immerse the Agrose gel electrophoresis gel using a TBE Buffer solution at a concentration of 1X, then close the relay cover and the bass relay device is operatedusing a current of 100 volts and 80 amperes for an hour.

Determination of estradiol hormone

The level of the hormone in the serum was measured for all women with vaginal candidiasis and the control group, according to the followers of the sisters mentioned in the leaflet attached to the ready-made test kit imported from the French companyBiomeriuex specialized in hormone testing.

Components of the scanning kit

Solid phase receptacle: ready for use, similar to the tip used in the micropipette, but marked at the wide end with a hormone code

Hormone Strips: These are ready-to-use strips consisting of 10 holes covered with a thin layer and marked with the symbol of the hormone examined.

Control(CI): Add 3 ml of distilled water and leave for 5-10 minutes.

CI))Calibrator:2 ml of distilled water was added to it and left for 5-10 minutes.

Dilutant(RI): It's ready.

MEL card : A ready-made card containing the basic encrypted information of calibration data used in the hormone level test calendar.

The principle of the test

The basic test principle involved mixing between immunoenzyme examination and immunofluorescent detection, for this reason it is called the enzyme binding test for immunofluorescence to make pipettes, the solutions are ready and distributed in the strips stone. All the steps followed work autonomously by the device as the reaction material enters the cones and exits many times, and its front is sensitive as a result of the presence of anti-monoclonal antibodies During the detection period as a final step, Methyl 4 - Umbelliferyl Phosphate rotates between the inside and outside in the cones, and the conjugate enzyme acts as an aid in hydrolysis of this substance into a flash product of 4 Methyl Umbelliferone, which is measured at a wavelength of 450 nm. The intensity of the flash is directly proportional to the concentration of antibodies present in the model. By self-determination by the device based on Standard 1 S1 solution)

Test steps

The method of work included the following steps:

Put the tubes containing serum in the incubator with a temperature of 37 ° C for 30 minutes.

Placing a special MLE card is a card used to identify tests on a minividas device through which it recognizes the test automatically , without which the device cannot show the results and is then printed.

Use one SPR & SSR strip for each standard solution serum sample and the concentration of the standard solution of the serum sample is determined in units per milliliter (unit/ml). Standard & Control The standard analyzed sample is placed in a designated place in the analyzer. As for control, it is used to verify the correctness and accuracy of the results provided by the device. Typically, the control sample is placed in a specific place in the device, and the test is run on it in the same way that it is operated Samples to be measured. The results obtained from the control sample are measured and compared with the previously known values to validate the measurements and ensure the accuracy of the device. .

I put 200 µL of serum in hole number one on the test strip. Now, in the same way, you can place the standard measurement solution in the hole assigned to it and place the control sample in the hole assigned to it on the same strip. These steps allow the device to make the required measurements and analyze the results based on the scales in the tape, standard solution and control.

I followed the appropriate steps and the device performed the calibration process automatically. The calibration duration may vary from one device to another and depends on the type of device and the analysis performed. If the calibration process takes 45 minutes.

After the calibration process was complete and the results were printed, I took out the SPR and STR chips from the machine and placed them in a special container. These chips are used only once.

Statistical analysis

The study data were analyzed statistically using the SPSS program to estimate the averages and standard error Std.error and the differences between the averages were compared based on the Dunkin' multi-range test at the probability level ($P \leq 0.05$) (Al-Rawi, 2000).

Results and discussion

Microscopic Examination

The results of microscopic examination of 200 samples show that 47% of these samples were positive for microscopic examination 94 out of 200 while 5 4% of the total samples were negative microscopic examination 106 samples out of 200 The percentages were distributed between 81 positive smear implants (40.5%) and direct microscopyand 13 Negative direct microscopy (6.5%), positive culture, 7 negative smesters (3.5%) and positive direct microscopy 94 swabs were negative (47%) for both culture and direct microscopy The reason for the appearance of negative results by microscopic examination and transplantation is due to the similarity in symptoms between fungal infections and other injuries or insufficient sample collected or may be the result of taking antibiotics randomly without consulting a doctor as it can return to the conditions of mushroom development on the culture medium (Arastehfar)The increased incidence of

vaginal candidiasis is attributed to wearing tight underwear, lack of attention to personal hygiene, and high levels of estrogen in the blood (Mikolajczyket al., 2006).

