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Pathogenesis and Diagnostic Challenges of Mucormycosis

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ABSTRACT

Mucormycosis is a fungal infection caused by the order Mucorales from the class Zygomycetes. Among the genera within the order Mucorales, Rhizopus sp. is the genus most frequently causing infections. Mucorales fungi enter the human body in three ways: through inhalation of airborne spores, through broken skin, and through ingestion of contaminated food. Within the human body, the process is angioinvasive, leading to thrombosis and resulting in tissue necrosis. Currently, establishing a diagnosis is crucial for determining the patient's prognosis, as it can reduce morbidity and mortality. The current diagnostic modes include direct examination, culture, histopathology, and molecular identification. Each of these methods has its advantages and disadvantages. However, for rapid diagnosis, molecular examination, either through PCR or RT PCR, is necessary.

KEYWORDS *Mukormikosis, Mucorales, network*

INTRODUCTION

Mucormycosis (previously known as zygomycosis) is a fungal infection caused by the order Mucorales in humans. Mucormycosis is a rare but life-threatening disease that causes high disability, especially in patients with uncontrolled diabetes mellitus (DM), neutropenia, long-term corticosteroid therapy, malignancies, and organ transplants. The incidence of mucormycosis is increasing globally, with the highest rise observed in India and China among the uncontrolled diabetes mellitus population. Diabetes is the most significant risk factor found in Asian countries, whereas hematologic malignancies and organ transplant recipients are important underlying diseases in Europe and America. [1]

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The current diagnostic modes for mucormycosis rely on conventional mycological laboratory examinations, including direct examination with 10% Potassium Hydroxide (KOH), culture, and histopathological examination. KOH 10% and histopathology examinations require highly trained personnel to recognize fungal elements, while culture examinations have low sensitivity due to the biological nature of the fungi. Understanding the pathogenesis of the fungus will significantly aid in establishing an early diagnosis of mucormycosis. [2, 3]

In this paper, we will discuss the pathogenesis and characteristics of the fungus that influence efforts to establish an early diagnosis of mucormycosis.

Mucorales

The order Mucorales, which causes mucormycosis, is a group of fungi classified in the subphylum Mucormycotina of the class Zygomycetes. Most mucormycosis cases manifest as invasive skin lesions and systemic infections. The most commonly found Mucorales genera causing the disease are Rhizopus, Lichtheimia (previously known as Absidia and Mycocladus), and Mucoraceae. Other genera such as Rhizomucor, Saksenaea, Cunninghamella, and Apophysomyces are rarely found. These fungi enter the human body in three ways: first, through inhalation of airborne spores, second, through skin trauma, and third, by ingestion of contaminated food. [5, 6]

Pathogenesis of Mucormycosis

Fungal spores in the environment enter the human body through inhalation into the respiratory tract, infect the skin through trauma, or are ingested into the gastrointestinal tract. [5, 7] Various risk factors or underlying diseases facilitate mucormycosis infection, including ketoacidosis in uncontrolled diabetes mellitus, hematologic malignancies such as lymphoma and leukemia, renal failure, organ transplants, neutropenia and immunosuppressive therapy, cirrhosis, burns, and HIV/AIDS. Another factor suspected of causing increased mucormycosis is prophylactic use of voriconazole, an antifungal drug that is the first-line therapy for invasive aspergillosis. .[5, 8]

Immunocompromised individuals generate an inadequate immune response to prevent infections, including Mucorales infection. The fungus can spread, causing infections in various organs, and the most frequently found form is an infection around the paranasal sinuses, eyes, meninges, and brain, or causing rhino-orbito-cerebral mucormycosis.

Asexual spores present in the air settle on the mucous membranes of the mouth and nose. In a healthy immune system, the spores entering the body are contained by the phagocytic response and eliminated or live as saprophytes. If the immune response fails, the spores will germinate and develop into hyphae, which are difficult to eliminate as polymorphonuclear leukocytes are less effective in destroying hyphae. When hyphae begin to invade arteries, they spread within the vessel walls and lumen, causing thrombosis, ischemia, infarction, and gangrene of the affected tissues. Dissemination to other organs can occur, such as the lungs and brain, leading to sepsis. .[5, 6]

