Current Tropical Medicine Reports
https://doi.org/10.1007/s40475-020-00222-1

TROPICAL MYCOSES (L MARTINEZ, SECTION EDITOR)

Host-Pathogen Molecular Factors Contribute to the Pathogenesis of *Rhizopus* spp. in Diabetes Mellitus

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Accepted: 20 November 2020

Abstract

Purpose of Review Infectious diseases represent up to 12% of all deaths in people with diabetes mellitus (DM). The development and progression of DM generate a chronic inflammatory state with unique characteristics that have been exploited by some pathogens; one of them is *Rhizopus* spp., a fungus considered the causative agent of mucormycosis. This disease has a poor prognosis with high mortality rates, and the apparition of resistant isolates each year has become a worrying concern. DM is an actual and continuing health problem, and for that reason, it is of foremost importance to study the pathogenesis of mucormycosis to generate new prevention and treatment strategies.

Recent Findings The worldwide incidence of mucormycosis has increased in recent years. The pathogenic mechanisms and factors identified in *Rhizopus* spp. are the cell wall, spore germination, proteins, and enzymes related to iron sequestration, CotH fungal protein, positive regulation of the GRP78 cell receptor, and immune evasion due to survival within phagocytes, among others. The physiopathology of DM offers favorable conditions for the successful replication of *Rhizopus* spp.

Summary The main reason for increase of incidence of mucormycosis caused by *Rhizopus* spp. has been associated with the rise of worldwide prevalence of DM. Knowing the fungal pathogenic mechanisms as well as the relationships between *Rhizopus* with the microenvironment found in the human body will undoubtedly help generate better antifungals to enhance treatment outcomes. Nowadays, some strategies to combat the fungus are based on the knowledge of its proteins, cellular interactions, and iron metabolism.

Keywords *Rhizopus* · Mucormycosis · Diabetes mellitus · Fungal infection · Evasion

Introduction

According to the World Health Organization, 422 million people worldwide live with diabetes mellitus (DM), 1.6 million die each year, and it has been estimated to be the seventh leading cause of death by 2030. Type 1 diabetes mellitus (T1DM) is an autoimmune disease and type 2 (T2DM) is related to environmental factors and represents almost 95% of cases. The American Diabetes Association estimated the cost of diabetes in $327 billion in 2017, the largest amount spent on treating comorbidities associated with a lack of glycemic control. Moreover, 12% of deaths in people with diabetes are attributed to infectious diseases [1–3]. A good example of DM vulnerability is the current global pandemic of SARS-CoV2, where many studies show DM as one of the most common comorbidities with an increased risk of disease complications [4, 5].

In general, DM is considered a risk factor for severe forms of soft tissue, urinary, and respiratory infections. The development and progression of DM generate a chronic inflammatory state that predisposes infection by *Rhizopus* spp., a fungus that is the causative agent of mucormycosis. The worldwide incidence of mucormycosis has increased in recent years, and some outbreaks of the disease have been reported recently [6–8]. The main reason for this increase has been associated

This article is part of the Topical Collection on *Tropical Mycoses*

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Published online: 22 January 2021

with the rise of immunodeficiency conditions in the population. It is an opportunist infection and several risk factors associated with mucormycosis are male sex, age between 39 and 61 years old, having factors that trigger immune dysfunction like the use of steroids and immunosuppressive therapy, and infectious, metabolic, and hematological diseases [7–10]. Mucormycosis is also classified as a nosocomial infection since a small percentage is present in hospitalized patients due to the use of contaminated material, such as medical adhesive tapes or vascular devices. Likewise, studies have shown the existence of this pathology in patients without comorbidities and a good immune response [8, 11].

Mucormycosis has a poor prognosis with high mortality rates (70–95%) primarily due to a late diagnosis from a deficient widespread availability of rapid and specific diagnostic assays (Soare AY, 2020). Treatment is aggressive and invasive because the removal of necrotic tissue is required to enhance the action of the antimycotic. Further concern involves the apparition of resistant isolates each year, representing an imminent public health problem [12]. These data sustain the fact that mucormycosis is a complex emergent disease that requires intensive studies. On the other hand, it should be noted that rhino-orbital-cerebral mucormycosis is the most frequent clinical presentation and the main risk factor is DM [6, 13]. Worldwide more than 60% of patient with DM (75% in México) have poor glycemic control, which makes them susceptible to opportunistic infections such as mucormycosis [14, 15]. Considering that DM is an actual and continuing health problem, it is of foremost importance to study the pathogenesis of mucormycosis to generate new prevention and treatment strategies.

Mucormycosis, a Challenging Emergence Disease

Respiratory infectious diseases are predominant infections in DM population. Viruses and bacteria are the most frequent causal agents. It has been shown that 7 to 14% of COVID-19 patients have DM as the main comorbidity [16, 17] . There is a risk factor up to 3 times higher for developing tuberculosis or SARS, 5 to 6 times for severe infection by *Influenzavirus*, 7 times for severe disease by MERS-CoV, and 12 times for severe melioidosis infection when DM is present as a comorbid condition [4, 18–20]. However, fungal pathogens are also important causal agents because mycoses are more frequent, recurrent, and severe in patients with DM in comparison with non-DM patients.

Mucormycosis is an emergent and opportunist fungal disease that includes six main clinical presentations, rhino-orbital cerebral mucormycosis (*ROCM*), and pulmonary, cutaneous, gastrointestinal, renal, and disseminated mucormycosis. The prevalence of cases per million inhabitants of mucormycosis is 0.6 in Oceania, 1.8 in America, 1.8 in Africa, 8.3 in Europe, and 29.9 in Asia. In the United States of America (USA), the reported prevalence is 3.0 cases per million inhabitants. Nonetheless, due to the diagnostic complexity, a significant number of individuals are not diagnosed, and therefore, the numbers may be higher. The estimated cases in the world are 10,000 per year (Fig. 1) [6, 13]. In developing countries, the incidence of mucormycosis is linked to diabetes and emerging risk factors like chronic renal failure and hospitalization in intensive care units [6].

