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The Influence of molecular Effects on Laser Nd:YAG and Diode on Trichophyton Rubrum fungi using RAPD marker

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ABSTRACT

This study was carried out to assess the morphological and molecular effects of the Nd: yag and Diode (semiconductor) lasers on Trichophyton rubrum Fungi using RAPD marke . Sixty samples of skin patches, nail clippings and parts of hair were collected from infected patients of both sexes (34 males and 26 females) for the age group (1-60) year of patients who have attended to Tikrit Teaching Hospital. The T.rubrum fungi was the most common among the skin fungi, T.rubrum radiated by using two lasers: the Nd: yag laser with a wavelength (530) nm and energy (300, 500, 700) mJ and for two times exposing 20 and 30 seconds by six coefficients and control sample, the low-density diode (5 mW) for 3 times 10, 20 and 30 seconds on distance 20 cm with three treatments and a control sample. DNA was extracted from the fungus after direct exposure and after leaving it to grow for a whole generation and then was used to complete RAPD reactions using five primers. The RAPD marker gave excellent results with all primers, It was noted that the exposure of T. rubrum to the Nd: yag and Diode lasers with different cards and times had different effects on DNA and caused significant changes in the RAPD patterns compared with the control group, new bands appear and others disappear. The energy affecting the fungus T.rubrum for the first laser Nd: yag is 500 mj at time 30 sec while the power of the laser Diode was 5 mw at time 20 sec. The results suggested that the diode laser is highly effective and has a great effect on the genetic material of fungi compared with the effect of the first laser. The conclusion that the use of laser can affect DNA of skin fungi and may lead to mutations which means that it can be used in the treatment of skin fungal infections and the RAPD was effective in detecting the effect of laser at the molecular level as a simple, and inexpensive.

Introduction

In recent years, human skin fungal can have strong connection with skin infections which have increased in recent years due to many factors including the environment concerns the growth of such fungi on the skin As a result of the high temperature and high humidity resulting from the secretion of sweat from the body, especially in the folds of the skin and where the presence of hair to contain keratin, which is food for most of the fungus in addition to the increasing numbers of patients with AIDS (AIDS, cancer, diabetes) due to the use of immunosuppressive drugs [1].

Dermatophytes, a group of interrelated fungi that can penetrate keratin-containing tissues of hair, skin and nails for human and animal [2]. These fungi form their colonies in keratinocytes, and inflammation occurs as a reaction to the body on the byproducts of fungus, and generally confined to the presence of these fungi on the stratum cornea of the skin because of the inability to penetrate the living tissue in the body of the host with active immunity, where the incidence of immune response in the body of the host ranging from moderate to severe and in rare cases invaded the tissue under the skin and cause Kerwin [3]. This group includes almost three species

Trichophyton, Microsporum, Epidermophyton [4]. Trichophyton is one of the most common types of fungal dermatitis, noted [5] that among the 10 cases of disease in Europe included T. rubrum and T.mentagrophytes, according to the various infections caused by dermatophytes has been a major development in the field of drugs and antifungal drugs, but some of these drugs have a serious side effect on immunity host such as of Amphotericin B, used in the treatment of fungal infections, As the buline and azules enter into the manufacture of fungal sugars [6].

The researchers tried to find other ways of treatment, they are repeated ionizing radiation therapy of various types and entry into various medical fields, as well as the treatment of skin fungus, where the laser used in the treatment of nail fungus, which is the most common, as well as ringworm of the body and the palm of the hand and the palm of the head [7]. The advantage of laser is safe and has little cost of the patient, its provide the doses with drugs that are taken continuously and the longtime of treatment that may take several months with a few and difficult healing rates, regardless of the side effects that can cause Including Terbinafine, Intraconazole, Fluconazole which is taken by mouth which may cause headaches with digestive and liver disorders[8]. In contrast, the laser has a clear effect in terms of rapid response by the patient with a successful and safe alternative to chemotherapy or other fungal antibiotics[9].

The (Random Amplified Polymorphic DNA) RAPD marker has been used extensively by many researchers to assess DNA damage, which is of great importance to widespread use. It is relatively simple, relatively cheap, fast and gives information on a large number of positions [10]. Changes in the pattern of RAPD marker after treatment or exposure to mutant material represent the change in intensity as well as loss or appearance of new bands by comparing the pattern of the RAPD marker between the treated or exposed non-exposed samples [11].