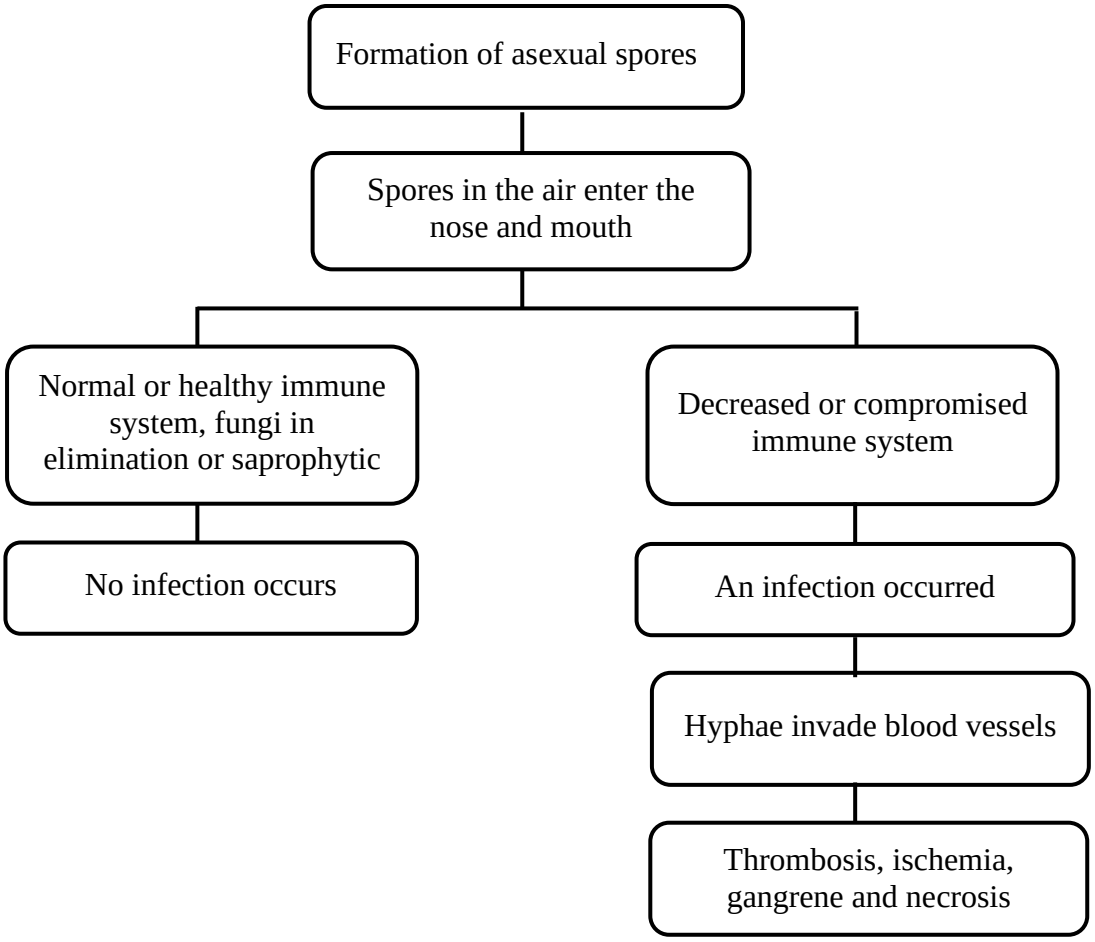

Figure 1 : Pathophysiology of mucormycosis, modification of Afroze SN, *et al*.[5]

Mucormycosis is aggressive and potentially fatal in individuals with diabetes due to disruption of host defense mechanisms and increased micronutrients such as iron. [9] Diabetes mellitus tends to alter the body's normal immunological response to any infection, including infection by *Mucorales fungi*.[5, 6] Mucor spores enter the body most often through inhalation, and inoculation on skin trauma. In a normal immune system, macrophages will phagocyte spores and prevent germination. In uncontrolled DM, phagocytosis is disrupted as well as polymorphonuklear chemotaxis (PMN) and macrophages are unable to phagocytosis of the spores so that spores are free to develop in the tissue. The spores will swell and form buds which then develop into senocytic hyphae.[10]

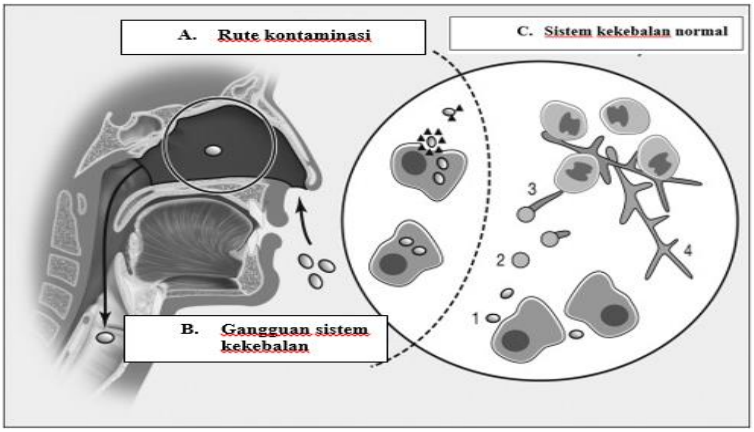

Figure 2: Pathogenesis of the cellular pathway of mucormycosis invasion in diabetic patients, (modified Rammaert *et al.*[10])