As an opportunistic disease, mucormycosis regularly occurs in immunosuppressed individuals. Regardless of the clinical forms of mucormycosis or its infection route (oral, respiratory or transcutaneous), *fungal* spores can frequently penetrate blood vessels and disseminate hematogenously and cause thrombosis and necrosis. The other route of transmission is by direct invasion of the cribriform lamina. Initial *ROCM* symptoms are similar to a sinus infection; the evolution of this pathology can lead to significant damage of the nervous system causing ocular alterations, such as blurred or double vision accompanied by pain. In addition, nerve injury may lead to paresthesia and other symptoms like fever and skin lesions within the nasal cavity. This fungus also causes a significant inflammatory response that could result in purulent arteritis, thrombosis, and tissue necrosis [21].

The immune system is capable of eliminating spores or hyphae easily by phagocytosis. In this context, any abnormalities in the immune system, especially in the innate response, constitute an opportunity for the growth of *Rhizopus* spp. and the development of mucormycosis. In fact, one of the first hematological findings in patients with suspicious lesions is neutropenia and, in this context, patients with neutropenia have nearly 2 times more risk of develop mucormycosis [22]. Other detection methods such as biopsies of lesions could be useful for the diagnosis of mucormycosis. The main biopsy findings include wide and coenocytic hyphae, inflammation, and polymorphonuclear infiltration, including plasma cells and eosinophils. Moreover, the presence of suppurative necrosis with infiltration of neutrophils, and giant or epithelioid cells is highly suggestive of mucormycosis [21]. On the other hand, some imaging techniques enable the diagnosis when the infection occurs through the direct invasion of the ethmoid sinuses. For instance, computed tomography (CT) and magnetic resonance imaging (MRI) have detected sinus opacification, bone erosion (osteomyelitis that may affect cranial nerves), and obliteration of deep fascia planes [21].

A timely diagnosis of mucormycosis is important for a good prognosis and disease resolution because treatment must focus on the patient's symptoms. Treatment usually consists of surgical treatments such as debridement accompanied by pharmacological treatment (amphotericin B) [21]. However, the mortality of mucormycosis remains high (approximately 70%). Antimycotic treatment is complicated because the main

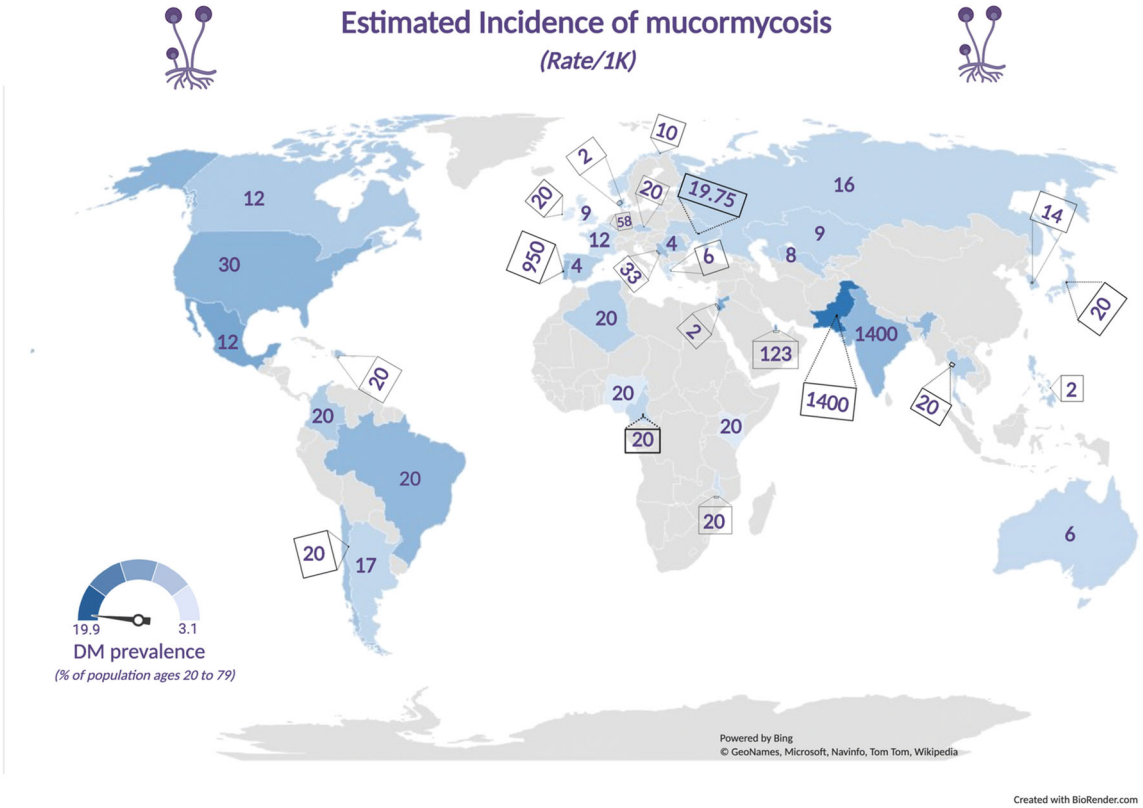

Fig. 1 Estimated prevalence of DM and incidence of mucormycosis. Data obtained from Prakash et al., 2019, and *Atlas from International Diabetes Federation*, 2019. It can be observed that in some countries with a high prevalence of DM they also present a high incidence of mucormycosis, such as Pakistan (19.9 and 1400 respectively), India (10.4 and 1400 respectively), Portugal (9.8 and 950 respectively), and Qatar (15.6 and 123 respectively) [6, 10]

underlying disease is diabetes mellitus often with kidney complications, and amphotericin B is nephrotoxic with a maximum tolerated dose up to 1 mg/kg/day. *Mucorales* species, including *Rhizopus* spp., are resistant to nystatin and triazoles; therefore, there are limitations in antifungal therapy [23]. In this regard, understanding the pathogenicity-associated factors that are involved in the development of mucormycosis is essential for the generation of new treatment strategies.

