Characterisation of the *Madurella mycetomatis* mycetoma granuloma tissue pigments and fibrous tissue.

Najwa Adam Mhmoud[1], Badr EL Din Margani Yousif[1], Ahmed Fahal[1*], Wendy van de Sande[2]

[1]*Mycetoma Research Centre, University of Khartoum*

[2]*ErasmusMC, Department of Medical Microbiology & Infectious Diseases, Rotterdam, The Netherlands.*

***Corresponding Author:** Prof. Ahmed Hassan Fahal, Mycetoma Research Centre, University of Khartoum. E-mail:ahfahal@hotmail.com – ahfahal@uofk.edu*

Abstract

Background: Mycetoma the unique neglected tropical disease, a mutilating chronic granulomatous inflammatory infection characterised by significant deformities and disabilities. It is characterised by the formation of brown and yellowish-brown pigments within the eumycetoma tissue granulomas, however, the nature of these pigments is not well characterized. This study was set out to determine the nature of these pigments.

Material & Methods: The study included 22 surgical biopsies from patients with *Madurella mycetomatis* eumycetoma. The biopsies were histopathologically examined using different stains. In addition to Periodic Acid Schiff (PAS) stain and Hematoxylin and Eosin stain, Prussian blue stain for haemosiderin identification, Masson-Fontana stain for melanin, Sudan black B and Sudan III for lipids, long Ziehl-Neelsen (ZN) stain for acid fast lipofuscin pigments and Masson's trichrome for collagen fibers were used.

Results: the *Madurella mycetomatis* eumycetoma tissue granuloma histopathological examination revealed two pigments; melanin and haemosiderin and collagen fibers. The dark pigment is melanin as it bleached with potassium permanganate oxidation. the iron-containing pigment probably related to haemosiderin. The fibrils stained with Masson's trichrome stain strongly suggest presence of collagen fibers. Lipofuscin pigments were not detected.

Introduction:

Eumycetoma remains a major public health and medical problem in many tropical and subtropical regions resulting in numerous negative impacts.[1,2] It is a chronic progressive granulomatous inflammatory disease, characterised by painless subcutaneous masses, multiple sinuses with purulent or seropurulent discharge containing grains.[3,4] Many organisms are incriminating in causing eumycetoma but *Madurella mycetomatis* is the commonest agent in many areas.[5,6] The causative agents are believed to exist in the soil and subcutaneous traumatic inoculation of these organisms is the popular theory for contracting mycetoma. After inoculation, the causative agent usually spreads along the different tissue planes and involves the deep structures and bone leading to massive deformities and disabilities.[7,8] In essence, mycetoma is a localised chronic granulomatous inflammatory disease but can spread by the lymphatics or the blood.

Brown or yellowish-brown granular pigments frequently present in the affected tissue are characteristic of eumycetoma. Melanin was isolated from the grains of eumycetoma and it may be the cause of these pigments.[9,10] furthermore, the true nature of the extracellular matrix and the collagen fibers in mycetoma are still enigma. However, it is not uncommon for the chronic granulomatous inflammation reaction to result in irreversible tissue injury and end-stage fibrosis, as evidenced by increased skin fibroblast proliferation and the deposition of collagenous material.[11,12] Therefore this study was carried out to determine the nature of these pigments and the tissue collagen fibers in mycetoma.

Materials and Methods:

This descriptive cross sectional study was conducted at the Mycetoma Research Center, University of Khartoum, Khartoum, Sudan. It included 22 surgical biopsies obtained from confirmed eumycetoma patients undergoing surgical treatment. The diagnosis of mycetoma was confirmed by careful history and meticulous clinical examination, the typical conventional X-ray and ultrasonic imaging appearance of mycetoma as well as cytological examination of the fine needle aspirates from the lesions. After informed consent a normal skin biopsy was taken as a control.

The patients were 20 males and two females, their mean age was 26.6 years (range 17- 55 years) and the mean duration of the disease was 5.6 years (range 0.2- 20 years). All patients had foot mycetoma.