Table(4) Results of microscopic examination and culture on SAD medium for Candida yeasts

Microscopy	Transplant on SAD	Percentages	Number of samples
+	+	**40.5%**	**81**
-	+	**6.5%**	**13**
+	-	**35%**	**7**
-	-	**47%**	**94**
Total number of samples		**100**	**200**

(+) Indicates the presence of spp Candida, (-) indicates that Candida spp is not observed

Isolation and diagnosis of Candida spp species

The results of swabs taken from women with vaginal candidiasis showed the isolation and diagnosis of four types of candidiasis, which were diagnosed based on cultured microscopy and biometric tests. Among some of the studied characteristics to differentiate between candida and other yeasts such as color and temperature appropriate for their growth in colonies, while other characteristics were adopted to differentiate between types of candida, such as the test of the representation of sugars and the formation of the germ tube, which was one of the characteristics of the type Candida albicans C. albicans in addition to the colors on the medium of chromium Akar (Chrom agar) isolated Candida Albicans by 65%, followed by yeast Candida calabrata C.glabrata, Candida tropics.C. trobicalis and Candida Justina C. lusitaniea by 21%, 7% and 3% respectively These results are consistent with several studies that confirmed that the type C. albicans is the most common type of Candida spp among women with vaginal candidiasis (Willems et al, .2020). It also agrees with Roudbarmohammadi et al, 2016 that yeast C. albicans The most prevalent compared to other types of candida and also coincides with the findings of Noman (2020) and Al-Hamdani (2020), who confirmed that C. albicans isolateswere 63%, which is considered the most frequent among other types

Schedule (5) Numbers and rates of laboratory testing of samples

Percentage	Number of isolates	Types of *Candida*
69%	**65**	***C.albicans***
22%	**21**	***C.glabrata***
5%	**5**	***C.tropicalis***
3%	**3**	***C. lusitaniae***
100%	**94**	**Total number of samples**

Also consistent with (Thiyahuddin et al, .2019) who also showed that the yeast C. albicans recorded the highest percentages and the prevalence of this type is natural due to the presence of types of Candida in the body as normal flora in 80% of cases and pathogenically from 5% - 20% of cases, but without the appearance of symptoms, it is believed that stimulating the fungus to turn into the form of the pathogen and the appearance of symptoms is due to several factors Changes in the vaginal environment result with reduced host immunity (Sharmaet al., 2017) Odds (1988) noted that C. albicans yeast is responsible for 80-90% of cases of vaginopathy and ovarian vulvar vulvar disease due to their high ability to adhere to epithelial cells as well as Germination tube formation in affected tissues and high susceptibility to the production of protein-digesting enzymes and Phosphlipase Markus (et al., 2022) Zeng et al. (2018) and Arastehfar et al. (2018) also revealed that eating high-sugar foods and drinksis one of thefactors Which contribute to the rise of infection by increasing the concentration of

glucose in vaginal secretions and antibiotics used, which enhances the adhesion of Candida to epithelial cells and stimulate their growth very significantly.

Candida spp diagnosis using PCR technology

of eight isolated Candida spp isolates from women with vaginal infections after being diagnosed using traditional methods after growing on the medium of SDA and Chrom Agar The diagnosis was confirmed molecularly for candida isolates because the traditional methods used in the diagnosis of yeasts are based on determining phenotypic criteria and are no longer sufficient due to the overlap of criteria with other species belonging to the same sex in addition to genetic variation. Among them, microbiology farms, even if they belong to a particular group, they differ in their growth characteristics and phenotypic qualities, and the difference does not necessarily mean that the genetic structures are necessary, especially between farms and isolations that belong to the same genus and species (Mohi Aldeen and Jigan, 2013).

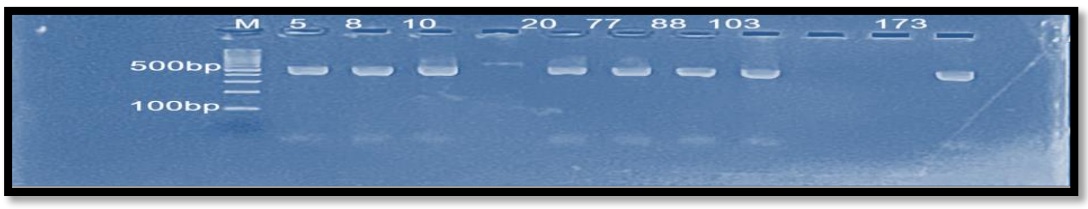
Results C Sequencing DNA analysis

Sequencing of the selected isolates was performed and entered into the NCBI BLAST program to find out the similarity between them and the results in the Global Genbank after obtaining the nucleotide sequence of the DNA beam of the local isolates and comparing it with the same region sequences of some local and global Candida spp isolates registered in NCBI BLAST. Where the yeast C. albicans showed A match of 94% to 100% with the results in the genebank, the type C. tropicalis showed a 99% match with the results of the alginate bank, while the yeast C. glabrata showed a match of 98%, and the type Clavispora lusitaniae gave a match between 98% to 97% with the results of the alginate bank, which indicates the widespread spread of this fungus in the world. Nucleotide sequence was inserted to the DNA package The program to find out the percentage of similarity between them and the results recorded in the Alginate Bank, the results showed after comparing them with the isolates registered in the Alginate Bank that all isolated isolates belong to the genus Candida spp, these results confirm the validity of the microscopic and phenotypic diagnosis.

