

RESEARCH ARTICLE

Correlation of Pathological Manifestations in Covid 19 Positive Cases Complicated by Either Isolated Mucormycosis or Co-Infection with Other Fungi: Experiences of a Tertiary Level Institute

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Abstract:

As a part of multi-disciplinary team of the institute in managing Covid positive patients as well as those admitted with its complications, Pathology department was reporting specimens of suspected fungal infection, received from clinical departments like neurosurgery, otorhinolaryngology, ophthalmology, oral and maxillofacial surgery, respiratory medicine, internal medicine etc. Simultaneous serology and various Covid associated blood parameters were being investigated during admission to hospital, as per the clinical scenario.

The **aim of this paper** is to discuss pathogenesis of fungal infections and bring out any significant pathological differences in Covid 19 positive cases afflicted subsequently by either mucor alone or mixed fungal infections.

Out of 274 tissue specimens received between April to November 2021, clinically suspected to be of Covid 19 associated mucormycosis, we found 14 cases of simultaneous co-infection with other species of fungi. 45 specimens were reported negative for fungal elements while 229 were confirmed by histo pathological examination.

Cases were grouped according to the presence of either only Mucormycosis on histology or mucor with co-infecting fungi. Various biochemical, hematological and histopathological parameters were compared and significance of difference analysed using student t test in the two groups.

Statistically significant difference was observed in mean values of serum **ferritin** (p value 0.005); C-Reactive Protein/**CRP** (p value 0.003); serum **creatinine**, Random Blood Sugar/**RBS**, **Haemoglobin**, Total Leucocyte Count/**TLC** and duration of hospital stay (p value of each being 0.00) while p value was **insignificant** in serum Lactate Dehydrogenase/**LDH** and InterLeukin 6/**IL6** values between the two groups.

Platelet count of patients in both the groups were within normal range. (1-4.5 x1000/cu mm). **None of the Histopathological parameters showed any statistically significant difference** in the two groups (p value of each was more than 0.05).

Introduction:

During the second wave of Corona virus pandemic, patients were being operated on both elective and emergency basis when

deteriorating, and transferred to Intensive care Units from isolation wards. Specimens of suspected fungal infection were routinely being sent to Pathology and

Microbiology departments for confirmation.

Mucormycetes or the group of fungi causing mucormycosis, are present in the environment in soil and with dead organic matter. The order they belong to is Mucorales which includes Rhizopus species (responsible for the majority of cases), *Mucor*, *Cunninghamella bertholletiae*, *Apophysomyces*, *Lichtheimia* (formerly *Absidia*), *Saksenaea* and *Rhizomucor*. [1]

Humans are regularly exposed to the spores from many species. The most commonly encountered fungal diseases are the superficial mycoses. Some fungi may elicit a range of host reactions from exudative, necrotizing to granulomatous whereas other fungi produce little cellular response to indicate their presence.

Close mimickers of the naso-sino-orbital area mucormycosis are invasive aspergillosis and malignancies. All such cases present with facial mass, proptosis and local inflammation. Once angioinvasion sets in, rapid tissue destruction ensues resulting in blindness and even cerebral mucormycosis. [2]

However, treatment may differ and unnecessary treatment with other non specific antifungals like voriconazole (with unpleasant side effects) should be avoided. The first line therapy for mucormycosis is liposomal amphotericin B which was given to patients in our hospital as well. [3]

Histopathological examination using Hematoxylin & Eosin as routine stain along with Grocott-Gomori's Methanamine silver (GMS) and Per-iodic Acid Schiff (PAS) as special stains of fixed tissue for fungi is useful, but it may be difficult to distinguish *Aspergillus* from *Fusarium* and *Scedosporium*, which are also filamentous. [4]

Characteristic Morphology and features on histopathology:

Mucormycosis species appear as broad, non septate hyphae, ribbon like linear elements of 5-25 micron diameter, branching at right to obtuse angles. These can be differentiated from *Aspergillus* which shows regular, septate, and acute angle branching hyphae, *Candida* as budding yeast and *Actinomyces* as abscess with grain formation and pink fillaments.[5]

Mucormycosis infections are characterized by extensive angioinvasion that results in vessel thrombosis and subsequent tissue necrosis. This angioinvasion likely contributes to the capacity of the organism to hematogenously disseminate to other target organs. Ischemic necrosis of infected tissues can prevent delivery of leukocytes and antifungal agents to the foci of infection.

