Sudan University of Science and Technology
College of Graduate Studies

Immunohistochemical Detection of CD20 Among Sudanese Mycetoma Patients

الكشف النسيجي الكيميائي المناعي لسي دي 20 لدى مرضى المايسيتوما السودانين

Adissertation submitted for partial fullfillment of the requirement of the M.Sc degree in medical laboratary sciences (histopathology and cytology**)**

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بسم الله الرحمن الرحيم

قال الله تعالى:

(وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا)

صدق الله العظيم

الاسراء – اية 85

Dedication

I dedicate this work to

Who gave me the meaning of the life My parents….

My sweet sisters and brothers….

My colleagues, my teachers and my friends…

Every one whom I respect and appreciate….

Acknowledgement

Firstly I am grateful to Allah for give me the knowledge, strength, patience to complete this work.

I am grateful to my Supervisor Dr. Mohammed Siddig Abdelaziz, for his perfect supervision, advice, encouragement and support from the early stage of this research as well as giving me opportunities of experiences throughout the project.

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A lot of thanks extended to Nada Salih for her help and support….

Abstract

This is a retrospective descriptive study was conducted in Soba university hospital during the period from January to December 2016. The study was aimed to detect the expression of CD20 in mycetoma.

A total of 30 fixed paraffin blocks of patients samples previously diagnosed as tissue affected with mycetoma were collected, 7 (23.7%) samples were actinomycetoma and 23 (76.3%) samples were euomycetoma. One section of 3 microns was cut from each block and stained by immunohistochemical method(avidin biotin technique) for CD20 detection. The obtained data was analyzed using SPSS computer program version 11. 5. frequancies, percent, mean and chi square were calculated.

The patients ages ranged between 12-87 years with mean age of 49 years, most of them 27(90%) were under 50 years and the remaining 3(10%)were above 50 years.

Regarding patient sex, the study revealed that 20(66.7%) patients were male and 10(33.3%) patients were female.

CD20 revealed positive result in 12(40%)samples, while 18(60%)were negative. 10(33. 3%) of euomycetoma cases were positive for CD20, while 13(43.3%)were negative for CD20. In actinomycetoma two cases (6.7%)were positive for CD20, while 5(16.7%) cases were negative for CD20, with insignificant relation between CD20 expression and mycetom (p.value 0.498). The study concluded that there is no relation between CD20 expression and causative agents.

المستخلص

اجريت هذه الدراسة الوصفية الاسترجاعية في مستشفى سوبا الجامعي خلال الفترة من يناير الي ديسمبر 2016. هدفت الدراسة لتحديد افراز سي دي 20 في المايسيتوما.

جمع ثلاثون قالب شمعي لهذه الدراسة. تم تشخيصها مسبقا كعينات مايستوما, منها 7(23.7%) عينة مايستوما بكتيرية و23(76.3)مايستوما فطرية, قطع من كل قالب مقطع بسمك 3 مايكرون وصبغت بطريقة الكشف النسيجي الكيمائي المناعي باستخدام تقنية الافيدين بايوتين للكشف عن CD20. وحللت البيانات التي تم الحصول عليها باستخدام برنامج الحزم الاحصائية للعلوم الاجتماعية اصدار11,5. تم حساب التردد والنسب المئويةوالمتوسط ومربع كاي.

تراوحت اعمار المرضى بين 12–87 سنة بمتوسط عمر 49 سنة, معظم اعمار المرضى كانت تحت 50عاما 27(90%) وكانت اعمار البقية 3 (10%) اكبر من 50عاما اما توزيع جنس المرضى فكان 20(66,7%) ذكور و10(33.3%) اناث.

ظهر التعبير الايجابي لافراز سي دي 20 وسط مجتمع الدراسة في12 (40%) عينة ونتيجة سلبية في18(60%) عينة. من مجموع 12عينة والتي اعطت نتيجة ايجابية كانت منها10عينات (33.3%) مايستوما فطرية وعينتان (6.7%) مايستوما بكترية. ومن مجموع 18عينة اعطت نتيجة سلبية كانت 13عينة(43.3%) مايستوما بكتيرية و5عينات (16.7%) مايستوما فطرية. مع عدم وجود علاقة بين ظهور السي دي 20 والعامل المسبب (القيمة الاحتمالية 0.498).

خلصت الدراسة الى عدم وجود علاقة بين ظهور السي دي 20 والعامل المسبب.

