Chapter one

Introduction

1.1. Introduction

Mycetoma is a chronic, specific, granulomatous, progressive subcutaneous and inflammatory disease. The disease is caused by true fungi or by filamentous bacteria and hence it is classified into Eumycetoma and Actinomycetoma respectively (Fahal , 2004) .

The Infection follows inoculation of organisms, frequently through thorn punctures, wood splinters, or pre-existing abrasions or trauma. After inoculation, these normally non pathogenic organisms grow and form grains (also called granules or sclerotic) and with excessive collagen accumulation surrounding it(fibrous capsule) (Hospenthal , 2009) .

Concerning the geographical distribution of the disease, mycetoma is worldwide distributed; endemic in tropical and subtropical regions with the highest prevalence in Africa (Fahal , 2011). Sudan seems to be mycetoma homeland .and the most cases are caused by *Madurella mycetomatis* (Belkum *et al*. , 2013) . The prevalence in Sudan is of 1.81 cases per 100,000 inhabitants (Sande , 2013) .

Disease is more common in agricultural workers and outdoor labourers, but it is not exclusively seen in rural areas. The increased numbers in tropical regions may also be explained by decreased use of protective clothing chiefly shoes (Hospenthal, 2009), also Neutrophils functions–related

polymorphisms IL-8 (CXCL8), its receptor CXCR2, thrombospondin-4 (TSP-4) , NO synthase2 (NOS2), and complement receptor 1 (CR1) seem to be act as predisposing factor (Sande *et al*. , 2007) .

Mycetoma clinically diagnosed depending on history of patients and examination of granules ,radio logically to check bone involvement , serologically in case of no grains discharge, microscopically and macroscopically in case of grains discharge, as well as histopathologically (Ramachandra *et al*. , 2013) .

Treating eumycetoma, required a combination of surgery and treatment with antifungal agents (Revankar , 2010).Using these antifungals, KCZ and Itraconazole, only facilitates surgical removal of mycetoma lesions as they induce encapsulation of the fungal grain with fibrous tissue (Fahal *et al*. , 2011) .

CD nomenclature is commonly used when referring to leukocyte surface molecules and antibodies against them. It provides an essential classification for diagnostic and therapeutic purposes (Zola *et al*. , 2005) . The CD1 family is a group of transmembrane glycoproteins , which are structurally related to the major histocompatibility complex (MHC) proteins (Bricard and Porcelli , 2007) The CD1 proteins mediate the presentation of primarily lipid and glycolipid antigens of self or microbial origin to T cells (Cao *et al.*, 2002) .The human genome contains five CD1 family genes organized in a cluster on chromosome 1 (Bruns *et al*. , 1990) .The CD1 family members are thought to differ in their

cellular localization CD1a is highly expressed by langerhans cells in dermis and epidermis (Shamshiev *et al.*, 2002) .

Although *Madurella mycetomatis* is the main causative agent of eumycetoma being responsible for more than 70% of all mycetoma cases in Sudan (Ahmed and Abugroun , 1998).There is no previous investigation has been conducted to verify the effect of KCZ therapy on the host tissue reaction to *Madurella mycetomatis*. Thus, this study is the first one that addresses these issues.

1.2. Objectives

1.2.1. General objective:

To examine the effect of long term KCZ therapy on CD1a expression in

Eumycetoma patients due to *Madurella mycetomatis*.

1.2.2. Specific objectives:

1- To asses CD1a expression among eumycetoma patients using immunohistochemical method.

2- To compare the quantitative expression of CD1a before and after chronic ketoconazol treatment.

CHAPTER TWO

Literature review

2.1. Scientific background:

Eumycetoma is a subcutaneous fungal infection in which the aetiological agent occurs in the form of more or less compact mycelia grains (Ahmed *et al.*, 2002) . Mycetomas are also frequently identified as ‘‘Madura foot’’. It is presumed that the first description of this entity occurred in 1842 in the Madras Medical Service of the British Army in India, and hence the term Madura foot (Fahal , 2004) . It primarily affects the poorer

populations and rural regions and produce considerable disability, disfigurement, and stigma and it may be fatal (Fahal , 2010) . Mycetomas are therefore listed as neglected tropical disease (Hotez *et al.*, 2008) .