Rhizopus spp. as an Etiological Agent of Mucormycosis

Until now, species from more than 10 genera have been identified as causative agents of mucormycosis, where members of the *Mucoraceae* family are the most frequent (*Rhizopus* spp., *Mucor* spp., and *Rhizomucor* spp.) following the *Lichteimiaceae* family and the single member of the *Cunninghamellaceae* family (*C. bertholletiae*) [24]. In general, *Rhizopus* spp. is the most frequent genus related to mucormycosis and it is mainly associated with the most frequent clinical presentation, ROCM. In addition, it is one of the three agents with the highest mortality rates (*C. bertholletiae* 77%, *Rhizopus* spp. 57%, and *Mucor* spp. 41%), primarily associated with diabetes as the comorbidity [12, 25, 26].

The genus *Rhizopus* belongs to the *Mucormycota* division and the *Mucorales* order and is one of the 19–21 genera from the *Mucoraceae* family. The *Mucorales* order is characterized by non-septate mycelium, abundant anamorphic sporangiospores in a diverse-shaped columella within a sporangium and teleomorphic zygospores, often with specific ornamentation [27]. *Rhizopus* spp. is aerobic, thermotolerant, fast-growing, saprotrophic, and ubiquitous in soil, animal excrement, or bread and rotting vegetables. In addition, it has an optimal growth temperature of 39 °C under conditions of low pH and high glucose concentration. On agar culture medium, *Rhizopus* spp. generates white cottony colonies that could change from gray or yellow and it also produces organic acids, ethanol, and hydrolytic enzymes [28].

Rhizopus genomes have an average size of 45 Mb, containing numerous simple sequence repeats, around 40% of GC content (guanine-cytosine content), which has a role in transposon biology and they are organized in 6 to 16 chromosomes [29, 30]. There are 11 to 13 species described in the genus *Rhizopus* grouped in four complex species: *R. microsporus*, *R. stolonifer*, *R. arrhizus* (or *R. oryzae*), and *R. delmar* (*R. arrhizus* var. *delemar*). Compared with other species,

R. arrhizus is most commonly isolated in clinical cases of mucormycosis with higher amylase and lipase activity and increased siderophore production.

The Immunopathological Mechanisms of DM2 Enhance and Fuse with the Pathogenic Mechanisms of *Rhizopus*

Despite the important role of *Rhizopus* spp. in agriculture, the food industry, biotechnology, and human medicine, the mechanisms involved in the growth, metabolism, and immune evasion have been understudied. The pathogenic mechanisms and factors identified in *Rhizopus* spp. include the cell wall, spore germination, proteins, and enzymes related to iron sequestration, CotH fungal protein, positive regulation of the GRP78 cell receptor, and immune evasion due to survival in phagocytes, among others. Most of them are successful due to immunosuppression or favorable conditions found in the host. The physiopathology of diabetes mellitus offers ideal conditions for the successful replication of *Rhizopus* spp., especially in those individuals with complications of uncontrolled hyperglycemia.

T2DM is a metabolic disease that causes persistent hyperglycemia and eventual insulin dysfunction. Hypertrophic obesity triggers the proinflammatory state broadly associated with the etiopathogenesis of T2DM. People with obesity and T2DM have an immunocompromised state characterized by dysregulated, dysfunctional, and/or unresponsive innate and adaptive immune cells [19, 31].

Some studies have shown dysfunctional components of the immune system due to a chronic inflammatory state. Patients with DM have low levels of the complement protein C4 associated with dysfunctional neutrophils and a decreased response to cytokines. Mononuclear cells and monocytes of DM release minor amounts of IL-1 and IL-6 in response to lipopolysaccharide (LPS), main component of the outer membrane of Gram-negative bacteria, that is activated in phagocytosis. Lymphocytes and macrophages have reduced IL-10 production due to increased glycosylation. Furthermore, it has been shown that patients with DM have a reduced major histocompatibility complex I (MHC-I) cell expression, which impairs cell-mediated immunity. Furthermore, DM reduces polymorphonuclear leukocyte mobilization, chemotaxis, and phagocytic activity [19, 31].

Phagocytic cells have an inflammatory role in poorly controlled DM since they cause the most frequent complications, such as infections or vascular damage. Mainly macrophages, but also monocytes, dendritic cells, neutrophils, and mast cells are phagocytic cells [32]. These cells by using metabolic pathways, such as glycolysis to produce energy in the form of ATP and pentose phosphate to generate free radicals, eliminate microorganisms that have the ability to invade the body (Fig. 2). The main function of these cells is phagocytosis, but multiple functions including inflammation have undoubtedly been identified [33]. As the prevalence of DM2 increases, the number of infections caused by fungi such as *Rhizopus* spp. also increases. Recent experimental studies have associated this risk with the malfunction of phagocytic activity and the low percentage of cells responsible for this activity. Conditions such as hyperglycemia and acidosis could cause phagocytic cell dysfunction, thus increasing the risk of *Rhizopus* spp. infections.

Macrophages are phagocytes by excellence due to the fact that they have a broad collection of receptors, such as Toll-like receptors (TLR) or pattern recognition receptors (PRR) that allow the initiation of the phagocytosis process. There are two main subsets of macrophages, classically activated M1 macrophages and activated M2 macrophages. Classical activation requires stimuli as a trigger, such as IFN-g, TNF-a, LPS, or GM-CSF, and once activated, macrophages produce pro-inflammatory cytokines (IL-1β, TNF, IL-12, and IL-18), synthesis of nitric oxide (NO), and the restriction of iron with an antimicrobial purpose. On the other hand, macrophages M2 are activated with the opposite pathway by CSF-1, IL-4, IL-10, IL-13, and TGF-b as they release IL-10 and low IL-12. In this context, M2 macrophages are called anti-inflammatory and they have been identified in allergic diseases, angiogenesis, and tissue remodeling and they are involved in the response against parasites and fungal cells [4, 34]. But, what happens in individuals with T2DM? (Fig. 2).