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List of abbreviation:

CD	Cluster of Differentiation
DPX	Distyrene Plasticizer Xylene
DAB	3, 3-Diaminobenzidene tetrahydrochloride
ELISA	Enzyme Linked Immune Sorbant Assay
ID	Immune Diffusion
SPSS	Ststistical Package for Social Siences
CIE	Counter Immune Electrophoriesis
MS4A gene	Membrane Spaning 4A gene
DLBCL	Diffuse Large B cell Lymphoma
ALCL	Anaplastic Large Cell Lymphoma
LFT	Liver Function Test
FNA	Fine Needle Aspiration

Chapter One

Introduction

Chapter One

1. 1 Introduction

Mycetoma is chronic, progressively destructive morbid inflammatory disease usually of the foot but any part of the body can be infected. infection is most probably acquired by traumatic inoculation of certain fungi or bacteria into subcutaneous tissue (Fahal,*et al*.1992).

Mycetoma was described in the modern literature in 1694 but was first reported in the mid-19th century in the indian town of madura. It commonly affects young adults, particually males, mostly in developing countries. People of low socioeconomic status and manual workers such as agriculture, labourers and herdsmen are the most affected (Rippon ,1988).

Early detection and treatment are important to reduce morbidity and improve treatment outcome. Distribution of disease is worldwide but are endemic in tropical and subtropical areas which includes the Bolivaria, Chad, Sudan , India, Mexico, Somalia and Yemen (Magana ,1984).

Transmission occurs when causative organism enters the body pricks. There is clear relationship between mycetoma and individuals who walk barefooted and the manual workers (Borion,*et al*.1998).

Disease is characterized by painless subcutanous mass, multiple sinuses and discharge containing grains. It usually spreads to involve the skin, deep structure and bone resulting in destruction, deformity and loss of function which may be fatal. Secondary bacterial infection is common (Borion,1998).

Diagnosis through tissue biopsy as well the lesion sinuses discharge, also through culture and imaging technique (Fahal,1997).

Treatment depends on the causative agent (bacterial or fungal) surgery is also used. Prevention is difficult, but people living or travelling to endemic areas

should be advised not to walk barefoot. Treatment may continue from few to many years(Mahgoub and Gumaa, 1984).

CD20 is CD marker-a molecule on the cell surface that can be used to identify and type particular cell in the body. CD20 is found on the surface of b cells. CD20 is not expressed on haemopoietic stem cells . Mainly it used to diagnose lymphoma, melanoma, multiple myeloma (Hardy, 2008).

There is three types of tissue reaction for mycetoma. In the third type of reaction (Type III), the grain material has largely or completely disappeared leaving a compact epithelioid granuloma with or without langerhans giant cells. This type is stained positive for CD20 antigen. however, is an uncommon reaction but represents spontaneous regression in some grains (ElHassan,*et a*l.2001).

1.2 Objectives:-

1. 2. 1 General objective:

To detect the expression of CD20 in mycetoma using immmunohisto-chemistry.

1. 2. 2 Specific objective:

To compare the expression of CD20 in euomycetoma and actinomycetoma.

Chapter Two

Literature Review

Chapter Two

Literature Review

2.1.Scientific background

Mycetoma is achronic granulomatous disease, infection caused by either true fungi or actinomycetes. Fungal mycetoma is known as euomycetoma, while the mycetoma caused by actinomycetes was known as actinomycetoma. The mass of infection and multiple sinuses draining pus, blood and grains characterize the mycetoma lesion. Grains are aggregate of fungal hyphae or bacterial filament sometimes embeded in hard cement like material (Boiron ,*et al*.1998).

mycetoma infection start subcutanously after traumatic inoculation of microorganism which are live as saprophyte in soil and other natural ecological niches. The infection progresses slowly over along period of time without painful manifestation. The mycetoma lesion may ultimately extend to the deep tissues and bones leading to deformity of the affected site and disability. Mycetoma infection is not self curing if untreated, always lead to massive lesion, which at the end leading to surgical amputation (Fahal, 2002).

The first case of mycetoma was reported by Dr. John gills as madura foot of the Madras Medical service of the british Army in India1842 (Rippon, 1988).

The instence of the disease was confirmed by colebrook more than acentury later in 1946. The term mycetoma was first used by vandyke carter in 1860 (Rippon,1988).

Mycetoma has an extermely uneven worldwide distribution, which is endemic in tropical and subtropical countries including Sudan, Somalia, Senegal, India, Yemen and Mexico. In addition mycetoma has been reported in many temperature regions as well as in Africa. Areas where mycetoma prevails are relatively arid with ashort rainy season of 4-6month. Rainfall was 50-1000mm per year, with relatively humidity of 60-80% and fairly constant temperature

of 30c to 37c for 24 hours aday,this was followed by dry season of 6-8 months with humidity (Mahgoub and Murry, 1973).