Diabetes as a Risk Factor

Diabetes is an important predisposing factor for mucormycosis. In individuals with uncontrolled diabetes, there are changes in immunity, making them highly susceptible to Mucorales infection. Several factors link the susceptibility of diabetes patients to mucormycosis. First, diabetes and ketoacidosis impair the function of phagocytic cells, such as neutrophils and macrophages, due to damaged chemotaxis mechanisms. [11, 12] Second, patients with diabetic ketoacidosis have acidic serum pH with increased levels of free iron, which is a crucial nutrient for the growth of Mucorales. Third, elevated glucose and iron levels are known to increase the expression of GRP-78. GRP-78 is a glucose-regulated protein located in the endoplasmic reticulum, which is translocated to the cell surface and acts as a receptor mediating endothelial cell penetration and damage by Mucorales. In diabetic ketoacidotic rat models, GRP-78 expression is known to increase in the sinuses, lungs, and brain. Administering anti-GRP-78 serum in these animal models has been shown to protect them against mucormycosis. [11, 14]

Innate and Adaptive Immune Responses to Mucorales

Innate immunity consists of physical barriers such as the skin and respiratory mucosa, which prevent microbes from entering host tissues. Mucorales can bypass these physical barriers, particularly through skin trauma or ingestion. After crossing the physical barrier, Mucorales encounter innate immune system cells such as macrophages, neutrophils, and dendritic cells (DC). The ability of Mucorales to cause mucormycosis in humans begins with the failure of the innate immune system, which inherently possesses the capability to kill spores and halt fungal spore germination. [15]

Macrophage ability is key to localizing the infection at an early stage and determining the adaptive immune response, which is more aggressive and specific in pathogenic cases. During germination, Mucorales sporangiospores exit

dormancy, swell, and increase metabolic activity before initiating filament or hyphal growth[16]. The innate immune response by healthy effectors is influenced by the developmental stage of Mucorales spores. Effector immune activity varies depending on the developmental stage of the sporangiospores. After successfully crossing the epithelial layer, spores face innate immune effectors such as macrophages and neutrophils. Macrophages suppress spore germination but do not kill dormant spores. In contrast, swollen spores and hyphae of Mucorales are vulnerable to damage and degradation by macrophages. Neutrophil cationic peptides do not invade dormant spores but induce damage to swollen spores. Additionally, neutrophil superoxide anions (O2) released cause hyphal damage. After crossing the endothelial layer through hyphal invasion and spore internalization, Mucorales invade blood vessels and enter the bloodstream. Here, Mucorales come into contact with platelets that attach to Mucorales spores and hyphae to suppress spore germination and cause hyphal damage.