A well-documented and accepted hypothesis about the pathogenesis of T2DM suggests that high levels of glucose cause cellular hypoxia, endoplasmic reticulum (ER) stress, increased release of reactive oxygen species (ROS), free fatty acids (FFA), and cytokine production in the liver, muscle, and adipose tissue. Hypertrophic cells in adipose tissue release pro-inflammatory cytokines like interleukin-1β (IL-1β), tumor necrosis factor (TNF), and chemokines like CC-chemokine ligand 2 (CCL2), CC-chemokine ligand 3 (CCL3), and CXC-chemokine ligand 8 (CXCL8). M1 macrophages are recruited by the action of TNF-α, and its activation releases more pro-inflammatory cytokines (mainly IL-1β) that generate persistent inflammation and the recruitment of more M1 macrophages (Fig. 2). In addition, at the cytoplasm level of tissue cells, FFA are recognized by TLR-4 activating JNK-AP-I and IKK-NFKB signaling [18, 19, 31]. By doing so, the expression and release of proinflammatory cytokines are increased promoting the local inflammatory state. Infiltration of diabetic tissue with M1 macrophages could contribute to the resistance of *Rhizopus* spp. to phagocytosis since M2 macrophages appear to be better able to activate and then kill fungal cells. For this reason, it is convenient to carry out specific studies that demonstrate the causes of these defects to further develop new therapeutic or preventive measures [35].

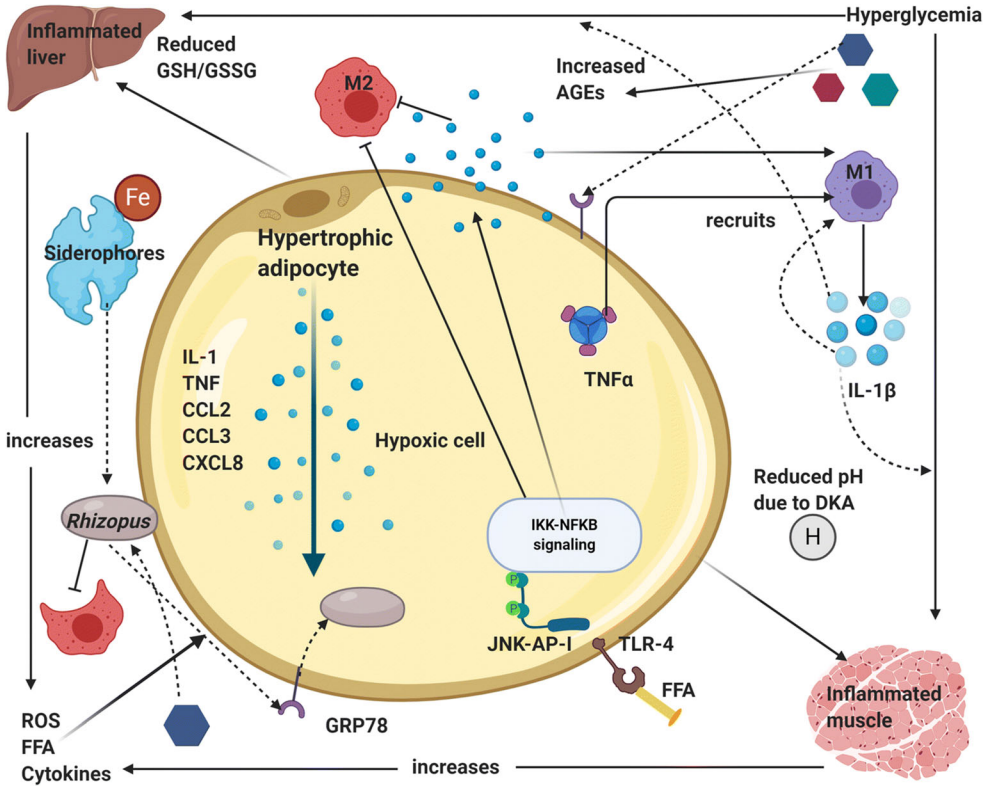

Fig. 2 Immunopathogenesis of comorbidity DM-mucormycosis. High levels of glucose cause endoplasmic reticulum (ER) stress and increased release of reactive oxygen species (ROS), free fatty acids (FFA), and cytokines in the liver, muscle, and adipose tissue. Hypertrophic cells in adipose tissue release pro-inflammatory cytokines like interleukin-1β, tumor necrosis factor alpha (TNF-α) and chemokines like CC-chemokine ligand 2 (CCL2), CC-chemokine ligand 3 (CCL3), and CXC-chemokine ligand 8 (CXCL8). M1 macrophages are recruited by the action of TNF-α and its activation releases more pro-inflammatory cytokines (mainly IL-1β) that generate persistent inflammation and the recruitment of more M1 macrophages. In addition, at the cytoplasm level of tissue cells, FFA are recognized by Toll-like receptors 4 (TLR-4) activating JNK-AP-I and IKK-NFκB signaling. Afterwards, the expression and release of proinflammatory cytokines promote the local inflammatory state. There is a tissue infiltration of M1 macrophages in diabetic patients, promoting an M1 macrophage pro-inflammatory response instead of an M2 macrophage regulatory response. *Rhizopus* uses iron (throughout siderophores) and glucose (which serum levels are increased in type 2 diabetes mellitus subjects due to the diminished cell catchment for insulin resistance) as growth factors and energy source, respectively. Low pH in patients with diabetic ketoacidosis (DKA) generates more susceptibility to mucormycosis because of the generalized oxidative environment which affects glutathione renovation through GSH/GSSG enzyme cycle. *Rhizopus* inhibits phagocytosis or survives to it. Advanced glycation end products (AGEs) and reactive oxygen species derived from the increased glucose metabolism accumulate in organs and tissues triggering the typical micro- and macrovascular alterations in DM patients leading to an increased susceptibility to a *Rhizopus* infection

Some studies have found that the immune system elicited by supplementing high-quality proteins in the diet known as nutritional immunity and triggered by iron restriction inside macrophages inhibits *Rhizopus* growth by a correct phagosome maturation. Patients with mucormycosis show an intracellular persistence in alveolar macrophages (AM), where it was found that *Rhizopus conidia* had melanin surface retention in order to arrest the maturation of LC3-associated phagosome, which also impact IFN-γ secretion. Iron uptake-related mechanisms are a promise for future therapeutics against the infection, since apoptosis is also triggered in *Rhizopus* when there are iron-deprived conditions [36].