2.2. The aetiology of eumycetoma:

There are many types of fungi that cause eumycetoma showing grains with different sizes, colours and consistency. The more common ones that cause black grain eumycetoma include, *Madurella mycetomatis, Madurella grisea, Leptosphria senegaliensis, Pyrenochaeta romeroi, Philolphore jenselmi , Curvularia lunata* (Desnos-ollivier *et al*. , 2006), *Madurella fahalii* (De hoog *et al.*, 2012) .

2.3. Epidemiology:

Worldwide, mycetoma prevails in the mycetoma belt that stretches between the latitudes of 15° South and 30° North (Fahal , 2004) . Sudan showed prevalence of 1.81 cases per 100,000 inhabitants (Sande , 2013) and more than 70% of cases are due to *Madurella mycetomatis* (Dlova *et al.*, 2015)

2.4. Route of infection:

There are many theories attempt to explain the infection ; the leading one states that organisms which are thought to live in soil; can be implanted into the host tissue through a breach in the skin produced by local trauma. However, many patients had no history of trauma at the site of infection (Fahal , 2011).The disease is not contagious from one person to another or from animal to human (Fahal , 2004) .

2.5. Risk factors:

There are many controversies on the infection susceptibility and resistance (Fahal , 2011). There are three main factors associated with the establishment of disease: inoculums size, immune status of the host, and hormonal adaptation (based on the observation that men typically develop the disease) (Bonifaz *et al.*, 2014).

Male predominance is common in mycetoma, although the actual sex difference is not universally equal. Epidemiological data from Sudan demonstrate that males are more affected (sex ratio 4:1) This is genuine sex difference is not related to the greater outdoor activities of males because in certain areas in Sudan, males and females go out to work in the fields side by side (Mufti and Aljhdali , 2015) . But it is explained by significantly elevated serum levels of 17β-estradiol in male patients (Sande *et al.,* 2010) .

On genetically base, IL-8 (CXCL8), its receptor CXCR2, thrombospondin-4 (TSP-4), NO synthase 2 (NOS2), and complement receptor 1 (CR1) polymorphisms were proved as predisposed factors towards eumycetoma . Further, the NOS2 Lambaréné polymorphism was clearly associated with lesion size (sande *et al.*, 2007) .

Polymorphisms in genes encoding for chemokine ligand 5 and interleukin-10 are associated with the development of the mycetoma granuloma (Mhmoud *et al.*, 2013) .

Referring to traumatic inoculation theory, in areas where mycetoma is endemic the walking barefoot is a habit as a result, the natural infection is expected to be more frequent than it actually is (Fahal , 2011). No age is exempted but, it commonly affects adults between 20-40 years old of age (Bonifaz *et al.*, 2014) . Nearly all cases affecting feet (70% of cases) or hands (12% of the cases) . Infection of other sites such as leg, knee, thigh, perineum, back, arms, thorax, shoulders, head, and neck are reported less frequently (Ahmed *et al.*, 2003) .

The nature of the patient's occupation also influences disease presentation, poor rural workers or homemakers that participate in outdoor activities, for example lumberjacks and sugarcane carriers but no occupation is exempted (Fahal *et al* ., 2015) .

2.6. Incubation period:

The incubation period in mycetoma is unknown due to the difficulty in establishing the time of initial infection, however, in experimental animals the formation of the mycetoma lesion was noted after a period of three weeks from the inoculation of the organism (Ahmed *et al*., 2003) .