There are a few studies on the effect of laser on fungi especially on the genetic material of fungi, therefore this study was carried out to assess the morphological and molecular effects of the Nd: yag and diode (Semiconductor) on Trichophyton Rubrum using the RAPD marker.

Materials and Methods

1– Isolated and identification of fungi

Sixty sample were collected through the peried between October 2017 and January 2018 from the patients who consulted the dermatologist of the Salahad Din General Hospital, samples collection from the patients group with (age 1-60 year), that residents in the city and the countryside, the initial examination and diagnosis was accomplished by the hospital's dermatologists. The residue of the crusts taken from the skin of the injured, infected hair and nail residues were culthred on Petri dishes containing the special medium for the growth of the fungal colony (sabouraud dextrose agar cychlohexamide chloromphenichol) and incubated at a temperature (25-28 C) for a period of (14-20) days, examined and observed every (3-4) days with the continuous purification of developing isolates and then examine by microscopy for diagnosis.

2- Radiancy of fungi

T.rubrum fungi was irradiated using Nd: YAG laser with different energy of (300,500,700) mj / cm3, and 6Hz frequency, and two times 20-30 sec. And Diode laser with 5Mw energy and three times periods (10,20,30) sec, where a part of the developing colony was taken by a 7mm diameter flange hole and placed with a new petri dish and placed under the device and exposed to radiation .

3- Isolation of DNA

It is important to Isolate the DNA from the T. rubrum fungi after direct exposure to the laser and after leaving the mushroom to grow for a one generation (20-14) according to the method mentioned [12], DNA integrity was identified using electrophoresis, the purity of DNA was estimated based on UV absorption at wavelength 260 and 270 nanometers using Nanodrop, the samples were diluted to obtain a concentration (25 ng) per microliter, which is the appropriate concentration for RAPD-PCR reactions.

4- Preparation of RAPD reactions:-

Reactions of RAPD were carried out according to [13] using five random primers (Table 1) shows the AccuPower PCR premix Kit prepared by Korean Bioneer and according to the attached instructions, initial experiments to reach the optimum concentration of the primers and DNA template were performed to obtain the best result of the amplification.

Table 1: Names and sequences of random primers used

No.	Primer code	Sequence 5 to 3	No.	Primer code	Sequence 5 to 3
1	OP G-05	CTGAGACGGA	4	OP Q-02	TCTGTCGGTC
2	OP J-01	CCCGGCATAA	5	OP V-20	CAGCATGGTC
3	OP P-04	GTGTCTCAGG			

A test tube (0.2 ml) was used to contain the basic components of the PCR reaction. Four ul (100 ng) of genomic DNA and one ul of 10-picomole of random primer was added to the tube, the volume was made up with distilled water to 20 µl, mix the reaction components well and then place the tubes in the thermocycler carefully after being programmed according to the program: One cycle for 5 minutes at 94°C followed by 40 cycles, each cycle (30) seconds at 94°C, (45) seconds at 36 °C and 45 seconds to 72°C with a final cycle of (7) minutes at 72 ° C for final elongation. Agarose gel was prepared with a 2%

concentration and then add (5 microliters) of Red Save dye before pouring the gel. The amplification products were carried out with the DNA Ladder for 90 minutes by 3 volts / cm, then the gel was examined under UV-light and images were obtained using the Gel Documentation System [14].

5- Recording RAPD results

The results of RAPD-PCR technique were recorded by examining the images of the electrical propagation patterns of each primers and recording the Bands and then comparing the results of the laser treatment samples with the control group based on the appearance of new bands or the disappearance of existing bands. The total number of RAPD bands shown by each primers of the studied samples and the identification of polymorphic bands was calculated and the percentage of multi-forms for each primers as in Table (2,3).

Results and discussion

It is significant to use the (RAPD) to assess the effect of two types of lasers (Nd: yag and diode laser) on genetic material to T. rubrym fungi using five random primers by direct exposure to the laser and exposing the fungus to the laser and leaving it for a generation to grow to detect the genetic changes that get to the fungus after the completion of the first generation.