The macroscopic examination of the surgical biopsies revealed the presence of brown to dark-brown pigments, (Fig. 1).

Histopathological Examination:

The specimens were fixed in a 10% formal saline, embedded in paraffin and processed for Hematoxylin and Eosin (H&E) and Periodic Acid Schiff (PAS) staining. The sections were additionally stained with Prussian blue for haemosiderin granules, Masson-Fontana stain for melanin granules, Sudan black B, Sudan III for lipid, long Ziehl-Neelsen (ZN) stain for acid fast lipofuscin identification and Masson's trichrome stain for collagen.

Results:

Tissue reactions detected

In the H&E and PAS stained sections, the typical eumycetoma granuloma was seen and it consisted of three zones. In zone I, the grains were surrounded and infiltrated by neutrophils, while zone II was composed mainly of macrophages and it was surrounded by zone III which was composed of lymphocytes, plasma cells and foreign body giant cells, (Figs. 2&3).

Numerous brown pigment laden histiocytes were present, diffusely scattered in the examined sections, (Fig.4).

The eumycetoma granuloma was surrounded by numerous collagen fibers as they stained strong green color with Masson's trichrome, (Fig.5).

Pigments detected:

Two types of pigments appeared prominent in the examined sections. Masson-Fontana stain confirmed that the dark brown or black pigment was melanin.

The other brownish blue pigment was iron-positive using Prussian blue, (Fig.3).

No lipofuscin was detected using long ZN and no fat was detected by Sudan black B and Sudan III stains.

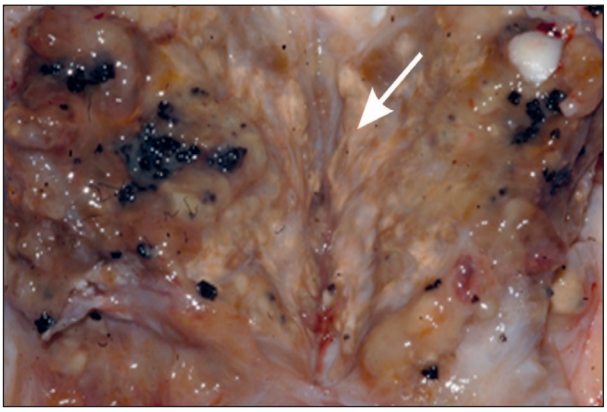

Fig. 1: The macroscopic appearance of a surgical biopsy specimen showing the yellow-brown pigment (arrow).

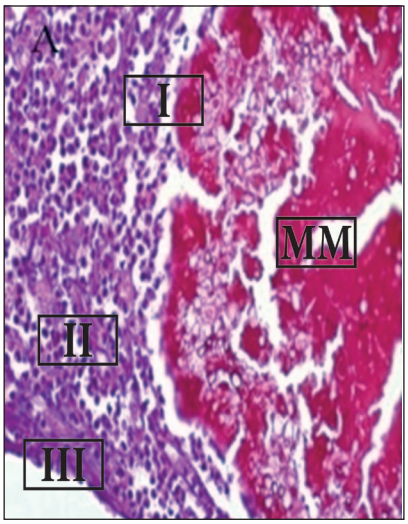

Fig. 2: Microphotograph showing the inflammatory reaction around the *M. mycetomatis* grain (MM) consisting of an inner zone (I) of numerous neutrophils, middle zone (II) consisting of macrophages and an outermost zone (III) consisting of lymphocytes and plasma cells. (H&E x200).

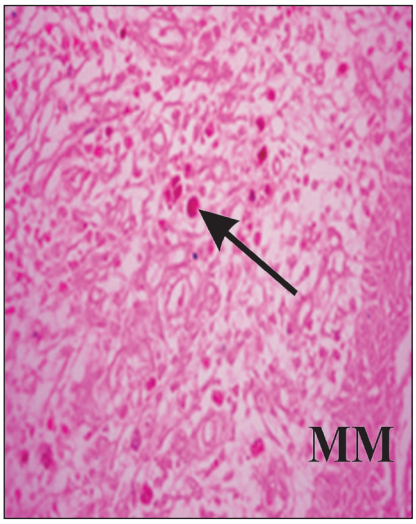

Fig. 3: Microphotograph showing *M. mycetomatis* eumycetoma lesion with numerous brown pigment laden histiocytes (arrow) diffusely scattered in the infiltrate (H&E x400).