The results indicate the diagnosis of four types of candidiasis, and the type C. albicans was the most frequent by 65 isolates and by (70%), followed by C. glabrata yeast by 21 isolates by (22%), followed by C. tropicalis by (7%), i.e. 9 isolates and finally C. lusitaniae yeast by (3 %) i.e. only 3 isolates These results coincide with many studies that confirmed that the type C. albicans is the most prevalent type among women with vaginal candidiasis (Willems et al, .2020)It also corresponds to what they said (Roudbarmohammadi et al, 2016) C. albicans are the most widespread species compared to species.

Others belonging to the genus Candida spp also correspond to the studies of Noaman (2020) and Al-Hamdani (2020).

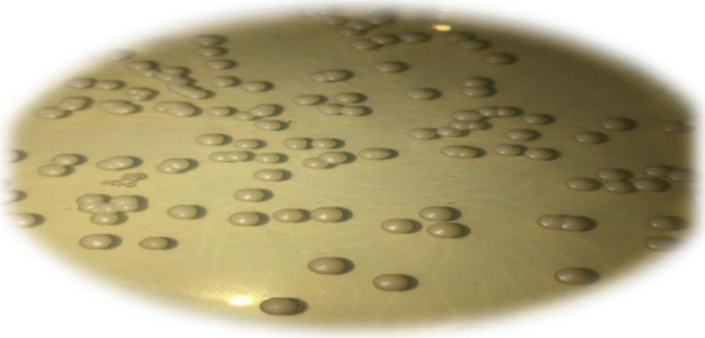
Table Global isolates and NCBI accession numbers compared with them through the BLAST website and the percentage of conformity with isolates under study



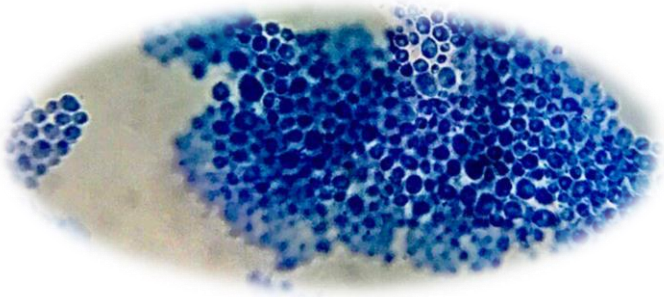
Shape(1) The separation of PCR products on the 1.5% agarose gel containing ethidium bromide was shown and photographed under UV light.

Phenotypic and Microscopic Characteristics

Diagnostic results of Candida spp based on culture and microscopic characteristics, biochemical tests and confirmation of diagnosis by PCR technique show that colonies growing on the culture medium Sabouraud Dextrose Agar with Chloramphenicol at a temperature of 37 for 24-48 hours appear in a circular white to creamy shape smooth surface and shiny and carry the smell of yeast as in Figure (2) These results match the findings of (Muslah, 2022) after the diagnosis of Candida SSP based on microscopic, implant and biochemical characteristics, as well as simple initial diagnosis using Chrom Agar Candida (CAC) medium and secondary diagnosis using PCR technique,their colonies appear when grown on Sabouraud Dextrose Agar medium. At a temperature of 37 ° C for 24-48 hours colonies appear white to milky, convex, smooth and shiny with an odor. As pointed out (Elliset al., 2017) that these characteristics such as color, shape and smell that resemble the smell of yeast shown by Candida SPP colonies when grown on SDA medium The cultivated colony was examined microscopically after being colored with crystal violet dye and cotton bluedye Lactophenol (where yeast cells appeared spherical to oval or In the form of single or budded elongated cells. These results are consistent with what Boon et al. (2013) report that yeast cells appear spherical or oval budding as in Figure (3).