2.7. Clinical Triad:

Eumycetoma shows three clinical characteristics: tumor, sinuses and grains (Kauffman *et al.*, 2011) . Mycetoma presents as a slowly progressive, painless and subcutaneous swelling. Mycetoma is usually painless in nature because it is assumed that, the organism produces substances which have an aesthetic

action. The pain; may be produced by the involvement of the bone ,or due to secondary bacterial infection (Ahmed and Abugroun , 1998) . At a late stage of the disease the pain may become negligent due to nerve damage (Gaspari and Tyring , 2008) .

Progress is more rapid in eumycetomas than in actinomycetomas, and bone involvement is more extensive. In eumycetomas, the lesion is firm and round, though it can be soft and lobular. It remains more localized and progression is slower than in actinomycetomas (Branscomb , 2003) .

Sinuses are a characteristic of the disorder; they can be absent in early stages, but later develop and drain pus, blood and grains (Desnos-ollivier *et al*., 2006) .

2.8. Diagnosis:

2.8.1. Microscopic examination:

The most definite modality relies directly on the examination of grains, looking at colour and texture, microscopic examination for hyphae or filaments and isolation of the etiologic organism. For the visualization of grains and fungal filaments, a sample of mucopus from an open sinus must be obtained. A variety of stains including potassium hydroxide (KOH),Gram stain could be used (Cheesbrough , 2006) . *Madurella mycetomatis*, microscopically: compact with brown-staining cement or a center of hyaline hyphae with brown cement on periphery only, hyphae 2-4 µm diameter (Sande *et al.*, 2007) .

2.8.2Culture:

A definitive diagnosis usually requires a positive tissue culture and identification of the etiologic agent.Isolation of the organism may be difficult.Grains are cultured onto media, such as Sabouraud agar, to isolate fungi (De hoog *et al.*, 2004).

2.8.3. Histopathology:

In H and E both grains types (filamentous and vesicular) contains cement material which is seen as homogenously pink material in which individual hyphae are embedded (Ibrahim *et al.*, 2013) .

2.8.3.1. Types of tissue reactions:

Three types of tissue reactions have been described, type I reaction, type II reaction and type III reaction (Fahal *et al* .,1995) .

2.8.3.1.1. Type I reaction:

In Type I reaction, the grains are usually surrounded by a layer of polymorph nuclear leucocytes. Outside the zone of Neutrophils there is granulation tissue containing macrophages, lymphocytes, plasma cells and few Neutrophils. The outermost zone of the lesion consists of fibrous tissue (Fahal *et al.*, 1995) .

2.8.3.1.2. Type II reaction:

In this type, the Neutrophils have largely disappeared and are replaced by macrophages and multinucleated giant cells (Fahal , 2004) .

2.8.3.1.3. Type III reaction:

At this stage there is formation of a well-organized epithelioid granuloma (Hospenthal , 2009) .

2.8.4. Cytology:

Mycotic mycetoma may also be accurately diagnosed by fine needle aspiration cytology (FNAC). FNAC smears allow the visualization of the characteristic histopathologic changes. This includes the polymorphous inflammatory cells intermixed with grains, Neutrophils, lymphocytes, plasma cells, histiocytes, macrophages, and foreign body giant cells. This technique allows differentiation between eumycetoma and actinomycetoma (EL hag *et al.*, 1996) .

2.8.5.Mycetoma imaging:

Radiographs may be normal, demonstrate soft tissue enlargement, bone sclerosis, bone cavities, periosteal reaction, bone expansion, extrinsic cortical scalloping, fanning of the rays or osteoporosis, mainly for bone involvement (Iffat and Abid , 2011) .

Ultrasound could be used to differentiate eumycetoma from actinomycetoma. In eumycetoma, the grains produce numerous, sharp, hyper-reflective echoes and there are single or multiple thick-walled cavities with no acoustic enhancement. In actinomycetoma, the findings are similar but the hyper-reflective echoes are fine, closely aggregated and commonly settle at the bottom of the cavities (Fahal *et al.*, 1997) .