Combined mucormycosis and aspergillosis infections in diabetic patients [6], a patient with Castleman disease [7], and renal transplant recipient [8] are available in literature.

Invasive mucormycosis causes severe morbidity, particularly and mortality in patients with the following:[9]

- hematological malignancies,
- diabetes mellitus,
- hematopoietic stem cell transplant and solid organ transplant patients,
- patients on corticosteroid-based therapy,
- iron overload and chelation therapy,
- intravenous drug use,
- trauma, burns, neonatal prematurity, and malnutrition

Fungi are often weakly hematoxyphilic and can be suspected on H&E stains. Fluorochrome-labeled specific antibodies to many fungi are available, and are in use in mycology laboratories for the identification of fungi on fresh and paraffin wax sections. These antibodies have not found widespread use on fixed tissue, where identification still relies primarily on traditional staining methods. H&E, PAS and GMS permit most fungal infections to be identified sufficiently for diagnoses. However, there is no substitute for microbiological culture. [10]

Material And Methods:

The various surgical specimens received for histopathology were:

- Tissue obtained on surgical debridement, scrapings/crust from nasal cavity/sinus,
- Eyeball/Orbital contents
- Brain abscess
- Maxilla/palate/alveolus/jaw
- Cell blocks prepared from secretions of respiratory tract and body fluids (like broncho alveolar lavage) and even nasal packs soaked in 10% formalin solution

Tissue processing and staining

- The specimens were processed, embedded in paraffin blocks, cut as 4-6 micron sections
- Stained with Hematoxylin and Eosin
- Special stains like Periodic acid Schiff (PAS) and Gomori Methanamine Silver (GMS) were used to highlight the hyphae as dark magenta and black colored structures respectively.

These observations can help confirm clinical suspicion at an early stage of disease

Hematoxylin and Eosin (H & E):

Standard protocol was followed. [11]

Non-automated Harris's hematoxylin and eosin stain for paraffin sections:

1. Dewax sections, rehydrate through descending grades of alcohol to water.
2. Remove fixation pigments if necessary.
3. Stain in hematoxylin for a suitable time.
4. Wash well in running tap water until sections 'blue' for 5 minutes or less.
5. Differentiate in 1% acid alcohol (1% HCl in 70% alcohol) for 5–10 seconds.
6. Wash well in tap water until sections are again 'blue' (10–15 minutes).
7. Or blue by dipping in an alkaline solution followed by a 5-minute tap water wash.
8. Stain in 1% eosin Y for 10 minutes.
9. Wash in running tap water for 1–5 minutes.
10. Dehydrate through alcohols, clear, and mount.

Results : Nuclei blue/black Cytoplasm varying shades of pink and fungal hyphae often stained deep blue-black.

Periodic acid Schiff (PAS): Standard protocol was followed. [11]

1. Dewax in xylene and rehydrate through graded [SEP]ethanols to distilled water. [SEP]
2. Oxidize with periodic acid for 5 minutes. [SEP]
3. Rinse in several changes of distilled water. [SEP]
4. Cover the sections with Schiff reagent for 15 [SEP]minutes. [SEP]
5. Rinse in running tap water for 5–10 minutes. [SEP]
6. Stain the nuclei with hematoxylin. Differentiate [SEP]and blue the sections. [SEP]

7. Dehydrate in graded ethanols and clear with xylene [11]

Result: The PAS technique is based upon the reactivity of free aldehyde groups within carbohydrates (as in wall of fungi) with the Schiff reagent to form a bright red/magenta end product.

Gomori Methanamine Silver (MGS): Standard protocol was followed. [11]

1. Deparaffinize sections and take to water.
2. Treat with 1% potassium permanganate solution for 2 minutes.
3. Rinse in tap water.
4. Bleach in 2% potassium metabisulfate solution.
5. Rinse in tap water.
6. Treat with 2% iron alum for 2 minutes.
7. Wash in several changes of distilled water.
8. Place in Coplin jar of silver solution for 1 minute.
9. Wash in several changes of distilled water.
10. Reduce in 4% aqueous formalin solution for 3 minutes.
11. Rinse in tap water.
12. Tone in 0.2% gold chloride solution for 10 minutes.
13. Rinse in tap water.
14. Treat with 2% potassium metabisulfate solution for 1 minute.
15. Rinse in tap water.
16. Treat with 2% sodium thiosulfate solution for 1 minute.
17. Rinse in tap water.
18. Counterstain as desired, van Gieson or eosin is suitable.
19. Dehydrate through ascending grades of alcohol.
20. Clear in xylene and mount medium.