The human body needs iron for many vital processes, such as cell growth and development, but this element needs to be bound to host carrier proteins (transferrin, ferritin, and lactoferrin) to avoid the toxic effect of free iron. On the other hand, *Mucorales* use free iron for their own processes; therefore, they must obtain it through multiple mechanisms to convert it into free iron from sources available in the host, or by taking advantage of the host's altered defenses, when there is an increased amount of free iron [37, 38]. In this context, patients with diabetic ketoacidosis (DKA) requiring hemodialysis with deferoxamine chelation treatment have augmented levels of serum free iron, and for that reason, they are more likely to develop mucormycosis [39, 40].

Several studies show that iron has an important role in *Rhizopus*, and like in other fungi, iron is obtained from the host by two mechanisms, either using *siderophores* (iron chelators) or *high-affinity* iron permeases [40–43]. Studies in *Rhizopus* have identified key molecular determinants of iron assimilation, one of them is called *high-affinity system*, which is composed of ferric reductase (Fre), ferroxidase (Fet3), and

high-affinity iron permease (FTR1) [44]. The high-affinity system reduces the free ferric ion Fe^{3+} to Fe^{2+} by ferric reductase (encoded by *fre* genes) to obtain a more soluble Fe form, then Fe^{2+} is oxidized by ferroxidase (encoded by *fet3* genes) to obtain again Fe^{3+}, which is recognized by the *high-affinity iron permease* (encoded by *ftr1* genes) that in the end concede the transport inside the cell [42, 45]. The *high affinity system* is also regulated by the environmental levels of iron and it is activated when there is low availability of it, which allows *Rhizopus* to obtain the highest amount of iron present in the environment. This system is considered a crucial virulence factor since the depletion of the high-affinity iron absorption system has been shown to reduce virulence, trigger growth defects, and upon iron starvation, even induce apoptosis in the fungus [46].

In the siderophore system, fungi compete with the host for the available iron. Fungi siderophore may be intrinsic or extrinsic and *Rhizopus* uses both. Rhizoferrin is the main intrinsic siderophore synthesized by *Rhizopus*, and it takes iron through a receptor-mediated and energy-dependent process. A genome-sequencing study of *R. oryzae* demonstrates 13 possible siderophore permeases that might act as receptors for siderophores. Through several protein crystallography assays, it has been shown that rhizoferrin has a diaminobutane backbone attached to two citric acid residues with an R, R-configuration around a chiral center. However, specific mechanisms of virulence have not yet described for rhizoferrin in contrast with a xenosiderophore desferrioxamine, which chelates iron from transferrin and transports it inside by the high-affinity iron complex [46].

In patients with diabetes, the fasting state generated by the lack of sufficient levels of insulin triggers the activation of the metabolism of amino acids and triacylglycerol (TAGs) stored in adipose tissue as an energy source. Serum concentrations of glycerol and free fatty acids are elevated due to restricted lipolysis, as well as alanine due to muscle catabolism. Glycerol and alanine are substrates for hepatic gluconeogenesis stimulated by excess glucagon that accompanies insulin deficiency. Glucagon also stimulates the mitochondrial conversion of free fatty acids to ketones. Under normal conditions, insulin blocks ketogenesis by inhibiting the transport of free fatty acid derivatives to the mitochondrial matrix, but in the absence of insulin, ketogenesis proceeds [19]. As a product of TAG metabolism, abundant ketone bodies are generated and thus influence serum pH, and this leads to the dysfunction of many serum enzymes. Some of them are transferrin and hemoglobin, which at a pH of 6.88–7.3 remain protonated and incapable of transporting Fe+3, so in patients with DM there is a greater amount of Fe+3 available in serum. In addition to use the available Fe+3 in these patients, *Rhizopus* have a ketone reductase enzyme that allows the development of the fungus in this acidic state [45, 47]. Other host enzymes are affected by the acidosis generated by *Rhizopus* spp. and this has a direct impact on phagocytosis and chemotaxis. Furthermore, it has been reported recently that reduced iron levels trigger the M1 proinflammatory LPS-induced response; thus, another mechanism adds to the polarization of an unfavorable response to fungal clearance [48].

It has been reported that ketoacidosis alone does not predispose to mucormycosis by Lichtheimia; however, DKA causes overexpression of Mucorales proteins and host surface receptors that augment the binding to endothelium and thus increase the risk of fungal invasion [49]. Main alterations in host enzymes caused by ketoacidosis are reversible after improvement in environmental pH; however, if hyperglycemia persists, irreversible dysfunction of many enzymes is possible [50]. For this reason, early detection of ketoacidosis is important in patients with DM. Timely treatment focused on reversal of the acidosis state could contribute to reduce the patient's susceptibility to Mucorales invasion and other infections.

There is another factor to consider for a good phagocytic response: reactive oxygen species (ROS). Oxidative stress is important in other virulence factors that will be discussed later. In individuals with diabetes, as a consequence of insulin resistance, hyperglycemia continues, and in order to reduce the glucose levels, there is an increased secondary lipolysis and glucose metabolism through oxidative phosphorylation. Advanced glycation end products (AGEs) and reactive oxygen species derived from the increased glucose metabolism accumulate in organs and tissues triggering the typical micro and macrovascular alterations in DM patients [51]. Oxidative stress is poorly controlled in these patients because the main antioxidant system of glutathione (GSH/GSSG) is affected by the deficiency of the cofactor NADPH needed for the regeneration of reduced glutathione. NADPH deficiency is due to its fast consumption by the polyol pathway for glucose metabolism. Oxidative stress activates inflammation mediated by NF-kB and TLR receptors; hence, a persistent chronic inflammatory state is eventually generated [52].

Overexpression of GRP78 Protein Caused by DM2 Is Suitable for Host Invasion by *Rhizopus*

Glucose-regulated proteins (GRPs) were first observed in transformed fibroblasts, in which the synthesis of these proteins increased when glucose depletion was induced [53]. The most abundant GRP is a 78-kDa glucose-regulated protein (GRP78), also known as immunoglobulin-binding protein (BiP), located in the lumen of the endoplasmic reticulum and expressed in mammalian cells. GRP78 is encoded by the *HSPA5* gene, assigned to chromosome 9q34. Structurally, GRP78 consists of two functional domains: a nucleotide-binding domain (NBD) and a substrate-binding domain (SBD), and its activity is regulated by the allosteric

ATPase cycle, where NBD binds and hydrolyzes ATP, and SBD binds to polypeptides [54–56]. While new features have come to light in recent years, GRP78 has been traditionally considered a molecular chaperone belonging to the HSP70 family, which acts to control ER stress through regulation of the unfolded protein response (UPR), and it plays a key role in the folding, assembly, and quality control of proteins and misfolded protein degradation [57, 58].