CT scan is the most sensitive diagnostic informative tool with conventional radiography and because of its direct multiplanner capability; it is the method of choice for pedal mycetoma in pretherapy and early stages of bone involvement

(Ali *et al* .,1997) .

2.8.6. Advance techniques:

Serodiagnosis is of a great help in identification and classification of the various organisms. TCTP (Translationally Controlled Tumor Protein) was isolated from *madurella mycetomatis* and used in ELISA, although it may not be the best diagnostic tool, but it could be useful in seroprevalence studies (Sande *et al*., 2006) . PCR tests are useful but do not provide a simple tool for bedside use (Ahmed *et al.*, 1999) .

2.8.7. Immunohistochemical techniques:

They are a techniques for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interactions .The site of the antibody binding being identified either by direct labelling of the antibody, or by use of a secondary labelling method (Linnoila and Petrusz , 1984).

2.8.7.1. CD1a:

Transmembrane glycoproteins, which are structurally related to the major histocompatibility complex (MHC) proteins and form heterodimers with beta-2-microglobulin (Bauer *et al.*, 1997) .

The skin immune system is characterized by the presence of two types of CD1a expressing cells: langerhans cells and dermal dendritic cells (dDC), which are professional antigen processing and presenting cells (Pigozzi *et al*., 2006) and they are located in the superficial and deeper layers respectively (Rozis *et al.*, 2008) .

CD1a-deficient cells had a selective functional deficiency in lipid Ag presentation to T cells , CD1a-deficient DCs were selectively

impaired in their ability to present lipid Ag to T cells, but showed no defects in endocytosis, cytokine secretion, or expression of costimulatory molecules (Seshadri *et al.*, 2013) .

2.9. Treatment:

The treatment of mycetoma is often difficult and it consists of combined medical and surgical therapy. As a consequence of the inability to define the accurate extension of the lesion during surgery and the increasing resistance to drug treatment by fungal organisms, an incomplete cure and recurrence after treatment are common (Abd elbagi , 2003) .

Madurella mycetomatis appeared to be most susceptible to the azoles group like KCZ, that have ability make lesions well localized, thickly encapsulated and easily excised surgically (Williams *et al*., 2013) .

A controversy exists regarding the immunosuppressive effect of KCZ. Several studies have investigated the effect of KCZ on the immune cell populations under different experimental settings, nevertheless the results were often inconclusive or conflicting (Cools *et al.*, 1992) . In 1983, there was a proposal that KCZ has a potent inhibitory effect on the DNA synthesis in mitogen-induced human lymphocytes (Buttke and Chapman , 1983) .Subsequently, Pawelec and colleagues showed that KCZ has a potent inhibitory effect on the proliferative response of human T-lymphocytes in vitro (Pawelec *et al.*, 1991) . In addition, it was reported that chronic administration of KCZ in mice causes

moderate, though statistically insignificant decrease in monocytes and lymphocyte counts (Bonhomme-faivre *et al.*, 2002) .

In another aspect, in vitro studies have shown that KCZ has either slight stimulatory or no effect on Neutrophil chemotaxis under different experimental settings (Yousif and Hay , 1987) . Similar conclusions have been drawn from in vivo human studies that involved testing the Neutrophil random migration in patients receiving KCZ treatment (Roilides et al , 1990) . Neutrophils were shown to influence the activation of DC (Schuster *et al.*, 2013) . Moreover, treatment of keratinocytes with of KCZ resulted in decrease in IL1 secretion (Zomorodian *et al.*, 2008) which is in turn responsible for activation of dendritic cells (Eriksson *et al.*, 2003) . Also, KCZ inhibits IL-8 production (Tsuji *et al.*, 2012) . Interleukin-8 (IL-8) thought to be the main cause of local Neutrophils accumulation (Baggiolini and Clark-lewis , 1992) .

There is hypothesis that the balance between proinflammatory and anti-inflammatory cytokines in the epidermal milieu plays an important role in control of LC motility (Wang *et al.*, 1999) . Hence cytokines concentrations which depend on their secreted cells number which in turn on are affected by KCZ administration.