Geographical distribution of mycetoma causative agents show considerable variation, which could be explanid on these and other enviromental factors, special rainfall (Mariat, 1963).

Many microorganism are capableof causing mycetoma, the most common actinomycete is *streptomyce*, *nocardia*, and *actinomadura*. most of euomycetoma in africa are caused by *madurella mycetomatis* (McGinnis, 1996).

All people living in the endemic area can be infected, but the most commonly infected were the farmers and other field workers who have direct contact with the open enviroment. Males are five times more often affecting by mycetoma than females, even in areas where both sexes due to work out door (Fahal and Suleiman, 1994).

Mycetoma is not considered to be transmissible from person to person or from animal to person. There are some conflicting reports about the role of the immune status of susceptible population. Some investigators reported partial impairment of the cell-mediated immune response in patient severely infected or not responds to medical treatment (Mahgoub,*et al.*1977).

Mycetoma initally present as slow progressive and painless subcutaneous swell combination with history of preceding trauma(Boiron,*et al.*1998).

Symptoms may required several months two years to develop. Investigations are complicated by the fact that most of the patient tend to present late, due to lack of health education, lack of clinical symptoms and fear from amputation (Boiron,*et al*.1998).

Theduration of the disease, the type of causative agent, site of the infection and possible host immune response, all affecting the clinical presentation of mycetoma (ElHassan,*et al.*1994).

Mycetoma can affect any site of the body, not only the feet or the lower limbs. however the majority of cases are usually seen in the feet followed by the hands and knee joints. In high endemic areas other parts of the body may be involved such as arm, head, neck and other parts of the body (Boiron,*et al.*1998).

The clinical trail of subcutaneous mass, sinus and discharge containing grain observed in patients from endemic areas is diagnostic for mycetoma. it is important to identify the causative agent in order to develop accorect plan of treatment.

Direct examination of grain may be useful in determining the type of mycetoma unlike fungal grains, crushed bacterial grain show fine filament. isolation of mycetoma agents can be difficult special in case of actinomycetoma (ElHassan,*et al*.1994).

2.2 Mycetoma causative agent:

2.2.1. M. mycotomatis:

It is the most common cause of eumycetoma in the Sudan. In clinical material, the grains in the tissue are black and numerous. In stained sections the grain is rounded, oval or trilobed. It has a more compact cortex, which is dark brown in colour due to pigment produced by the organism and has a lighter medulla. In some grains the division into cortex and medulla is not evident. The grain filaments are usually embedded in a hard brown cement matrix (ElHassan,*et al*. 1994).

Two main morphological types of grains are identified. The filamentous, which is the commonest type, consists of brown septate and branched hyphae that may be slightly more swollen towards the periphery. In the cortex, the filaments are arranged radially while in the medulla they tend to run multi-directionally. Round or oval cells, 7-15 um in diameter, are seen, particularly in the periphery. The second type of grain is the vesicular one. It is less common than the filamentous and is composed of unusually large cells that look like vesicles. Both types of the grain can be found in the same lesion. Grains that

are partially vesicular and partially filamentous are not uncommonly seen (ElHassan,*et al*. 1994).

. There are three types of tissue reaction. In type I, there is a zone of neutrophils in the vicinity of the grain which stained positive for CD15. These are sometimes found within the grain substance causing its disintegration. Some histocytes may also be seen among the neutrophils but they are more numerous outside the neutrophil zone. Some of the histocytes have a foamy cytoplasm and give a positive reaction for fat and stain positive for CD 68 and CD3 antigens. In type II reaction, the neutrophils largely disappear and are replaced by histocytes and multinucleated giant cells. Some of the latter contain fragments of grain or pigmented cement substance without any hyphae. The macrophages may contain a black pigment derived from the grain. At this stage the grain itself is usually small and fragmented. This type of histocytes/giant cell reaction follows on the earlier neutrophil response, which causes fragmentation of the grain(ElHassan,*et al*.2001).

In the third type of reaction (Type III), the grain material has largely or completely disappeared leaving a compact epithelioid granuloma with or without langerhans giant cells. This type is stained positive for CD20 antigen. however, is an uncommon reaction but represents spontaneous regression in some grains (ElHassan,*et a*l.2001).