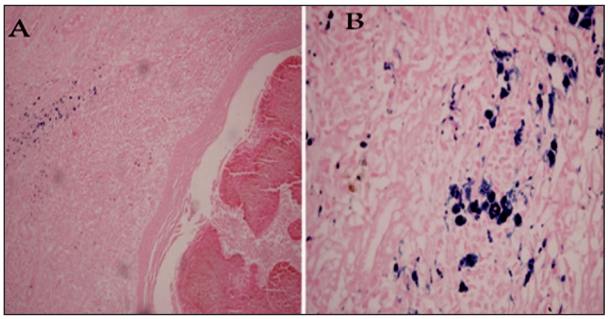

Fig. 4: A & B: Microphotograph showing iron-positive pigment. (Prussian blue stain x100 and 400x).

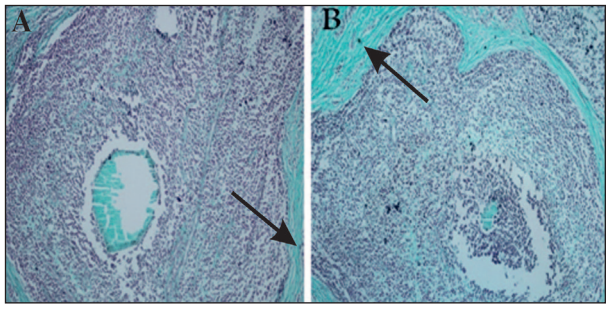

Fig. 5 A & B: Microphotograph showing collagenic fibers (arrow) stained positive with Masson's trichrome stain, (100x).

Discussion:

A substantial knowledge gap remains in the pathogenesis of mycetoma and in particularly eumycetoma. Likewise, the role of pigments seen in the granulomatous tissue in mycetoma. The *Madurella mycetomatis* grains are composed of cement substance in which the fungal hyphae are usually embedded. The cement substance is composed of melanin pigments and other constitutes, and it plays important roles in protecting the fungus against the available antifungal drugs and the host defense mechanisms.[5]

This study showed that, the brown pigment seen in mycetoma granulomatous tissue was melanin pigment. The melanin pigment is possibly released from the fragmented destroyed mycetoma grains. This is in line with Van de Sande and associates report which stated that, the melanin pigment is produced by the grains and the phagocytic cells surrounding the grains.[5]

The melanin pigment proved to be protective to the melaninised organisms against various antifungals in spite their low in vitro MIC.[11,12] Melanin pigments also protect microorganisms against stresses caused by cell damage due to solar UV radiation or generation of toxic oxygen radicals.[11,12] Many studies confirmed that, the melanin pigment can protect *Cryptococcus neoformans* and other organisms against the host immune responses.[13 -17] However, the role of these pigments in the mycetoma granulomatous tissue need further in depth studies.

The H&E stained tissue sections, in this study showed numerous brownish fine granular pigment-containing histiocytes which were diffusely scattered in the mycetoma lesions. This is in line with El Hassan and colleagues report on the histiocytes deposited haemosiderin and melanin found in enlarged inguinal lymph nodes of patients with secondary mycetoma from the foot primary lesions.[18] This pigment proved to consist of a major pool of iron which most probably of host origin. This haemosiderin is possibly responsible for tissue

damage seen in mycetoma. Many studies showed that it has an important role in the microorganisms' resistance against many antimicrobial agents.[19- 23]

van de Sande and associates also reported on the association between macrophages with haemosiderin deposits and high CXCL8 (IL-8) expression.[24] Recent studies indicated that, iron-loaded macrophages impair the ability of these cells to inhibit the growth of *Candida albicans.* [25] In this study, collagen fibers with their staining characteristics were demonstrated in several sections. These collagen fibers may contribute to the encapsulation and fibrosis seen in the mycetoma granulomatous lesions. Many studies showed mononuclear cells release mediators capable of initiating the fibrotic response.[26 -29]

Similarly, other studies showed that, cytokines and chemokines levels have a major role in the degree of extracellular matrix deposition around mycobacterial and *Schistosoma mansoni* egg during their infection.[30] All these observations may explain the formation of fibrous tissue seen in the mycetoma lesion. This fibrous tissue tends to localise the infection and limit its spread but on the other hand hinder the diffusion of the antifungal drugs into the lesions.