Results : Fungal hyphae and spores are stained black.

Report format for histopathology [12]

Each section was thoroughly searched for evidence of the following details:

- Soft tissue invasion (mucosal penetration),
- Destruction of cartilage or bone (nasal turbinate),
- Necrosis and inflammation,
- Mucosal metaplasia from columnar to sturdy squamous epithelium
- Angio invasion, and
- Mycelia/Hyphae and/or spores

Simultaneous serology and various Covid associated blood parameters of these patients were analyzed, done under strict protocols with quality controls, during their admission to hospital.

OBSERVATIONS:

- Total cases: 274 (including 12 Recurrent/repeat biopsies)
- Mucor only: 215
- Co-infection: 14 (aspergillus, actinomycosis, candida)
- HPE negative for any fungal element: 45

All the patients were adults. There were no cases of juvenile diabetes admitted for mucormycosis.

Minimum hospital stay of patients was 4 while the maximum was 70 days in the present study.

All the patients were non-reactive for HIV I & II, HCV and HbsAg. There was no significant difference in any laboratory or histological parameter amongst male or female patients.

Cases were grouped according to the presence of either only Mucormycosis on histology or mucor with co-infecting fungi. Various biochemical, hematological and histopathological parameters were compared and significance of difference analysed using student t test in the two groups.

Necrosis and bone invasion were not evident in all biopsies as the tissue specimens were received from different surgical specialities and hence specimen type and size were variable.

The inflammatory infiltrate was seen in all biopsies; **mixed** acute and chronic inflammatory cells (polymorphs and

mature lymphocytes together) were predominant in **147 cases**, closely followed by chronic inflammatory cells in **123 cases** and only neutrophilic/ infiltrate in **4 cases**. Simultaneous neutrophil count in blood of the patients ranged between 60-80% out of total leukocyte count, in patients of both the groups being compared.

Table 1: Hematological and biochemical parameters

Parameter (mean value)	Mucor Only (n=215)	Co-infections (n1=14)	Reference range used in our laboratory	P value
Ferritin (microg/L)	1140.00	570.75	30-220 (males); 20-110(females)	0.005
CRP (mg/dl)	77.09	39.74	0-6	0.003
LDH (IU/L)	727.13	866.33	240-480	0.09
IL6 (pg/ml)	43.58	44.53	0-16.4	0.116
Creatinine (mg/dl)	0.86	0.93	0.6-1.5	0
RBS (mg/dl)	214.00	292.56	79-149	0
Hemoglobin (g/dl)	10.5	10.0	13-17	0
TLC (cells/cu mm)	13,257	11,616	4000-11000	0
Duration of hospital stay	28 days	20 days	4-70 days in our patients	0

Table 2: Histopathological parameters

Parameter	Mucor only(n=215)	Co-infections (n1=14)	P value
Fungal hyphae	186	14	0.489
Necrosis	198	14	0.498
Bone invasion	91	05	0.528
Granuloma	41	02	0.540
Angioinvasion	65	07	0.428
Epithelial metaplasia	67	04	0.519

Using paired t test, **statistically significant difference** was observed in mean values of serum **ferritin** (p value 0.005); C-Reactive Protein/**CRP** (p value 0.003); serum **creatinine**, Random Blood Sugar/**RBS**, **Haemoglobin**, Total Leucocyte Count/**TLC** and duration of hospital stay (p value of each being 0.00) while p value

was **insignificant** in serum Lactate Dehydrogenase/**LDH** and InterLeukin 6/**IL6** values between the two groups.

Platelet count of patients in both the groups were within normal range. (1-4.5 x1000/cu mm)

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significant difference in the two groups (p value of each was more than 0.05)

Discussion:

While the second wave of Covid 19 was taking toll of human lives, each medical specialist was engaged somehow or the other in the service of mankind. Amidst the diagnostic challenges in their laboratories, pathologists and microbiologists were also managing wards and ICU resources in various hospitals.

Mucormycosis is an opportunistic infection that caused significant morbidity and even cost many lives during and post second wave of Covid19. The two most important reasons responsible are the fast pace of infection spread from one to another region of head and neck, and the shortage/crisis of liposomal amphotericin, the drug of choice, because of sudden surge in demand.