On the other hand, evidence of immunosuppressive effect of KCZ in human is meagre, and most often based on assumptions rather than empirical studies. It was observed that when cyclosporine is given in conjunction with KCZ, lower doses of the former are required to achieve the same immunosuppressive effect in transplant patients (Treĭvish *et al.*, 1998) . In contrast, in an in

vivo human studies, Rensburg and associates reported that no significant inhibition of mitogen-induced lymphocytes transformation in six adult subjects following ingestion of therapeutic dose of KCZ (Rensburg *et al* ., 1983) .

Chapter three

Materials and methods

3.1. Materials:

Blocks of 30 patients previously diagnosed as eumycetoma patients caused by *Madurella mycetomatis* before and after treatments. An abstraction form was used to record data about the demographic and clinical profile of the patients.

3.2. Study design:

This was a Quasi experimental study aimed to determine the impact of KCZ therapy on expression of CD1a. The study was conducted during the period from January to September 2015.

3.3. Study population:

The study subjects were patients who previously diagnosed with eumycetoma infection due to *Madurella mycetomatis*.

3.4. Selection criteria:

Selected blocks were from male or female (not pregnant, not suckling mother), with an age of 18 years old and more, also shouldn't undergo previous surgery or prior antifungal treatment .Patients ' sample with significant compromise or on long term medical treatment for other condition (e.g. Diabetes , chronic liver disease) were excluded from the study.

3.5. Study samples:

Two blocks were obtained from all patients before and after they received KCZ (4 tap per/day) for a period of 6 months.

3.6. Study area:

The study was conducted in the mycetoma Research Centre (MRC),Khartoum state, Sudan.

3.7. Sample processing:

One section was cut from each paraffin block using rotary microtome for immunohistochemistry which then taken in coated slides and dried in a hot oven at 60.c for 1 hour.

3.7.1. Immunohistochemical staining:

Antigen retrieval was performed on rehydrated sections prior to the immunostaining procedures. for 20 minutes in water bath at 95c and then equilibrated in phosphate buffer saline(P.B.S PH 7.4) at room temperature for 10 minutes. Then sections are stained as mentioned in biogenex protocol: endogenous peroxidase blocking for 10 minutes ,washed in P.B.S for 1minutes,then

primary antibody CD1a applied for 30 minutes, washed in P.B.S for 10 minutes ,secondary antibody applied for 20 minutes, washed in P.B.S for 10 minutes and finally DAB for 7 minutes for colour developing and after washing counter stained using mayers' haematoxylin for 1 minute .blued in running tap water for 10 minutes ,dehydrated, cleared and mounted in D.P.X.

Because of the localized distribution of DCs within the skin, each slide was scored by counting positively stained cells in five high-powered fields (hpfs) under × 400 magnification. Results were reported for each section as the mean 5 hpfs (Poindexter et al., 2004).

3.8. Ethical consideration:

All samples were taken from authorized centre for research purposes.

3.9. Result interpretation:

All quality control positive and negative measures were adopted during assessment of the results.CD1a expressed on DC as membranous and cytoplasmic brown granules.

3.10. Data analysis (Statistical methods):

The data was analyzed using Statistical Package for the Social Sciences (SPSS) software 16.0. Frequencies, means and paired t _ test were calculated.

Chapter four

Results

4.1. Results

A total of 60 samples with mycetoma were investigated by conventional histopathology and immunohistochemical methods.

Out of 30 patients, the age ranged between 18 to 69 years old with the mean of 33 years old. Most patients were aggregated in less than the age of 40 years representing 22(73.3%) patients and remaining 8(26.7 %) patients were older than 40 years as indicated in table (4.1).

In this study, males were 24(80%) of participants and females were 6 (20%) of participants as indicated in table (4.2).

Among study subjects, 25(83.3%) of patients developed mycetoma in their left feet and the remaining patients 5(16.7%) developed mycetoma in their right feet as indicated in table (4.3).