The results of the RAPD-type electrode transfer of the five primers presented a clear difference in the number of DNA bands that were duplicated and "distinct" in their molecular weight depending on the primer used, Results were analyzed separately by comparing the results of laser-exposed samples with control samples (non-exposed) based on the disappearance of bands within the control group or the emergence of new bands of laser-exposed samples resulting from the multiplication of certain sites on the fungus genome used and on the molecular weight of bands that depend on the number and locations of the sequence of each primers on the template DNA tape, The difference in inteansity between exposed and non-exposed samples was not calculated because the intensity of the inteansity may reflect differences in a number of copies of the conjugation sites in the sample and also represent variations in the DNA concentration between the samples taken [15].

The significant variation in the DNA level between the treated samples compared to the control samples indicates that the laser treatment has caused changes in the DNA structure of the samples resulting in the emergence of new bands and other disappearance that existed, The mark (+) For the emergence of a new link site (band) and the mark (-) for the disappearance of a previously existing site, and the total number of different locations and the percentage of variation between transactions compared with control group as in figure (1,2) and Table (2,3).

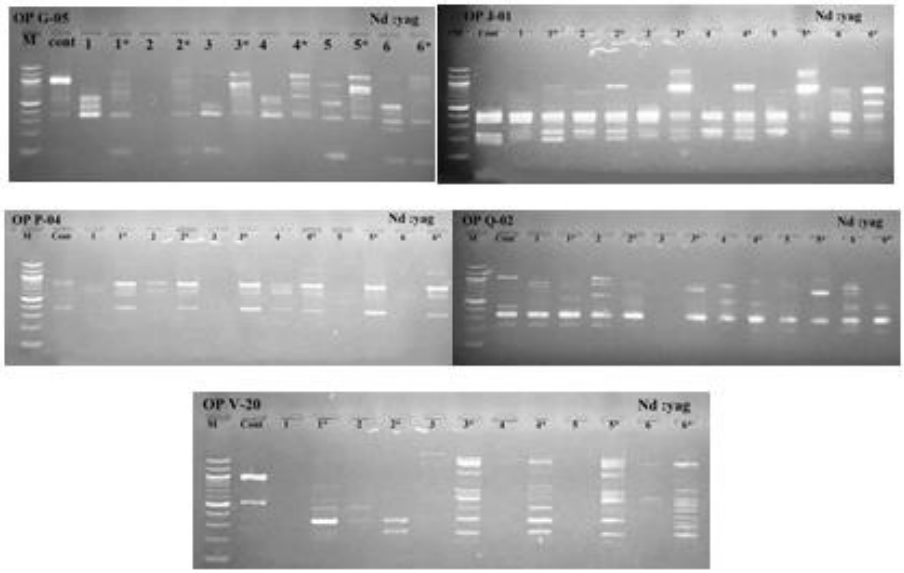

Fig. 1: Electrophoresis of the Random Amplified Polymorphic DNA (RAPD-PCR) of the five primates on the agaros gel 1.5% for fungus samples (T.rubrum) treated with Nd: yag laser