The three types of tissue reaction may be found in the same lesion. Viable grains are nearly always present in biopsy material. It is not known if spontaneous regression of all the grain ever occurs in mycetoma. The unique feature of M. mycetomatis is the formation of a capsule around the lesion. The lesion grows by expansile growth in the tissue plains. In the bones there is usually no capsule formation, the organism usually forming cavities that are filled with the grains. This gives the bone support and may explain the rarity of pathological fractures in mycetoma (ElHassan,*et al*. 1994).

. Ultrastructural studies of the host reaction show neutrophils adherent to the grain). The cytoplasm of the neutrophil is stretched over the grain and the neutrophils granules are concentrated in the part of the cytoplasm adjacent to the grain. This is an immune adherence, which is mediated by immunoglobulins and is an example of antibody dependent cell mediated cytotoxicity. Immunoglobulin and complement can be demonstrated in the grain (ElHassan,*et al.* 1994).

. Bones are frequently involved in advanced mycetoma of the soft tissue. Occasionally primary bone mycetoma in the absence of soft tissue involvement is seen. M. mycetomatis produces lytic lesions which arelarge in size, few in number and have well defined margins; this is well seen radiologically (ElHassan,*et al.* 1994).

2.2.2. Streptomyces somaliensis:

The grains are yellow in colour and hard in consistency. During surgery it may be difficult to distinguish the grains from fat which makes radical excision of the lesion difficult. This is especially so since the lesion is not encapsulated. In sections the grain is rounded to oval, dense and homogenous. Characteristically marks of the microtome knife are seen in the grain in the form of parallel cracks. The grain stains a light purple or pink colour in haematoxylin and eosin stained sections. The grain varies in size from 30 to 200 um. Hyphal elements embedded in cement can be visualized by gram stain (Fahal,*et al.* 1994).

The grain is surrounded by an intense neutrophil polymorphonuclear leucocyte infiltrate (Type 1). Outside this zone, there is a vascular layer containing macrophage, lymphocytes, plasma cells and giant cells. The giant cells usually contain fragments of the grain. Some macrophages have a foamy cytoplasm. It looks as though the fragmentation of the grain induced by neutrophils is less severe than in M. mycetomatis. This may be due to the more compact and hard grains of S. somaliensis. Small grains surrounded by macrophages and giant cells (Type 11) are occasionally seen but pure epithelioid granuloma (Type

111) apparently does not occur. Giant cells containing viable actinomycetes are believed to aid the spread of the organism in the tissue and to the regional lymph nodes. Despite the invasive nature of S. somaliensis and other actinomycetes, tendons and nerves are resistant to invasion(Fahal,*et al*.1994).

. Ultrastrcturally the grain consists of heterogenous and amorphous matrix arranged in an irregular and reticulate structure surrounding electron lucent areas between 1 and 5 um. In some of these spaces bacterial filament are found. The organisms are usually unicellular and coccoid and the cell wall is electron dense (Fahal,*et al*.1994).

2.2.3. Actinomadura pelletierii:

The grains in clinical material are tiny and red in colour. In section the grain is rounded, oval or semilunar. It stains a purple colour and compact hyphae give it the appearance of "Iron filings". The periphery of the grain has a narrow deeply eosinophilic band. The grain is usually surrounded by a zone of neutrophils which causes fragmentation of the grain. The other layers are similar to those seen in S. somaliensis but the giant cells are less conspicuous. The ultrastructure of the grain is quite distinctive. The hyphae are septate, compact without cement substance and under low magnification the hyphae have a starry sky appearance because of the vacuoles in the hyphae. These are probably fixation artifacts. Neutrophils usually adhere to the grain and degranulate. Grain material is phagocytosed by the neutrophils and destroyed (Mahgoub and Murry ,1973).

2.2.4. Actinomadura madurae:

Macroscopically the grains are yellow or white. They are difficult to distinguish from the surrounding fat. Histologically the large grains have a characteristic variegated pattern. The periphery of the grain is dense, homogenous and deep purple while the centre is less dense or even appears hollow. Not infrequently the grain fragments into geometric fragments. The periphery shows a brightly

eosinophilic material forming clubs. This, material contains immunoglobulins. Smaller grains are more homogeneous and are difficult to distinguish from *A. pelletierii*.

However, even the small grains of A. madurae have a more deeply stained purple fringe, which is not seen in A. pelletierii. The inflammatory reaction is similar to that of *A. pelletierii* (Fahal,*et al.*1995).

2.3.1.Pathological changes in mycetoma:

The arteries and veins in the mycetoma lesion show hypertrophy of the muscles. The lumen is narrowed but is not occluded completely. Grain fragments are occasionally seen within the blood vessels. These may explain the rare haematogenous spread to distant sites (Fahal,1996).