All sections before treatment with KCZ stained for CD1a showed expression with mean 62.33± 33.446, and sections after treatment showed expression with mean 17.63 ± 20.184. These results revealed significant statistical association between treatment with KCZ and depleted CD1a expression with p.value 0.000 as indicated in table (4.4).

Table (4.1) : Distribution of age among the study sample.

Age group(year)	Frequency	Percent (%)

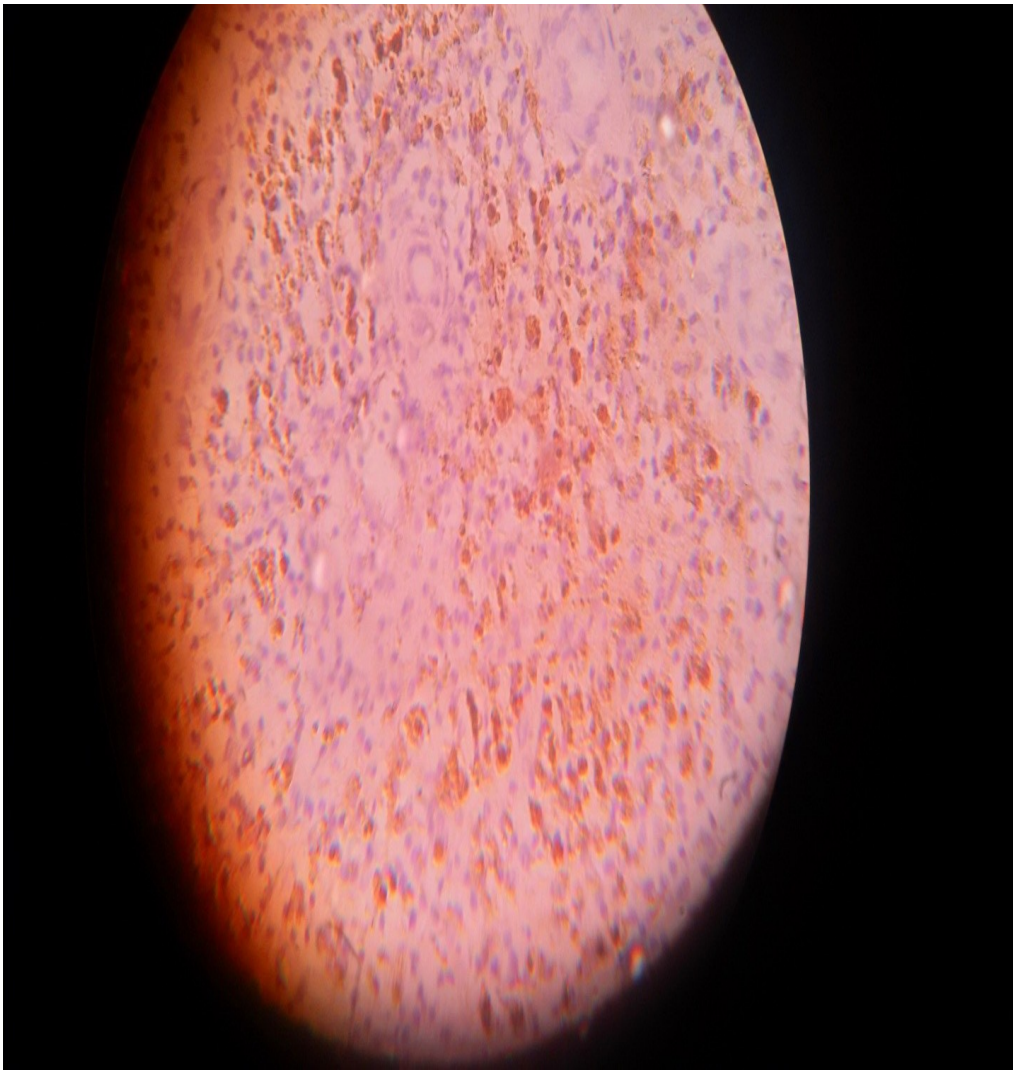
Less than40	22	73,3
More than 40	8	26.7
Total	30	100.0

Table (4.2): Distribution of gender among the study samples.