As mentioned earlier, GRP78 is mainly found in the ER, but it also has the ability to translocate and accumulate in other intracellular locations [59]. In the cytosol, GRP78 protein is found as GRP78va, an isoform generated by alternative splicing (retention of intron 1) and alternative translation, which lacks the ER signal peptide. GRP78va has cytoprotective properties and the potential to regulate signaling to the UPR, promoting cell survival under stress from the cytosol [60]. The expression of GRP78 in the intermembrane space, internal membrane, and the matrix of mitochondria is triggered by ER stress, and it participates in UPR signaling inside the organelle [61]. The recent relevance of GRP78 expression is its translocation to the surface of the cell membrane, where it has receptor and regulatory functions in cell signaling by the formation of complexes with extracellular ligands and proteins anchored to the cell surface [59, 62]. In 1997, the GRP78 protein was identified in the cell surface of malignant lymphocytes in patients with acquired immunodeficiency syndrome (AIDS) and cutaneous lymphoma or leukemia [63]. Since then, this protein has been constantly analyzed and is now known to be involved in the proliferation of diverse types of cancer, chronic or inflammatory diseases, as well as the invasion by fungi and some viruses [59]. Thus, the stress in the ER, caused by many pathologies, is known to trigger the GRP78 translocation to the cell surface, where it is called csGRP78, and it can even have antigenic properties inducing the production of anti-GRP78 autoantibodies and it also acts as an associated signal receptor to the membrane [64].

Recent studies have shown that hyperglycemia is a stress trigger in the endoplasmic reticulum, which consequently induces the overexpression of the GRP78 protein depending on glucose concentrations and the persistence of high glucose levels. It has been proposed that GRP78 is translocated to the cell surface from the ER through a mechanism regulated by the MTJ-1 chaperone. Once this protein is located on the cell surface, it interacts with the β1 integrin in charge of mediating phosphorylation of kinases. The activation of the Kinase of Focal Adhesion (FAK) and downstream protein kinase Akt causes fibrosis through the expression of extracellular matrix proteins (ECM), such as fibronectin and type I collagen [65]. On the other hand, studies have shown that the overexpression of csGRP78 plays an important role as the entry receptor of some pathogens, like the *Dengue virus*, *Ebola virus*, *Coxsackievirus*, and the novel *SARS-CoV2*, among other viruses and *Rhizopus* spp. [66–68]. A mouse mucormycosis model has shown that high glucose levels increase 2- to 5-fold higher levels of *Grp78* mRNA in the sinus, lungs, and the brain compared with normal mice and it increased endocytosis up to 40% of *R. oryzae* by human endothelial cells [66]. These first assays suggested that csGRP78 is crucial for invasion but not for adhesion because it is bound by germlings but not by spores. However, it is highly probable that local factors, such as stasis and dehydration of the mucous secretion, superficial lacerations, or retentive anatomical niches, allow the establishment of sporangiospores and their subsequent germination into hyphae.

In addition, it was shown that *Rhizopus delemar* interacts with different receptors in nasal and alveolar epithelial cells. csGRP78 is overexpressed in nasal epithelial cells but not in alveolar epithelial cells when they are infected with *Rhizopus* spp. in vitro. Moreover, it was identified that *Rhizopus* spp. interacts with alveolar epithelial cells by bounding with integrin-β1 and not with csGRP78. Therefore, microenvironmental conditions drive the pathogenicity of *Rhizopus* spp. where glucose, iron, and diabetic ketoacidosis (DKA) trigger csGRP78 overexpression only in nasal epithelial cells, but they do not trigger integrin-β1 overexpression in alveolar epithelial cells. It is possible that this phenomenon is the explanation of the major clinical manifestation of mucormycosis in DM patients known as the rinocerebral presentation, in contrast with other immunodeficiencies where the pulmonary presentation is most frequent [69]. The diversity on cell receptor usage by *Rhizopus* spp. is mediated by the second component in this interaction; csGRP78 specifically interacts with homologous spore coating proteins (CotH) present in the *Mucorales*, allowing invasion and damage to endothelial cells [66, 67, 69, 70].

CotH is a protein kinase belonging to the spore coating protein family, localized and required for the assembly of proteins in the inner layer of the spore coat; it is expressed during sporulation; and its activity is regulated by autophosphorylation with ATP and subsequent phosphorylation of CotB and CotG on serine residues. CotH has a short half-life of 4 to 5 h and its concentration falls shortly after the transcription of the structural gene has been deactivated. This protein has been recently identified and is a required component for spore germination in many bacterial and eukaryotic species, including human pathogens, such as spore-forming bacteria *Bacillus anthracis* and spore-forming fungi *Rhizopus oryzae* [67, 71, 72]. When GRP78 was identified as the necessary receptor for Mucorales invasion [66], the search for the putative ligand allowed the identification of CotH in *Mucorales* [67]. Thus, it has been possible to identify the expression of the *CotH1*, *CotH2*, and *CotH3* genes in a variety of members of the *Mucorales* order. However, in the DKA context, evidence has been shown that *CotH3* is mainly expressed in the germinations of *R. oryzae* and shows a greater capacity to adhere and, therefore, invade endothelial and nasal epithelial cells. Some studies have established that when

exposing endothelial cells to germination of *R. oryzae*, the proteins that specifically reflect a greater expression on these include CotH2 to a lesser extent and CotH3 with a relatively higher proportion, which were recognized as the specific fungal ligands that bind to the GRP78 protein, promoting the invasion of the host endothelial/nasal epithelial cells [67, 69, 70, 73]. (Fig. 3). In contrast, in pulmonary mucormycosis, CotH7 is the major ligand that interacts with integrin-β1 of alveolar epithelial cells and it is not closely related with CotH3 (~ 50% amino acid identity) [69].