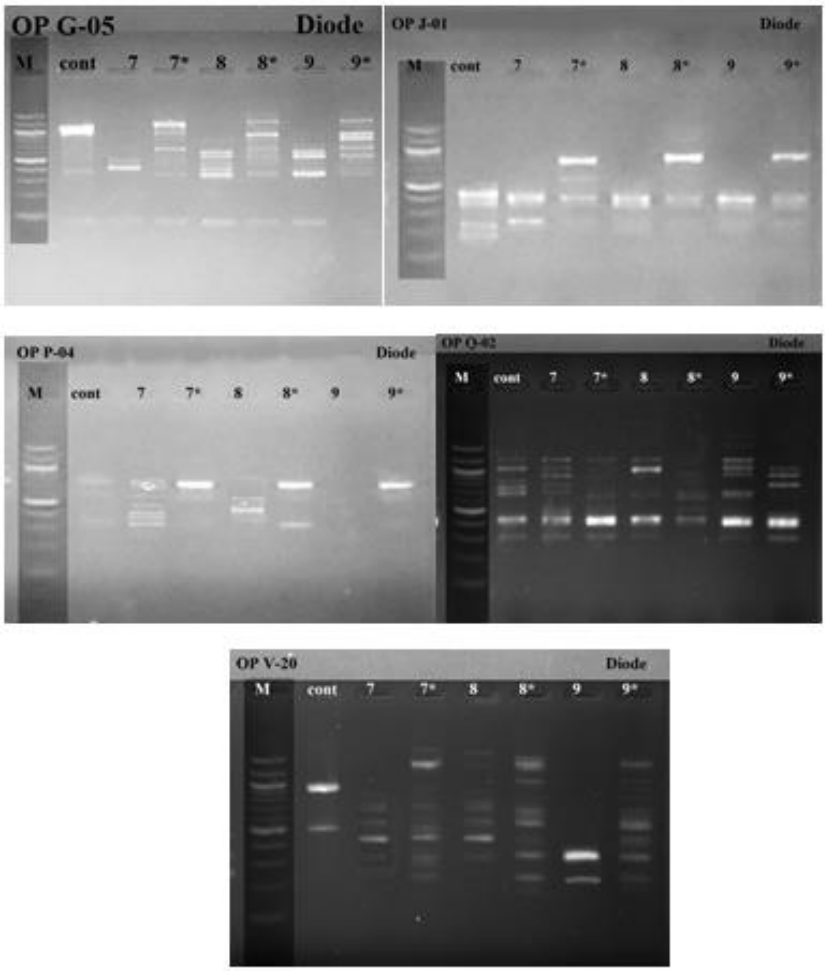

Fig. 2: Electrophoresis of the Random Amplified Polymorphic DNA (RAPD-PCR) of the five primates on the agaros gel 1.5% for fungus samples (T.rubrum) treated with Diode laser

Table (2) shows the number and molecular weight of visible and lost bands and the percentage of polymorphism of RAPD pattern for fungus and T. rubrum exposed to Nd: yag laser cards and different times

T. rubrum															
Energy and exposure time												Number of sites for control group	Number of sites			The name of the primers
T \ 30 sec. Ene\700 mj		T\ 30 sec. Ene\500 mj		T \ 30 sec. Ene\300 mj		T \ 20 sec. Ene\700 mj		T \ 20 sec. Ene\500 mj		T \ 20 sec. Ene\300 mj			A generation later	Immediately after exposure	Total	
- absence	+ occurrence	- absence	+ occurrance	- absence	+ occurrance	- absence	+ occurrence	- absence	+ occurrence	- absence	+ occurrance					
1000, 590 380 590, 380	490, 300 50 300 , 50	1000, 590	900, 500 100, 1050 800, 700	1000 1000	500 , 400 1100 , 900	1000,590 1000,590	1150, 450 100 1100, 850	1000, 590 380	1100 , 900 100	1000	450 1100 , 700 100	3	20	17	40	OP G-05
220	800, 750 800, 500	220, 220 370, 250	1200, 800	220	900, 190	220	490 ,1200 900, 190	220	750 900 ,190	220	900 ,190	4	29	25	58	OP J-01
800, 390	1050, 500	800, 390	_	_	690, 650 580	800, 390	650	390	650 650	800	790, 650 550	2	18	9	29	OP P-04
1050 1050, 230	750	1050, 420 420, 230	650	1050, 420 230, 1050 420	900, 550 1000, 490	1050,420 320,230 1050,420	700	420, 1050 420, 230	900, 600 900, 490	1050, 420 1050, 420	900, 800 900, 800	4	17	19	40	OP Q-02
520,1000 1000	1150, 300 1150, 700 350, 420,250	1000,520 520	1200,320 1050,700 600, 250	1000,520 1000,520	1200, 700 600, 450 320, 250 720	1000,520 1000,520	1300,250 1200,320 1050,700 600	1000, 520 1000, 520	450, 320 320, 250	1000, 520 1000, 520	720, 450 320	2	34	4	40	OP V-20
8+6=14	7+12=19	7+8=15	3+12=15	6+6=12	6+14=20	10+7=17	5+13=18	7+6=13	6+10=16	6+5=11	5+11=16	15	118	74	207	Total
18 , 15		20 , 10		20 , 12		20 , 15		16 , 13		16 , 11		-	-	-	-	Total of different packets
120%	100%	133.3%	66.6%	133.3%	80.0%	133.3%	100%	106.6%	86.6%	106.6%	73.3%	-	-	-	-	% Polymorphism