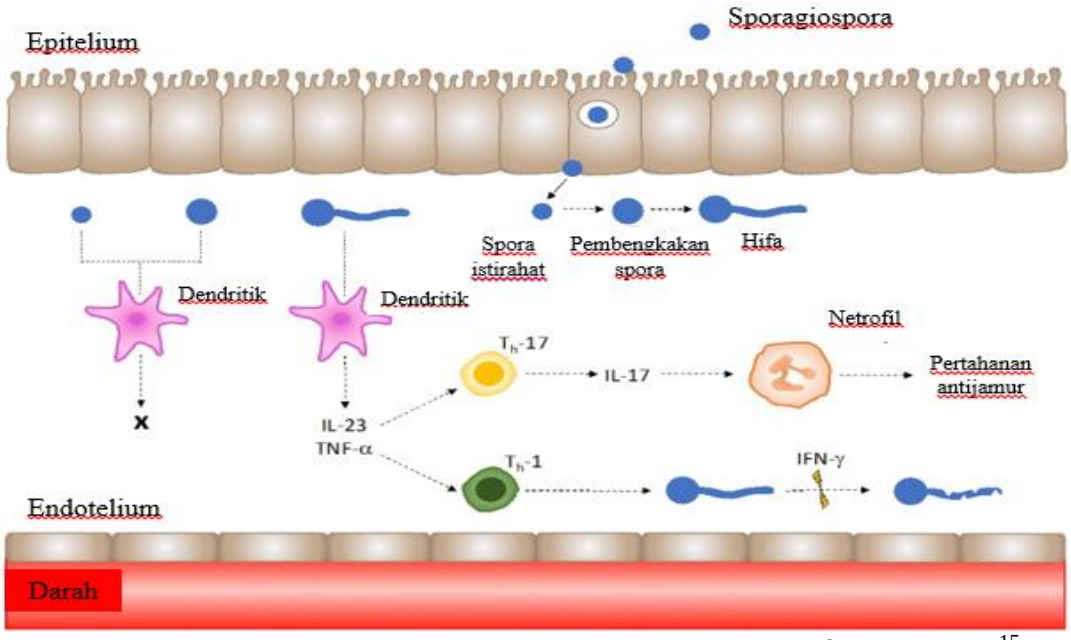

Figure 3. Macrophage response to *Mucorales,* Ghuman modification, *et al*.[15]

Neutrophils

Neutrophils are the most abundant leukocytes found in the blood and are quick to arrive at infection or inflammation sites. They phagocytize and nonspecifically destroy pathogens, playing a crucial role in combating pathogen invasion, including Mucorales. Neutrophils destroy pathogens using cationic and oxidative peptides, and they also mediate acute inflammation and maintain hemostasis. NK cells are lymphocytes that contribute to immunity against pathogens. Mucorales hyphae penetrate epithelial and endothelial tissues, causing extensive tissue damage. NK cells limit tissue damage by inducing cell cytotoxicity. Dendritic cells play a role in the host response to pathogen invasion by acting as

antigen-presenting cells for adaptive immune effectors and triggering the adaptive immune system. Dendritic cells migrate to infection sites in response to the release of microbial antigens, enhancing the immune response. When dendritic cells recognize Mucorales hyphae, they trigger a helper T cell response. Dendritic cells are not activated by Mucorales spores, but exposure to hyphae results in dendritic cell activation and the release of IL-23 and TNF-α. IL-23 promotes a Th-17 response, while TNF-α enhances a Th-1 response. Th-17 produces IL-17, aiding in neutrophil recruitment and antifungal defensin release, while Th-1 secretion of IFN-γ damages Mucorales hyphae.

Platelet and Vascular Endothelial Response to Mucorales

Platelets not only play a role in hemostasis, thrombosis, and inflammation but also in pathogen recognition. Platelets adhere to fungal spores and hyphae, causing damage to hyphal structures and inhibiting hyphal growth in response to fungal contact. Endothelial cells form the inner lining of blood vessels and are crucial for pathogen recognition and maintaining physiological function. Endothelial cells can phagocytize and destroy Mucorales fungal spores. Glucose-regulated protein 78 (GRP78) is a chaperone molecule, a member of the heat shock protein 70 (HSP70) family, and a receptor on the surface of endothelial cells that specifically recognizes Mucor spp. Additionally, GRP78 mediates endothelial cell invasion and damage by Mucorales. Increased concentrations of iron and glucose in diabetic ketoacidotic rats result in increased GRP78 expression on endothelial cell surfaces, particularly in the brain, lungs, and sinuses, compared to normal rats. This can be observed through immunohistochemical examination of ethmoidal sinus tissues infected with Mucorales. Besides endothelial cells, this protein can also be found on the surface of macrophages in necrotic tissue.

Clinical Manifestations of Mucormycosis

Tissue necrosis due to vascular invasion and thrombosis is a hallmark of mucormycosis. Mucorales infections are progressive and have high mortality rates (40-70%), depending on the infection location and host condition[21,22]. The classification of mucormycosis is determined by the anatomical site of infection. Spores enter the body through the respiratory tract, broken skin, or contaminated food consumption. The disease can manifest as rhino-orbito-cerebral, pulmonary, cutaneous/subcutaneous, gastrointestinal, or other organ infections such as the kidneys. Rhino-orbito-cerebral mucormycosis originates in the paranasal sinuses and extends to the brain, starting from the nose, sinuses, eyes, and then the brain. Early symptoms include sinus pain, nasal congestion, fever, soft tissue swelling, and headache. The disease progresses rapidly and, if untreated, leads to surrounding tissue invasion, thrombosis, and necrosis, causing painful black scars on the palate or nasal mucosa. It can also extend to the eyes, causing blurred vision or even total vision loss. From the eyes, the disease can progress to the central nervous system, resulting in decreased consciousness, cranial neuropathy, and brain abscess.