The mechanisms through which the interaction between the invading fungi and the endothelial/epithelial cells are promoted begin to have a solid foundation and represent an important step in the pathogenesis of diabetes-associated mucormycosis [66, 67, 69]. High glucose, iron, and B-hydroxy butyrate (BHB) as the predominant ketone body improve the growth of the fungus through inducing the expression of CotH3 in the DKA context [74]. The surface relocation of the GRP78 protein that copes with the stress of the endoplasmic reticulum caused by hyperglycemia and an acid microenvironment promotes a tissue stage predisposed for the establishment of *Rhizophus* spp. In this same tissular niche, the iron release from sequestered protein transferrin occurs through glycosylation or protonation processes. Therefore, high concentrations of glucose, the available free iron [36], and an acid microenvironment enhance CotH expression on the fungal cell surface, resulting in GRP78/CotH3 interaction and allowing endothelial/epithelial invasion and fungal dissemination [74]. After invading the superficial nasal epithelium, the fungus must interact with its basement membrane, since spores and stem cells from germ tubes bind to components of the extracellular matrix. This is supported through the observation of *Rhizopus* spp. attaching to plates coated with laminin and collagen IV [75].

In neutropenic mice infected with *R. delemar* strains, polyclonal antibodies of the peptide regions of CotH3 (anti-CotH3) showed reduction in their fungal load, by preventing invasion and decreasing their subsequent hematogenous

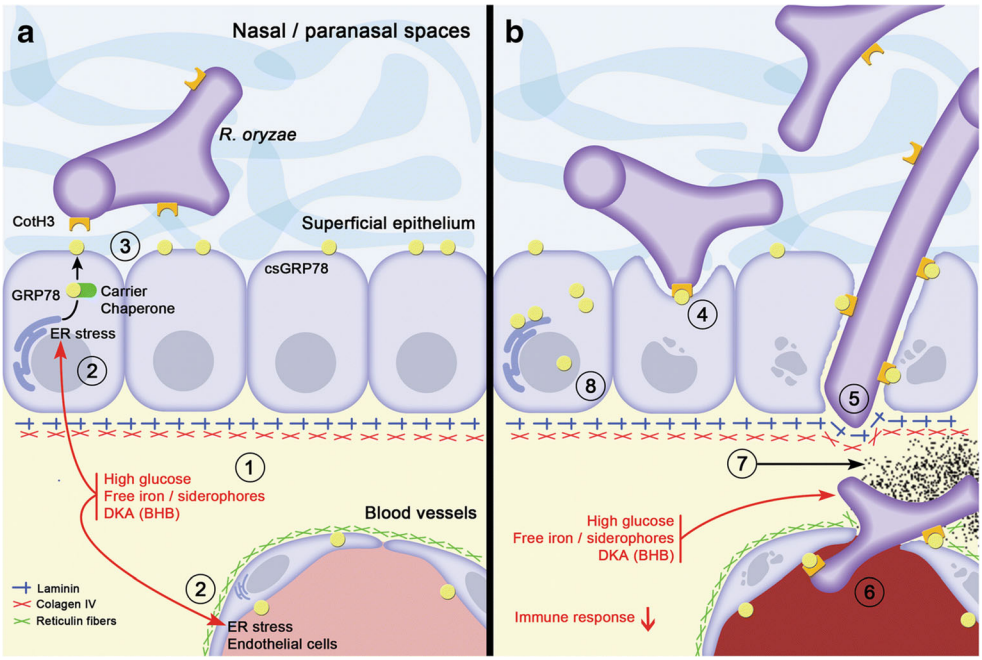

Fig. 3 Proposed mechanisms for the establishment of rhino-orbital-cerebral mucormycosis in diabetic patients. **a** (1) Tissular microenvironment in a diabetic patient is altered; there are high levels of glucose, free iron, and ketone bodies (BHB, B-hydroxy butyrate); and these conditions cause stress on the endoplasmic reticulum in the adjacent epithelial/endothelial cells. (2) In response to ER stress, GRP78 is overexpressed and relocated in diverse cellular compartments, particularly on the cell surface, carried by several co-chaperone proteins like MTJ-1 and Par-4. (3) Once GRP78 is exposed on the cell surface (csGRP78), it favors the possibility of interaction with the hyphae of *R. oryzae* through the expression of their CotH3 proteins. **b** (4) The interaction between the GRP78 and CotH3 proteins promote hyphae that can damage cells and penetrate the epithelium. (5) The association of *R. oryzae* hyphae to laminin and type IV collagen in the basement membrane allows their adherence and entry into the interstitium where the fungi can approach blood vessels. The microenvironmental imbalance offers favorable conditions for fungus growth: energy (high glucose), free iron for its metabolic requirements, as well as a conducive acidic environment generated by ketone bodies. (6) Meanwhile, endothelial cells continue to produce GRP78 in all compartments and the hypha can associate with these proteins on the basal side where the presence of reticulin fibers are exceeded, which allows it to anchor and outflank this area to later interact with GRP78 expressed on the luminal surface of endothelial cells. Once internalized in the lumen of the blood vessels, the fungi consequently induce the extrinsic coagulation pathway activation, consequently cell damage occurs, and all this triggers the formation of thrombus. (7) This results in ischemia and sustained hypoxia, which generates infarction of the tissues and its consequent necrosis. (8) Finally, the altered microenvironment in diverse tissular compartments of these patients generates GRP78 overexpression, allowing *Rhizopus* spp. to find this protein in any type of epithelial or endothelial cell, establishing its interaction and continuing its invasive behavior

spread and recruiting phagocytes and the elimination of fungi through opsonophagocytosis. Unfortunately, the presence of natural anti-CotH3 antibodies generated in exposed volunteers and patients infected with mucormycosis are too low to exert a protective activity as the one provided by anti-CotH3 in a murine model [67]. Nonetheless, other cellular and fungal proteins may be involved in mucormycosis pathogenesis because in vitro assays with different epithelial cells by using anti-CotH antibodies did not completely block *R. delemar*-mediated damage of host cells [69]. However, improving the generation and use of anti-CotH3 antibodies represents a promising immunotherapeutic alternative.