The black number indicates the location of the band at direct exposure, and the red number indicates the band location after a generation

Table (2) shows a number of sites visible to each individual primer, which includes control sample sites and direct exposure, In addition to a number of identical packets and the ratio of polymorphisms for each transaction, where the largest number of different packets was the treatment time of 20 sec and the card 700 mJ, where the number after direct exposure 15 sites by 100% and 20 sites of samples after a generation of 133.3%.

Table 3: shows the number and molecular weight of visible and lost bands and the percentage of polymorphism of RAPD pattern for fungus and T. rubrum exposed to laser diode cards and different times

T. rubrum										
Energy and exposure time						Number of sites				The name of the primer
T \30 sec. Ene/5 mW		T \20 sec. Ene/5 mW		T \10 sec. Ene/5 mW		Number of sites for control group	A generation later	Immediately after exposure	Total	
- Absence	+ occurrence	- Absence	+ occurrence	- Absence	+occurrence					
1000 1000,90	500,550, 450,900 1100,800, 450	1000	500,450, 390,550 1100,800,600	1000, 390	500,400, 1100,600	3	14	13	30	OP G-05
250,190 190	800	250,190 390,250, 190	800	390,250 390,250, 190	800,550	4	10	8	22	OP J-01
800,300 300	600	300,800 ,300	500,400	300	600,500,400 350,600	2	5	9	16	OP P-04
700,600 1050,600	750,500 750	700,600 1050,900 700,600,250	500,500	1050, 900 700,600	800,500	6	10	18	34	OP Q-02
900,500 ,500	320, 220,1150 600, 450,320 220	900,500 900,500	1200,650, 450,320,1150 1000,700,600 320,220	900,500 900	650,450 320,1250,1150 450,320,250	2	20	9	31	OP V-20
9+7=16	9+10=19	9+11=20	11+11=22	6+9=15	10+11=21	17	59	57	133	Total
17,18		22,20		20,16		-	-	-	-	Total of different packets
100%	105.8%	129.4%	117.6%	117.6%	941%	-	-	-	-	% polymorphisms

The black number indicates the location of the band at direct exposure, and the red number indicates the band location after a generation

Table (3) shows a number of sites visible to each individual primer, which includes control sample sites and direct exposure, In addition to a number of identical bands and the ratio of polymorphisms for each transaction, where the largest number of different bands was the treatment time of 20 sec and the card 5 mW, where the number after direct exposure 20 sites by 117.6% and 22 sites of samples after a generation of 129.4%.

The results pointed out that the primer OP G-05 gave (70) bands, the primer OP J-01 gave (80) bands, the primer OP P-04 gave (45) bands, The primer OP P-04 gave (45) bands, and the primer OP V-20 gave (71) bands as a total number of samples which were treated directly and indicated in the image (number without the mark *) and the samples after the passage of the generation and the reference (number with the marker *) with control samples for both lasers used in this study as shown in Figure (1) for the Nd: yag and Figure (2) of the Diode laser and in table (2) for the Nd: yag laser and Table (3) for the Diode laser.

A total number of visible and lost bands (red numbers) in Table (2) for this fungus Parameter Nd: yag laser (112) bands was (72) bands appeared and (40) bands disappeared, the strongest effect on DNA occurred at (500) mJ and at time (30) sec in general, The number of hidden bands was 8 bands as a total of all the primers used for this energy, This effect included the disappearance of a specific band after generation that was present only in the control sample or in the control sample and in the directly treated sample.

In the treatment of the fungus T. rubrum, the diode laser was affected by its genetic material at different times of exposure (10, 20, 30) sec at most of the primers used, the total number of visible and lost bands (red numbers) in Table (3) for this fungus Parameter Diode laser (59) bands was (32) bands appeared and (27) bands disappeared, the greatest effect on fungus DNA was at 5 mw and 20 sec in general. A number of hidden bands was 11 bands as a total of all the primers used for this energy, This effect included the disappearance of a specific band after generation that was present only in the control sample or in the control sample and in the directly treated sample.