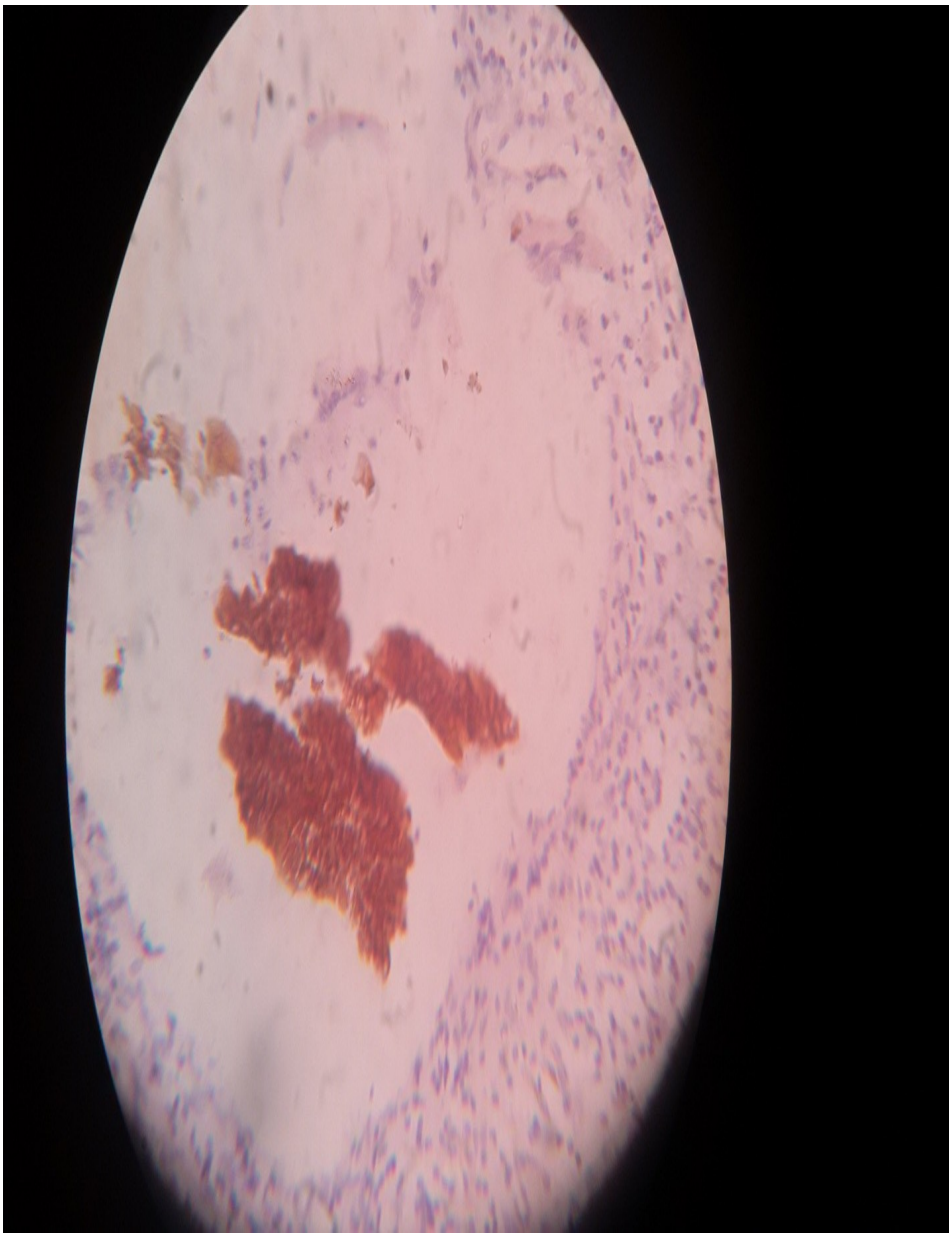
Gender	Frequency	Percent (%)
Male	24	80.0
Female	6	20.0
Total	30	100.0

Table (4.4) : Distribution of CD1a among the study samples.