The study, demonstrated the presence of collagenous like protein in the fungal cell walls. This was supported by the grains itself been stained with Masson's trichrome stain for collagenic fibers, (Fig. 5).The function of these collagenous like protein is unclear, however, it was suggested that the collagen molecule contains a chemotactic signal for neutrophils and that chemotactic factors are released during the process of collagen degradation. [31 -33] These findings may be supported by the strong presence and adherence of neutrophils and the grains.

In conclusion, this study confirmed that, the mycetoma granulomatous tissue pigments are melanin and haemosiderin in nature which are most probably of host origin and released from the damaged grains. We also postulate that the fibrous tissue characteristic of mycetoma is induced by certain mononuclear cells, cytokines and chemokines. Further studies are required to confirm these suggestions and bridge the knowledge gaps in the pathogensis of mycetoma in order to improve the management of these patients.

Acknowledgements:

We would like to thank Mrs. Zenab Abdellah for preparing the histological specimens.

References:

1. Fahal AH. Mycetoma. Review article, Khartoum Med J. 2011; 4: 514-523.

2. Fahal, AH. Management of mycetoma. Expert Rev Dermatol. 2010; 5: 87-93.

3. Fahal AH. Mycetoma thorn on the flesh Review article. Trans R Soc Trop Med Hyg. 2004; 98:3-11.

4. Ahmed A, Adelmann D, Fahal A, Verbrugh H, van Belkum A, de Hoog S. Environmental occurrence of Madurella mycetomatis, the major agent of human eumycetoma in Sudan. J Clin Microbiol. 2002; 40: 1031-6.

5. Van de Sande WW, de Kat J, Coppens J, et al. Melanin biosynthesis in Madurella mycetomatis and its effect on susceptibility to Itraconazole and Ketoconazole. Microbes Infect. 2007; 9: 1114-23.

6. Fahal AH, EL Toum EA, EL Hassan AM, Gumaa SA, Mahgoub ES. Host tissue reaction to Madurella mycetomatis: New classification. J Med Vet Mycol. 1995; 33: 15-17.

7. Standiford TJ, Rolfe MW, Kunkel SL, et al. Macrophage inflammatory protein-1 expression in interstitial lung disease. J Immunol. 1993; 151:2852 -2863.

8. Jose PJ, Griffiths-Johnson DA, Walsh DT, et al. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea-pig model of allergic airways inflammation. J Exp Med. 1994; 179:881- 887.

9. Zhang K, Gharaee-Kermani M, Jones ML, et al. Lung monocyte chemoattractant protein-1 gene expression in bleomycin-induced pulmonary fibrosis. J Immunol. 1994;153:4733- 4741.

10. Findlay GH, Vismer HF, Kiebenberg NW. Black grain mycetoma: the ultrastructure of Madurella mycetomi. Mycopathologia. 1979;67: 51-54.

11. Gomez BL, Nosanchuk JD. Melanin and fungi. Curr Opin Infect Dis. 2003;16: 91-96.

12. Gomez BL, Nosanchuk JD, Diez S, et al. Detection of melanin-like pigments in the dimorphic fungal pathogen Paracoccidioides brasiliensis in vitro and during infection. Infect Immun. 2001; 69: 5760-67.