Diode laser is the most powerful effect on the T.rubrum fungus compared with the results of the Nd: yag laser depending on the number of hidden bands of the treated samples, Which is the desired effect by the transfer of the effect of the treatment to the passing of a generation taking into account the number of transactions per laser where the number of transactions in the laser Nd: yag (6) parameters and the laser diode (3) transactions. The effect of the T. rubrum genome after exposure to laser directly and after a generation indicates the efficiency of the laser species used to influence the fungus used as shown in previous forms and tables.

Genetic markers, including DNA markers, have been used to determine the chemical and physical effects and changes in the DNA level Because of those genes with genetic toxicity and from these markers the (RAPD) which can determine the change that happens to DNA after exposure to the chemical or physical effect and can be used in studies of genetic toxicity and detection of carcinogens[16,17]. The RAPD markers is one of the most widely used modern techniques for detecting changes in the genome or dysfunction of DNA because of its easy and no need for a large amount of DNA also can analyze a large number of samples in a timely manner in addition to not having to know the sequence DNA template used, and using the RAPD marker, damage to DNA can be broadly assessed starting from point mutations to large changes (large rearrangements) [18].

The RAPD marker was used in this study to assess the genetic effects of the T. rubrum genome as one of the most common medical fungi in fungal infections after laser exposure to highlight the use of laser in the treatment of medical fungi, especially that many of these fungi show great resistance to fungal treatments, which causes the continuation of infection. The results of this study canfirmed that the exposure of the fungus to the laser has led to different events of the genetic material indicated by differences in the pattern of RAPD marker of the samples of laser-treated fungi compared to samples of control non-treatment where bands that existed and new bands were missing. The disappearance of one of the bands appearing in the pattern of the RAPD due to exposure to the laser can result in DNA damage Such as fracture of the single or double-stranded DNA chain or changes in the nucleotide sites complementing the initiatory sequences that may be due to rearrangement or by mutation and chromosomal reorganization (chromosomal rearrangements) [19].

The effect of the fungus on laser radiation can be in this case in the first two forms led to the absence of the band at direct exposure and emergence after a generation of the same sample was caused by the effect of the protein found within the DNA and thus the inability of the two chains to circumvent the protein and then show the band (when exposed directly), The second form of effect was a mutation in the fungus and evidence that the bands did not appear after a generation of the treated sample the reason for this is either a change in the nucleotide sequence due to the effect of laser beams or because of the transposons within the DNA that are vector agents that can influence nearby genes thus creating a mutation and absence of the band at the first generation [20,21,22], the disappearance of sites from laser-exposed samples can result in damage to DNA due to radiation. This damage involves the breaking of the single or double DNA, changing the location of the initiator link, the oxidation of the nitrogen bases, the influence of the proteins within the chromosomes and point mutations[23].

Laser is a physical episodic factor that separates or dissociates water molecules and produces hydroxyl radicals that cause oxidation cracking (oxidative damage) [16], Free liberated radicals interact with biomolecules including DNA and remove electrons, This breaks down the structure of DNA. During polymerase chain reaction (PCR) when the polymerase enzyme (Taq polymerase) is met with DNA crusher this will close the link sites, which means that the enzyme can not bind which means loss of sites existed before exposure to radiation. The other effect of radiation is that it affects the homeostasis of calcium ion, Cell regulation, programmed apoptosis and DNA synthesis[24,25].

The emergence of new bands can be due to the presence or appearance of new link bands have become suitable for the association of primers after exposure to stimuli such as chemicals and physical factors or because of the deletion of a region of DNA[19], The appearance of new bands may result

in a change in complementary sites due to mutations (new collisions, large deletions, homologous recombinations). Genetic influence or changes, such as mutation, do not occur only because of a change in the sequence of nucleotides, since chromosome and transposon proteins in DNA can also be inherited [26].

The physical principle of laser is the mutual effect between light and matter and that the energy produced by this effect can be precisely guided, which makes use with biological materials causes tissue changes can be used in the treatment methods, one of the most important principles to be considered when using laser is to know the coefficient of absorption and spread of laser and the intensity of radiation on the living material and the duration of exposure to laser and addition to the size of the irradiated area, the most important of which is to determine the type of laser is it pulse or continuous with the wavelength determination of the type used[27].