Sample	Mean	Standard deviation	P .value
Pre treatment CD1a readings	62,33	33.446	.000
Post treatment CD1a readings	17.63	20.184	



Microfigure1: pre treatment eumycetoma .Shows membranous and cytoplasmic expression of CD1a, X 40.



Microfigure2: post treatment eumycetoma .Shows negative expression of CD1a, X 10.

Chapter Five
Discussion

Considerably little is known about the immunopathology of eumycetoma lesions due to *Madurella mycetomatis*. Eumycetoma caused by *Madurella mycetomatis* is treated surgically and with high doses of KCZ .Therapeutic responses are poor and recurrent infections are common (Sande *et al*., 2005) .

The studied patients were aggregated in less than the age of 40 .This study agrees with previous prevalence that showed high frequency among adults between 20 – 40 years old and this explained by high exposure rate to the fungi among this age group due to outdoor activities (Klueken et *al*., 1965) .

In fact, male predominance is common in mycetoma (Sande *et al*., 2010) . And in this study, males were 24(80%) of participants and females were 6(20%) of participants. These findings similar to those showed by (Mufti *et al*., 2015).And it'not related to the greater outdoor activities of males because in certain areas in Sudan, males and females go out to work in the fields side by side (Mufti and Aljhdali , 2015) . But it is explained by significantly

elevated serum levels of 17β-estradiol in male patients (Sande *et al.,* 2010) .

In this study 30 (100%) of patients developed mycetoma in their feet. and these results agrees with Fahal and his colleagues who reported that feet are the most commonly affected sites (Fahal *et al*., 2015) .

dDC are numerically higher in pre treatment CD1a stained sections which showed positive results with mean 62.33 ± 33.446, but they are low in post treatment ones that showed positive results with mean 17.63± 20.184. So there is a significant association between chronic administration of KCZ and depleted dDC.

Chapter Six

Conclusion and Recommendations

6.1. Conclusion:

From this study we conclude that:

The CD1a numerical reduction - as label for dendritic cells- is correlated significantly with the chronic administration of KCZ. And this is the first study that makes evidence that KCZ is immunosuppressant.

6.2. Recommendation:

Further studies should be done using large sample size co mpiling with markers labelling other leukocytes like T-

lymphocytes subgroups, so as to identify the mechanism through which ketoconazole suppress the immunity.

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Appendix I:

Immunohistochemical solutions:

- **citra – base solution PH6.0:**

▪ Citra base 10 ml

▪ Distilled water 90 ml

- Hydrogen peroxide.

- Phosphate buffer saline(PH 7.4):

▪ Stock(A)

0.2 Sodium dihydrogen orthophosphate:

Sodium dihydrogen orthophosphate 3.2 g

Distilled water 100ml

▪ Stock(B)

Disodium hydrogen orthophosphate 2.83g

Distilled water 100 ml

Take 9.5 ml from stock(A) and 40.5 ml from stock (B) and made up to

100mlwith distilled water.

- **DAB chromogen kit:**

▪ 3.3 Diamino benzidinetetra hydrochloride.

▪ Substrate buffer

- **Other material and Reagents:**

-Waterbath.

-Microtome.

-Stanadrd light microscope.

- Coblin jars.

- Measuring clinder.

-Plastic pasture pipettes.

-Timer.

-Sterile gloves.

-Humid plate.

-Staining jars.

- Slides.

- Coverslips.

-Positive control tissue blocks.

-Xylene.

-Absolute alcohol.

-Mounting media(DPX).

Appendix II:

Kits leaflets