13. Hamilton AJ, Gomez BL. Melanins in fungal pathogens. J Med Microbiol. 2002; 51: 189-191.

14. Langfelder K, Streibel M, Jahn B, et al. Biosynthesis of fungal melanins and their importance for human pathogenic fungi. Fungal Genet Biol. 2003; 38: 143-58

15. Jacobson ES. Pathogenic roles for fungal melanins. Clin Microbiol Rev. 2000; 13: 708-717.

16. Kwon-Chung KJ, Polacheck I, Popkin TJ. Melanin-lackingmutants of Cryptococcus neoformans and their virulence for mice. J. Bacteriol. 1982;150:1414-1421.

17. Guillot L, Carroll SF, Homer R, et al. Enhanced innate immune responsiveness to pulmonary Cryptococcus neoformans infection is associated with resistance to progressive infection. Infect Immun. 2008 76:4745-56. doi: 10.1128/IAI.00341-08. Epub 2008 Aug 4.

18. EL Hassan AM, Mahgoub ES. Lymph node involvement in mycetoma.Trans R Soc Trop Med Hyg. 1972; 66:165.

19. Salyers AA, Whitt DD.Virulence factors that damage the host. In: Bacterial Pathogenesis. A Molecular Approach. New York: ASM Press 1994, pp 47-62.

20. Griffiths E, Chart H. Iron as a regulatory signal in Iron and Infection. 2nd ed. Edited by JJ Bullen and Griffiths E. 1999, pp 213- 54.

21. Kochan I. Role of iron in the regulation of nutritional immunity. Bioorganic Chem. 1976; 2:55-57.

22. Braun V, Hantke K, Koster W. Bacterial iron transport: mechanisms, genetics and regulation. In: Metal Ions in Biological systems. Sigel A, Sigel H, editors. Iron transport and storage in Microorganisms, Plants and Animals. New York: Marcel Dekker. 1998, pp 67-145.

23. Ratledge C, Dover LG. Iron metabolism in pathogenic bacteria. Annu Rev Microbiol. 2000; 54:881-941.

24. Van de Sande WW, Fahal A, Verbrugh H, et al. Polymorphisms in genes involved in innate immunity predispose towards mycetoma susceptibility. J Immunol. 2007; 179: 3065-74.

25. Romani L. Innate and adaptive immunity in Candida albicans infections and saprophytism. J. Leukoc. Biol. 2000; 68:175-179.

26. Whiteside U, Worrall JG, Prince RK, et al. Soluble mediators from mononuclear cells increase the synthesis of glycosaminoglycan by dermal fibroblast cultures derived from normal subjects and progressive systemic sclerosis patients. Arthritis Rheum. 1985;28:188-197.

27. Postlethwaithe AE, Snyderman R, Kang AH.The chemotactic attraction of human fibroblasts to a lymphocyte-derived factor. J Exp Med. 1979; 144:1188-1203.

28. Estes JE, Pledger WJ, Gillespie GY. Macrophage derived growth factor for fibroblasts and interleukin-1 are distinct entities. J Leuk Biol. 1984; 35:115-29.

29. Geneugelijk K, Kloezen W, Fahal AH, van de Sande WWJ. Active matrix metalloprotease-9 is associated with the collagen capsule surrounding the Madurella mycetomatis grain in mycetoma. PLoS Negl Trop Dis 2014;8:e2754

doi: 10.1371/journal.pntd.0002754. e Collection 201.

30. Chensue SW, Warmington K, Ruth J, et al. Cytokine responses during mycobacterial and schistosomal antigen-induced pulmonary granuloma formation: production of Th1 and Th2 cytokines and relative contribution of tumor necrosis factor. Am J Pathol. 1994;145:1105-1113.

31. Postlethwaite, AE, Seyer JM, Kang AH. Chemotactic attraction of human fibroblasts to type I. II. and III collagens and collagen- derived peptides. Proc. NatI. Acad. Sci. USA.1978; 75:871.

32. Chang C, Houck JC. Demonstration of the chemotactic properties of collagen. Proc. Soc. Exp. Biol.Med. 1970; 134: 22.

33. Riley Di, Kerr IS, Guss HN, et al. Intratracheal instillation of collagen peptides induces a neutrophil influx in rat lungs.Trans Assoc Am Physicians.1984;97:290-5.