2.3.2. Bony changes in mycetoma:

Bones are frequently involved in advanced mycetoma of the soft tissue. Occasionally primary bone mycetoma in the absence of soft tissue involvement is seen. M. mycetomatis produces lytic lesions which arelarge in size, few in number and have well defined margins.

Actinomycetoma destroys bone and also evokes new bone formation. The cavities produced are usually smaller in size, numerous and have no definite margins (ElHassan,*et al.* 1994).

2.3.3. Lymph nodes in mycetoma:

Lymph nodes draining a mycetoma focus are frequently enlarged. Most of the node shows only reactive hyperplasia with an intense plasma cell infiltration. The plasma cells often contain Russell bodies. These changes may be due to antigens reaching the node from grains at the primary site (ElHassan,*et al.* 1994).

2.4.Diagnosis of mycetoma:

2.4.1.Radiology:

A series of radiological changes are seen in mycetoma. This is due to the fact that all mycetoma agents are osteophilic and it may be due to the effect of the granuloma on both the affected bone and its blood supply. In the early stage, there is a soft tissue granuloma. Calcification and obliteration of the fascial planes may sometimes be seen .Late in the disease, there may be multiple punched out cavities through the normal density of the bone. These cavities are large in size, few in number with well-defined margins in eumycetoma. Whereas, the bone cavities in actinomycetoma are usually smaller in size, numerous and have no definite margins (Fahal, 1992).

2.4.2.Ultrasonic imaging of mycetoma:

The mycetoma grains, its capsule and the accompanying inflammatory granuloma have characteristic ultrasonic appearances.In eumycetoma lesions, the grains produce numerous sharp bright hyperreflective echoes, which are consistent with the black grains. In actinomycetoma lesion the findings are similar but the grains are less distinct. The ultrasonic diagnosis of mycetoma is more precise and accurate in lesions with no sinuses. The size and extent of the lesion can be accurately determined ultrasonically and this is useful in planning surgical incisions and procedures (Fahal,1997).

2.4.3.Fine needle aspiration cytology of mycetoma:

Mycetoma can be accurately diagnosed by Fine Needle Aspiration (FNA) cytology. Mycetoma lesion has a distinct appearance in a cytology smear characterised by the presence of polymorphous inflammatory cells consisting of an admixture of neutrophils, lymphocytes, plasma cells, histiocytes, macrophages and foreign body giant cells and grains. In sections, the grain is closely surrounded by and occasionally infiltrated by neutrophils causing its fragmentation. Outside the neutrophil zone, monocytic cells and giant cells are

seen. This is surrounded by granulation tissue rich in fibroblasts and blood vessels. FNA allows morphological identification of mycetoma and its classification into eumycetoma and actinomycetoma, this is important as the treatment depends mainly on the aetiological agents (Fahal, 1996).

The technique is simple, cheap, rapid, sensitive and can be tolerated by patients. It can be used not only in routine diagnosis but can be used as an effective mean in collection of material for culture and immunological studies. Due to the simplicity of the technique it can be used in epidemiological survey of mycetoma and for detection of early cases in which the radiological and serological techniques may not be helpful (Fahal, 1996)

.2.4.4. Cytopathology of mycetoma

Mycetoma has distinct cytological features characterized by the presence of polymorphous inflammatory cells consisting of neutrophils, lymphocytes, plasma cells, histocytes and foreign body giant cells.To distinguish between actinomycetoma and eumycetoma, fine needle aspiration cytology was found to be as accurate as histopathology when grains are present. The technique is simple, quick and cheap. It can be introduced for routine diagnosis of mycetoma and for epidemiological surveys (Fahal,*et al.* 1997).

2.4.5.Culture:

A large variety of microorganisms are capable of producing mycetoma. They can be identified by their textural description, morphological and biological activities in pure culture. The biological activity may include,acid fastness, optimal temperature, proteolytic activity, utilization of sugars and nitrogenous compounds . The grains are the source of the culture and they should be alive and free of contaminants. Many culture media are in use e. g. Sabouraud, blood agar and malt extract agar. The culture technique is cumbersome, time consuming and chance contamination may give a false positive result. It also requires experience to identify the causative organisms (Mahgoub, 1985). .

2.4.6.The histological technique:

Stained sections usually show the grain morphology and the tissue reaction to the organisms. The technique is attractive in that it requires neither aseptic procedure nor the rigid time schedule required for culture, however it lacks the precision of culture (El Hassan *et al* 1994).