Several studies have examined the effect of lasers on living matter. These studies show that the dynamics of the biological processes of a living cell are affected by the exposure of an organism such as bacteria, fungi and algae to laser light at a specific wavelength, the energy emitted by the laser light can affect the electrochemicality of cellular membranes as it can affect the movement of protons in mitochondria and produce morphological changes of cells and organism, The laser can also break the double band of DNA. The organism can repair the affected DNA using several mechanics, including re-elongation (excision repair) and photoreactivation [28], Low-energy laser therapy is a sophisticated medical technique where exposure to laser light may prevent a cellular function as this technique is known as laser therapy[29].

Conclusions

The study suggest that the use of laser can affect the DNA of the fungus, including T. rubrum and may lead to mutations and the effect of the T. rubrum genome after exposure to laser directly and after a generation indicates the efficiency of the laser species used to affect fungi, Which means the possibility of using laser in the treatment of skin fungal infections, On the other hand, the RAPD proved that it was limited in detecting laser effect at the molecular level as a simple, fast and inexpensive technique.

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تقييم التأثيرات الجزيئية لليزر Nd: YAG وليزر Diode على فطر Trichophyton Rubrum بإستخدام مؤشر ال RAPD

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الملخص

اجريت هذه الدراسة لتقييم التأثيرات المظهرية والجزيئية لليزر النديميوم- ياك والدايود (شبه الموصل) على فطر Trichophyton Rubrum باستخدام مؤشر التضاعف العشوائي المتعدد الأشكال لسلسلة الدنا ال (RAPD). جمعت ستين عينة كقشطات جلدية، قصاصات الأظافر واجزاء من الشعر من المرضى المصابين من كلا الجنسين (الذكور 34 والأناث 26) للفئة العمرية (1 – 60) سنة من المرضى المراجعين لمستشفى تكريت التعليمي. زرعت العينات وتم تنميتها على الاوساط الزرعية ثم شخصت الاجناس والانواع الفطرية, تم شعع الفطر T.rubrum كونه الاكثر انتشاراً بين الفطريات الجلدية باستخدام ليزرين هما ليزر النديميوم- ياك بطول موجي (530)nm وطاقة (700 , 500 , 300) ملي جول ولزمنين تعريض 20 و 30 ثانية لكل بواقع ست معاملات وعينة سيطرة، وليزر الدايود ذو القدرة الواطئة (5) ملي واط لثلاثة أزمنة 10 و 20 و30 ثانية على مسافة 20 سنتيمتر بواقع ثلاث معاملات وعينة سيطرة, استخلص الدنا من الفطريات بعد التعرض المباشر وبعد تركها لتنمو لمدة جيل كامل ثم استخدم لانجاز تفاعلات مؤشر ال (RAPD). اعطى مؤشر ال RAPD نتائج ممتازة مع البادئات الخمسة المستخدمة وتبين ان تعرض فطر T.rubrum لليزر النديميوم- ياك والدايود بطاقات وازمنة مختلفة قد اثر على (DNA) وسبب تغيرات كبيرة في انماط مؤشر ال RAPD بالمقارنة مع مجموعة السيطرة من ناحية ظهور حزم جديدة واختفاء حزم كانت موجودة وان الطاقة المؤثرة على الفطر T.rubrum لليزر الأول النديميوم- ياك هي 500 mj عند الزمن 30 sec والطاقة المؤثرة لليزر Diode كانت 5 mw عند الزمن 20 sec ، كما تبين ان ليزر الدايود ذو فعالية عالية وتأثير كبير على المادة الوراثية للفطر بالمقارنة مع تأثير الليزر الاول. يمكن ان نستنتج ان استعمال الليزر يمكن ان يؤثر على ال (DNA) للفطريات الجلدية وقد يؤدي الى حصول طفرات مما يعني امكانية استخدامه في علاج الاصابات الفطرية الجلدية كما ان مؤشر ال RAPD كان كفوء في الكشف عن تأثير الليزر على المستوى الجزيئي باعتبارها تقنية بسيطة, سريعة وغير مكلفة.