Cutaneous mucormycosis occurs when fungi inoculate through open wounds or burns. An acute inflammatory response is observed as abscesses, skin swelling,

and necrosis. Initially, lesions appear red and then develop into black scars. The infection can progress into deeper tissues, reaching muscles, tendons, or bones, and may cause systemic infection.

Gastrointestinal manifestations occur due to ingestion of food or drinks contaminated with Mucorales, though this is relatively rare. In some cases, infections are due to contaminated herbal and homeopathic medicines. Symptoms depend on the infection site and usually include abdominal pain, nausea, and vomiting. [23, 25]

Diagnostic challenges

Mucormycosis is a disease that causes disability and high mortality. Early diagnosis plays a very important role in the treatment of this disease because the progression of the disease towards worsening takes place very quickly. Establishing early diagnosis faces challenges due to the low sensitivity of direct and cultural examinations.

The clinical material required is usually a biopsy tissue of necrotic lesions taken from the site of infection. The thing that must be considered in handling clinical materials is the manipulation of biopsy tissue to a minimum. Senocytic hyphae are hyphae without partitions/valves so that cytoplasmic leakage is easy which results in the death of the fungus so that it does not grow in culture. This is related to the low sensitivity of the culture method.[26]

Detection of Mold in Tissue

Direct Examination

The direct examination involves inspecting clinical specimens prepared in a 10% KOH wet mount. This method is simple, easy, inexpensive, and provides quick results. Using this examination method, only fungal elements, such as coenocytic hyphae or non-septate hyphae with relatively thick walls, can be identified. Although species identification is not possible, a positive result indicating the presence of coenocytic hyphae is sufficient for initiating antifungal treatment. One limitation of this method is that it requires experienced personnel to obtain quick and accurate results.

Culture Examination

Culture examination is the gold standard for diagnosing mucormycosis. Although Mucorales are fast-growing fungi, it takes 3-5 days for the fungi to grow well on standard media such as Sabouraud dextrose agar. Cultures are incubated at room temperature (25-30°C). Direct examination with 10% KOH is commonly paired with culture examination. Both methods complement each other: direct examination ensures that the fungi growing in culture are not contaminants, and culture allows for the isolation of species for identification, which can predict appropriate antifungal treatments and, if necessary, perform drug susceptibility testing. However, the sensitivity of culture is low, ranging from 25-40%. Fungal identification can be performed morphologically or using molecular-based methods.

Histopathology Examination

Histopathology examination, like direct examination with KOH, is important because Mucorales can be found as contaminants in clinical samples, and finding fungi in tissue confirms the diagnosis. Additionally, tissue reactions can be clearly observed. The stains used in this examination include hematoxylin-eosin (HE) and Gomori methenamine stain (GMS) or Grocott. HE staining reveals coenocytic hyphae—ribbon-like hyphae—and clear tissue responses. Grocott staining shows hyphae as black due to silver impregnation, which is highly contrasted with the background. The background appears green when using light green and reddish when using HE as a secondary stain. Histopathological examination can show tissue changes such as neutrophilic infiltrates, necrosis, thrombosis, and septic infarcts, as well as vascular invasion.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is a method increasingly used to detect Mucorales. This method uses primer-mediated enzymatic amplification to synthesize a new complementary DNA strand to the targeted template strand. The specificity of the PCR reaction allows it to be used directly on clinical specimens containing large amounts of fungal nucleic acids without obtaining pure colonies grown in culture. The limitation of PCR in diagnosing fungi is the low amount of fungal DNA in clinical samples compared to bacteria. This limitation drives variations to increase PCR sensitivity.

One variation of PCR used to detect Mucorales is real-time qPCR, which targets the ITS1/ITS2 regions for identifying Rhizopus oryzae, Rhizopus microsporus, and Mucor spp. Real-time qPCR uses specific primers designed to amplify parts of the b cytochrome gene and specifically target two different regions, 18S and 28S, enabling the detection of DNA from various Mucorales species.

In addition to real-time qPCR, there is a PCR RFLP-based molecular technique for diagnosing and identifying Mucorales by targeting the 18S region. This method involves adding restriction enzymes to the amplicons and then comparing the results with standard Mucorales strain band patterns using electrophoresis, allowing for specific results. In Indonesia, the limitations of this method lie in the availability of laboratories/equipment and laboratory personnel capable of performing it.

CONCLUSION

Mucormycosis is an invasive fungal infection that can occur in patients with various risk factors such as uncontrolled diabetes, organ transplantation, neutropenia, burns, etc. Due to the high destructive power of tissues, early diagnosis should be established as soon as possible. Understanding pathogenesis is invaluable for seeking early diagnosis so that fast and accurate therapy can be provided.

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