2.4.7.Serology:

In the absence of the classical triad of mycetoma, the demonstration of significant antibodies titers against the causative organism may be ofdiagnostic value. The common serodiagnostic tests for mycetoma are the immunodiffusion (ID) and counter-immuno-electrophoresis (CIE) (Mahgoub and Gumma 1975). The (ID) test is not sensitive,it can be negative in early cases. (CIE) test remains reliable, simple, economical, rapid and a sensitive test. Recently the indirect fluorescent antibody test was used in diagnosing mycetoma and was found to be specific and sensitive (Joshi and Sighvi 1988). Enzyme linked immunosorbent assay (ELISA) was also used in the diagnosis of mycetoma. ELISA may be a useful tool in community studies as sero-epidemiological survey could give valuable information on the distribution and prevalence of exposure to mycetoma (Taha 1983).

2.5.CD20:

It is also called MS4A gene. This gene encodes a member of the membrane-spanning 4A gene family. Members of this nascent protein family are characterized by common structural features and similar intron/exon splice boundaries and display unique expression patterns among hematopoietic cells and non lymphoid tissues. This gene encodes a B-lymphocyte surface molecule which plays a role in the development and differentiation of B-cells into plasma cells. It use in diagnosis of chronic lymphocytic leuckemia, B cell leuockemia, non Hodgkin lymphoma, melanoma, mycetoma and multiple myeloma. Also it use to differentiate between diffuse large B cell lymphoma (DLBCL) which be

positive and anaplastic large cell lymphoma(ALCL) which be negative.Also used in treatment of mantle cell lymphoma (Tedder,1994)

2.6.Treatment of Mycetoma:-

The mycetoma treatment needs a long period of time. Eumycetoma treated by anti fungal agents such as ketonasol (ketonase 200 mgwith folic acid) or Nesoral. Actinomycetoma was treated with antibacterials example strepmycine although in some patients are resistant. The newly discovered (Amikacine) is the best for treatment Amikacine injection 4 cycle. During this period the patient is check liver function test (LFT). Surgery is also used (Mahgoub and Gumaa,1984).

2.7.Prevention and control:-

Farmer and other people who work in farms should wear suitable shoes, if any person suffering from thorn prick inshore the thorn take out and clean this site. In condition of any mass or lesion work to the near clinic to observed and follow up (Fahal and Suleiman,1994).

Chapter Three

Materials and Methods

Chapter Three

Materials and Methods

3.1.Study design:

This is a reterospective descriptive study aimed to study the detection of CD20 in mycetoma among Sudanese patients.

3.2.Materials:

Formalin fixed, paraffin wax embedded tissue blocks were selected from tissues of patients sample previously diagnosed with mycetoma were used in this study.

3.3.Study population:

Thirty blocks were randomly selected for this study from patients their samples were reffered to the department of mycetoma center in Soba university hospital (January 2016-December 2016). A clinical data were obtained from each patient's file and recorded in request form.

3.4.Sample collection:

Sections of 3 microns in the thickness were cut from formalin fixed, paraffin wax embedded tissue blocks using rotatory microtome and dewaxed in ahot plate oven.

3.5.Immunohistochemical staining:

Sections required for immunohistochemistry were retrieved in water bath for 20 minutes at 97c, then treated in buffer for 5 minutes then treated by endogenous enzyme blocker for 5 minutes, then treated in buffer solution for 5 minutes, followed by primary antibody for 20 minutes and washed in two changes of buffer solution (5min for each),then biotinlyated secondary antibody, then treated by substrate chromogen for 10 minutes and rinsed in buffer solution then tap water. Lastly counter stained in Mayer's haematoxylin. Then dehydration, clearing, mounting and examination under light microscope for results interpretation.

3.6.Result interpretation:

All quality control measure were adopted during sample collection and processing for the assessment of immunohistochemical results. Positive staining for CD20 appeared as cytoplasmic brown colour in reaction reagion.

3.7.Satitical analysis:

The data was analyzed using SPSS computer program. Frequancies, mean, percent and chi squre were calculated.

3.8.Ethical consideration:

Samples were collected after taking ethical approval from Soba university hospital to use the tissues blocks for research purposal.

Chapter Four

Results

Chapter Four
Results

Results

Thirty samples were collected from Soba University hospital, the patients ages ranged between 12-87 years with mean age 49 years, most of them27 (90%) were less than 50 years and the remaining 3(10%) were more than 50 years as shown in table 4. 1.

The patients sex revealed that 10(33.3%) patients were female and 20(66.7%) patients were male as shown in table 4. 2.