Shape (2) Candida spp on SDA with a temperature of 37 °C



Shape (3) Candida spp yeast dyed cotton blue in Lactophenoland under the power of X40

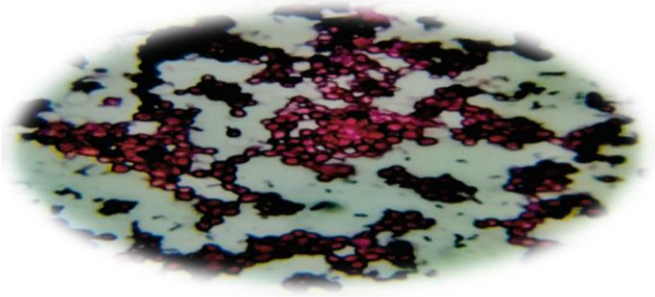


Figure (4) Candida spp dyed with Crystal violet dye

The results also coincided with a study by Sudbery et al. (2004) who showed that yeasts appear more clearly when colored with violet crystal pigment compared to cotton

blue.The appearance of yeast cells in blue is due to their retention of the Peptidoglycan layer located in the yeast cell wall because it is positive for the Cram pigment (Soll and Anderson, 1986) In addition,cotton blue pigment is of great importance for viewing chlamydia boards and fungal filaments. Webb et al., 1998.

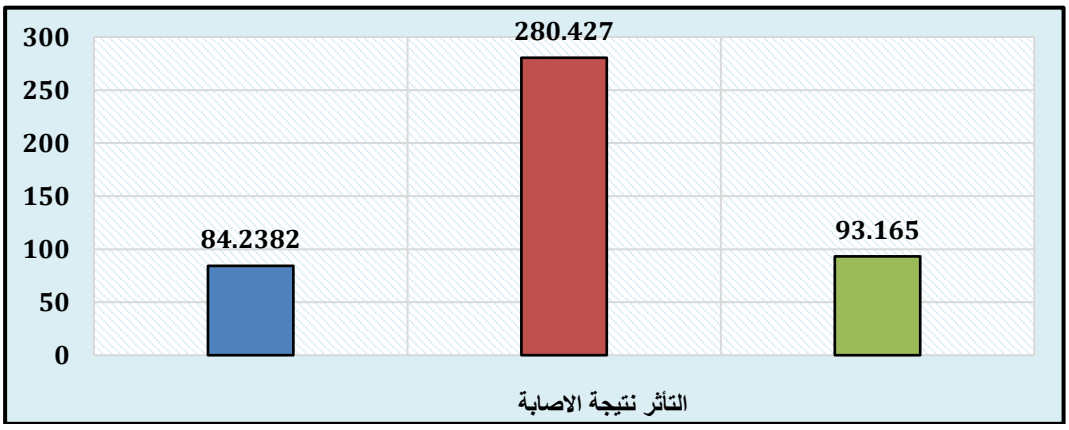
Effect of high estrogen level on various vaginal candidiasis

The effect ofestrogen level on the outcome of candidiasis was classified into three categories: negative, positive and control. negative = 84.238 positive = 280.427 control = 93.165). We note through the statistical test for analysis of variance (ANOVA) that there is a significant difference between the three categories where the significance value (p value) is equal to 0.046 which is less than 0.05 as shown in Table 2 Glycogen stored in the blown epitheliumacts as a basic substrate for Nutrition for Candida) Dennerstein and Ellis,2001) Estrogen is responsible for the formation of inhibitors by epithelial cells that inhibit the anti granulocyte function and thus cause leukocyte energy (Yano et al.,2018؛ Willems et al.,2020؛ Naglik et al.,2019) . The energy of leukocytes in the vaginal tract is explained by the fact that under certain conditions including elevated estrogen levels, epithelial cells produce inhibitors such as heparan sulfate which prevent the related receptors of granulocytes from interacting with the corresponding bonds of yeast (Yano et al.,2018 ؛ Naglik et al.,2019(Most often in the middle of the menstrual cycle due to increased levels of estrogen with a high glycogen content, and during the luteal phase While there is a rapid decrease in symptoms after low estrogen levels during menstruation) Dennerstein and Ellis,2001 .(Women who use combined oral contraceptives also have higher levels of estrogen. In addition, postmenopausal women use Hormone replacement therapy Both groups have increased the risk ofvaginal candidiasis epidemic in addition to these factors. (Fischer and Bradford .2011) Hormonal status in women may also have a direct impact on immune response and injury (Kalo-Klein and Witkin.,1989).

Schedule (6) Comparing estrogen to the categories Negative, positive, control

Injury Impact	**N**	**Mean**	**Std. Deviation**	**Std. Error**	**95% Confidence Interval for Mean**		**$LSD_{0.05}$ p value**
					Lower Bound	**Upper Bound**	
Negative	34	84.2382 **b**	129.14013	22.14735	39.1791	129.2974	**3.557**
Positive	55	280.427 **a**	515.47247	69.50629	141.0755	419.7790	**0.032**^{S}
Intact	20	93.1650 **b**	107.36594	24.00775	42.9162	143.4138	

(p value < 0.05)



Shape (5) Comparing estrogen to the categories Negative, positive, control

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