Rhizopus spp. Interactions: New Approaches for Treatment

A recent study found that it is possible that bacterial endosymbionts of *Rhizopus* spp. have an effect on the virulence by innate immune evasion. The bacterium *Ralstonia pickettii* in endosymbiosis with *Rhizopus microspores* increases its ability to survive within macrophages, which attack and trigger a profuse pro-inflammatory cytokine release. *R. pickettii* is a Gram-negative bacillus that is considered a nosocomial pathogen frequently found contaminating medical solutions as sterile water, disinfectants, and saline solution. It is possible that *R. pickettii* participated in the last mucormycosis outbreaks reported in hospitals [8].

Patients with diabetes and allogeneic hematopoietic stem cell transplantation (another common mucormycosis risk group) have recently shown oral dysbiosis characterized by an increment of *R. pickettii* proportions [76]. This finding could have an impact in the incidence of nosocomial mucormycosis. An increased presence of *R. pickettii* in patients with diabetes and obesity has been reported. Therefore, *Rhizopus* infection in these patients could be due to the possibility that both microorganisms coincide and benefit each other, precisely in this type of patients. New approaches based in re-establish microbiota in DM patient could be useful.

Amphotericin B, posaconazole, and isavuconazole are the best available antifungals for the treatment of mucormycosis. A recent analysis of matching cases showed that new formulations of posaconazole were associated with lower mortality rates and favorable responses of up to 80% in invasive mucormycosis compared to amphotericin B [77]. Voriconazole, a polyene derived from triazole and better tolerance than amphotericin B showed that in 25 Mucorales strains (of which 18 were of the genus *Rhizopus*), voriconazole/amphotericin B combinations were more effective at inhibiting the growth of *Rhizopus* spp. in vitro and using the *Galleria mellonella* mucormycosis model compared to amphotericin B [78]. However, the results of the few analyzes performed in recent years with small size samples should be considered with caution. Therefore, the study of competitive interactions between *Rhizopus* and other microorganisms have been of interest for the search of new antifungals.

Evidence of competitive interactions between fungi and bacteria has been used for the development of antifungals. A recent study described use of tanzawaic acids from hot spring-derived *Penicillum* sp. as a lethal dose-dependent agent against *Rhizopus oryzae*, with similar efficacy to that obtained with amphotericin B and with no effect against other fungi such as *Candida* spp., *Aspergillus* spp. or *Cryptoccoccus* spp. [79]. Other study has shown that bacterial strains such as *Serratia marcenses*, *Serratia proteamaculans*, *Bacillus subtillis*, and *Neurospora crassa* significantly inhibit the growth of *Mucor circinelloides*, *Actinomucos elegans*, and *Rhizopus stolonifer* within 24–72 h after interaction with these bacterial strains or with derived volatile compounds [80].

On the other hand, the fungus cell wall is a mechanism of pathogenicity with the ability to evade interactions, mainly with immune system cells. There is a replication of some gene families in the genome of Mucormycotina subdivision lineage that suggests a whole genome duplication event as an evolution response for adaptation to the environment [29]. Chitin deacetylase and chitin deacetylases families of proteins are widely and diversely represented in the *Rhizopus oryzae* genome. These two enzymes are involved in chitosan or chitin synthesis, which is the main component of the fungal cell wall, and thus are involved in evading the immune system [81]. However, there are still no antifungal against this potential target.

Spore germination is the crucial mechanism associated with the pathogenic mechanism of angioinvasion, thrombosis, and tissue necrosis; some triggers of this mechanism are changes of nutrients, light, osmolarity, and pH among others. Experiments using *R. delemar* have shown that ungerminated spores have more than 6000 significantly expressed genes from germinated ones. Within 6 to 12 h of hyphal growth, the most relevant genes with augmented expression were those involved in mitochondrial enzymes, organophosphate and sulfur metabolism, transposase, ATPase, nucleoside triphosphatase, and a vast repertory of stress response genes [24]. Phase differences in proteome make difficult to generate vaccines or identify important antigenic compounds.

R. oryzae is a complex fungus to be manipulated in the laboratory and presents many challenges for the in vitro experimentation, thus limiting the research about virulence mechanisms. The inefficiency of null mutant retrieval because *R. oryzae* appears to have multiple copies of its genes has recently been resolved using state-of-the-art techniques such as genome editing using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9

(Cas9), where by this technique a point mutation was induced in *R. oryzae* [82]. Although the RNA interference and CRISPR/Cas9 approach have been more used in other fungi, this first relatively easy manipulation performed in *Rhizopus* will undoubtedly bring new findings about virulence mechanisms in the following years.

Conclusion

Fungal pathogens such as *Rhizopus* spp. have been little studied. The uncontrolled increase in metabolic diseases in recent years has generated favorable microenvironments for the development of mucormycosis; *Rhizopus* spp. is a fungus whose genome contains information for its adaptation to different environmental conditions, so the human body offers a very favorable microenvironment in patients with immunocompromised conditions and chronic inflammation, such as those caused by diabetes mellitus. Although the treatment of mucormycosis is based on the control of DM characterized by immunosuppression, there is a critical need to identify new prophylactic measures or therapeutic targets against *Rhizopus* spp.

However, progress in this area has been very limited in recent years. There are still no approved vaccines against fungal pathogens and the availability of drugs for the treatment of mucormycosis is limited to a few. Although promising treatment alternatives such as the use of in vitro-reactivated T-specific lymphocytes or the use of heat-killed yeast, *Saccharomyces cerevisiae*, as a candidate vaccine, have recently been reported, there have been no other approaches in recent years. It is necessary to continue deepening knowledge about the pathogenic mechanisms, the relationships between *Rhizopus*, and the microenvironment found in the human body. This knowledge, as well as the use of cutting-edge techniques such as CRISPR/Cas9 for the genetic modification of the complex genome of *R. oryzae*, will undoubtedly contribute to develop better antifungal agents, treatment alternatives, and vaccine development.

Acknowledgments We appreciate the training support for Anaya-Ambriz EJ and Sánchez-Nuño YA, received from the Maestría en Microbiología Médica at UDG and the scholarship that they receive from CONACYT. Also, the training support for Morales-Franco B received from Doctorado en Farmacología at UDG.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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