The study involved 30 blocks, previously diagnosed as mycetoma, 7 (23. 3%) samples were actinomycetoma and 23 (76.7%) samples were euomycetoma as shown in table 4. 3. The results of CD20 were positive in 12(40%), while 18(60%) of cases were negative as shown in table 4. 4. 10(33.3%) of euomycetoma cases were positive for CD20, while 13(43.3%) were negative for CD20. In actinomycetoma cases 2(6.7%) were positive for CD20, while 5(16.7%)were negative for CD20 with in signifcant correlation between CD20 and mycetoma(P value 0. 498) as shown in table 4. 5.

Table (4.1):Distribution of age groups among study population:

Age groups (years)	Frequency	Percent
50 ≥	27	90%
50<	3	10%
Total	30	100%

Table(4.2): Distribution of sex among study population:

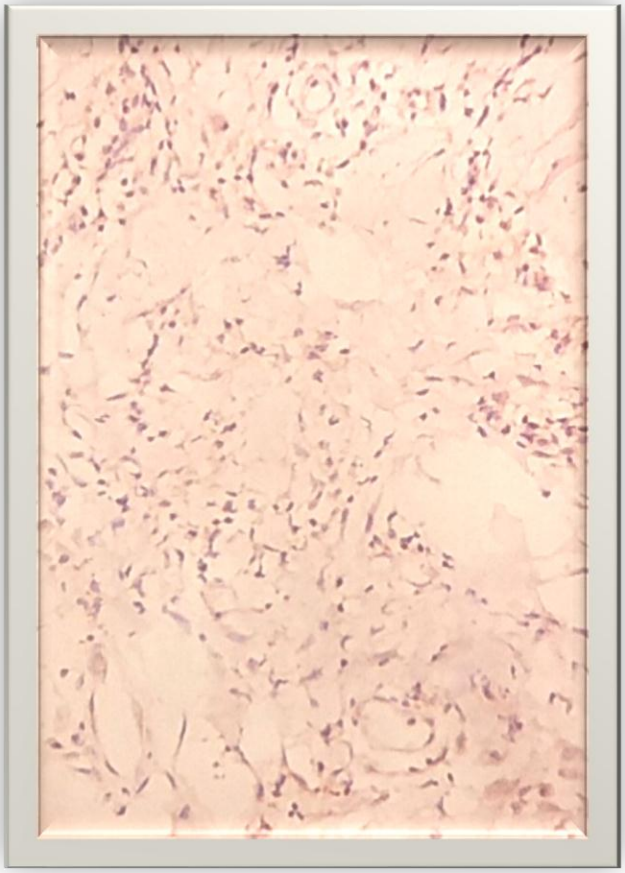
Sex	Frequency	Percent
Male	20	66. 7%
Female	10	33. 3%
Total	30	100%

Table(4.3) :Expression of CD20 among study population:

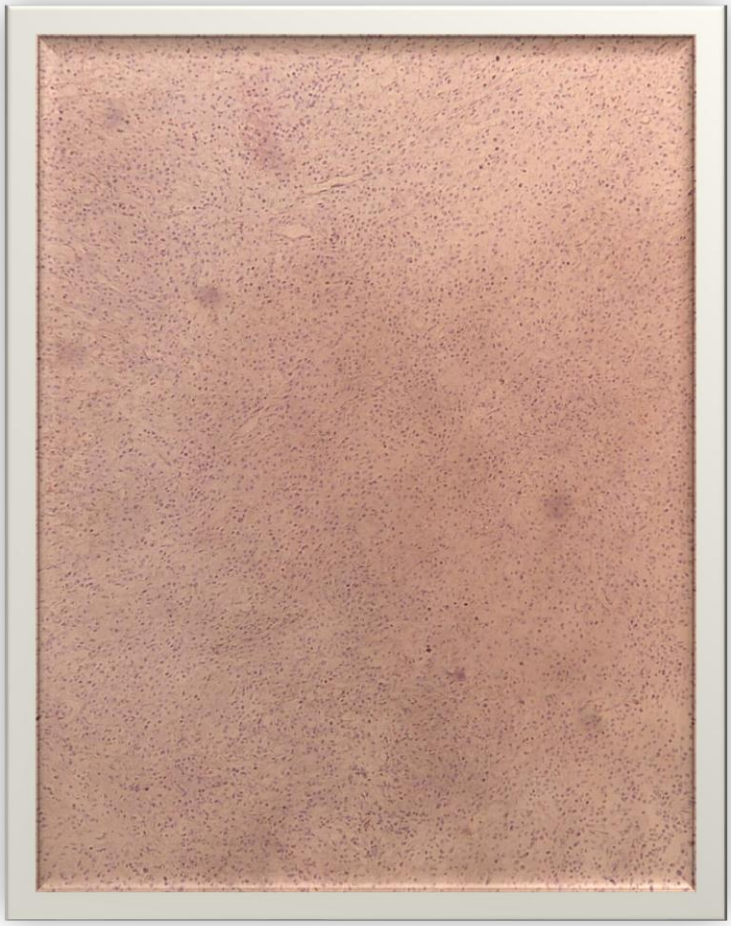
CD20 Expression	Frequency	Percent
Positive	12	40%
Negative	18	60%
Total	30	100%

Table(4.4): Relation between CD20 expression and mycetoma types :

Mycetoma types	Expression of CD20 marker		Total	P. value
	Positive	Negative		
Euomycetoma	10(33. 3%)	13(43. 3%)	23(76. 6%)	0. 498
Action mycetoma	2(6. 7%)	5(16. 7%)	7(23. 4%)	
Total	12(40%)	18(60%)	30(100%)	



Photograph(4.1) Section show positive expression of CD20 in mycetoma.



Photograph (4.2) Section show negative expression of CD20 in mycetoma.

Chapter Five

Discussion

Chapter Five

Discussion

5. Discussion:

The study involved 30 blocks, previously diagnosed as mycetoma were used in this study. Regarding the age of the study population the study revealed that most of patients were less than 50 years. this result compatible with Bonifaz, *et al*(2014), they reported that the number of patients was increased in agriculture or rural patients from 20-40 years more than other groups of age.Also this result is agreed with Fahal,*et al.* (2015) who reported that 64% of mycetoma cases under 30 years.

The study revealed that most of patients were men, with male:female ratio2:1, differences in nature of work might explain the lower female:male ratio. This result agreed with Bonifaz, *et al.*(2014), who reported that mycetoma incidence and mortality with sex ratio 3:1 higher in male than in female. Also Fahal ,*et al*.(2015) reported that 76% of mycetoma cases were detected in male . In this study most cases of mycetoma infection were euomycetoma, this result disagree with Fahal,*et al*.(2015), who reported that most of cases is actinomycetoma. The expression of CD20 in mycetoma were positive in 10(33.3%) in euomycetoma cases, while in actinomycetoma 2 (6.7%) cases were positive with in significant correlation between CD20 and mycetoma (P value 0. 498). this result disagree with Elhassan, *et al.(*2001).

Chapter Six

Conclusion and Recommendations

Chapter Six

Conclusion and Recommendations

6.1.Conclusion:

On basis of this study we conclude the following:

The age of the mycetoma patients in our study is commonly less than 50 years. The sex of the mycetoma patients in our study is commonly male. There is no association between CD20 expression and mycetoma infection.

6.2.Recommendations:

On basis of this study we recommended the follow:

Further studies should be done with large sample size should be done. Anthor adhesion molecules associated with mycetoma should be applied .

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Appendices

Appendices:

Appendices(1):

Instrument and material:

Instrument:

Gloves.

Rotary microtome.

Microtome knife.

Coplin jars.

Oven.

Staining racks.

Water path.

Coated slides.

Dako pen.

Humidity chamber.

Cover glass.

Materials:

Ethyl alcohol(absolute, 90%, 70%, 50%).

Xylene.

Distell water.

Mayer's heamatoxylin.

Peroxidase blocker.

primary antibody(CD20).

Secondary antibody(biotinylated secondary antibody).

3, 3 di amino benzidine tetra hydrochoride in substrate buffer.

DPX mounting media.

Phosphate (PH 7. 4) component:

Solution A (0. 2 M sodium di hydrogen orthophosphate, 3. 12g disodium hydrogen orthophophate, 100 ml DW).

Solution B (2. 1g citric acid, 100 ml DW)(72. 7mlfrom solution A+22. 8 ml from solution B).

Citrate buffer(PH 6. 8)component:

Solution A (0. 2 M sodium di hydrogen orthophosphate, 2. 83 g disodium hydrogen orthophophate, 100 ml DW).

Solution B (2. 1g citric acid, 100 ml DW)(72. 7mlfrom solution A+22. 8 ml from solution B).

Mayer's haematoxlin:

Haematoxlin powder1gm

Potassium alum or ammonium alum50gm

Sodium iodate0.2gm

Citric acid1gm

Chloral hydrate50gm

DW1000ml

Ammoniated water:

Concentrated ammonia0.05ml

Tap water99. 95ml