The immunocompromised hosts, ie patients of hematological malignancies (one case of promyelocytic leukemia in our study), uncontrolled diabetes mellitus (approximately 15 cases), and transplant recipients (two of our cases) were worst hit. Rhino-orbito-cerebral and maxillary/frontal sinusitis was the predominant form of clinical presentation.

Early diagnosis (by histopathology and culture) and treatment which includes surgery and antifungal drugs may improve outcome and survival. This may avoid unnecessary treatment with voriconazole and other antifungal drugs.

When fungi grow in tissue, they may display primitive asexual (imperfect) forms which appear as either spherical yeast or spore forms. Some may produce vegetative growth which appears as tubular hyphae which may be septate and branching. These features are important

morphologically for identifying different types of fungi. A mass of interwoven hyphae is called a fungal mycelium. Only rarely, when the fungus reaches an open cavity, the body surface, or a luminal surface such as the bronchus, are the spore-forming fruiting bodies called sporangia or conidia, produced. [12]

Impairment of phagocytic function increases risk of mucormycosis as in neutropenic patients.

It has been shown that neutrophils, but not necessarily T lymphocytes, inhibit fungal spore proliferation. Furthermore, both mononuclear and polymorphonuclear phagocytes of normal hosts kill Mucorales by the generation of oxidative metabolites and the cationic peptides, defensins, and [13] up-regulation in Toll-like receptor 2 expression[14].

Not only the inhalation of sporangiospores but also their germination, hyphae formation and unique virulence traits are critical for establishing infection. One significant capability of mucorales is to acquire iron from host. In humans, iron is usually not in free form, rather bound to proteins like transferrin, and ferritin which by default is a strong defence. However, this fails in cases of diabetic ketoacidosis (DKA), where besides dysfunctional phagocytes and impaired chemotaxis, elevated serum levels of free iron has been found. [15]

However, why patients with uncontrolled type I diabetes or on corticosteroids (like organ transplant recipients) are the worst affected, is yet to be explained.

Patients on dialysis and receiving iron chelator deferoxamine are also uniquely susceptible to a lethal form of mucormycosis. The bacterial siderophore, strips ferric iron from transferrin and attaches itself on the mold through an inducible receptor, thus facilitating intracellular transport by an active

reduction of the ferric form into the more soluble ferrous form. [16]

Transplantation patients, have iron overload due to repeated blood transfusions and hence underlying myelodysplastic syndrome may predispose them to mucormycosis. [17]

Biochemical and blood parameters have been reported by Deval *et al* on 200 Covid positive patients admitted in our institute. They found significant differences in TLC which was $13,200 \pm 6,999.2$ in severely ill patients as compared to cases presented with mild and moderate symptoms ($12,100 \pm 6,488.41$ & $8,788.20 \pm 4,954.32$, $p = 0.001$). There was statistically significant differences in serum ionic calcium, random blood sugar, C-reactive protein, fibrinogen, prothrombin, International Normalized Ratio, ferritin, urea, fibrinogen, and procalcitonin and Lactate Dehydrogenase (LDH) levels as well between these three groups.

They suggest hematological and serum biochemistry parameters could be used as a screening tool to stratify patients as to whether they be admitted in intensive care unit or isolation wards, right at the time of first presentation.[20]

When mucor invades blood vessels, thrombosis and ischemic necrosis result. Necrosis further prevents delivery of leukocytes and antifungal agents to the foci

of infection; which in turn facilitates hematogenous dissemination. [17]

Raised blood sugar level, that plays a major role in post covid mucormycosis may also be due to self medication/ telephonic advice/inappropriate use of steroids in Covid 19 afflicted patients during home isolation.

An understanding of these mechanisms may help us develop new strategies to prevent and/or treat mucormycosis.

Conclusion:

Histomorphology with special stains, culture on fungal sensitive media at 37 degree Celsius for at least 5 days, molecular assays to detect ribosomal RNA which may be PCR based or MALDI-TOF, are the currently advised modalities for confirmation of mucormycosis. [18]

Since, molecular methods are still not standardized, all these investigations must be carried out in light of clinical findings and history of Corona virus infection as well as co-morbidities.[19,21]

Patients admitted in our institute and hospital were managed according to recommendations made by the institutional multi-disciplinary expert committee. [22]

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Figures

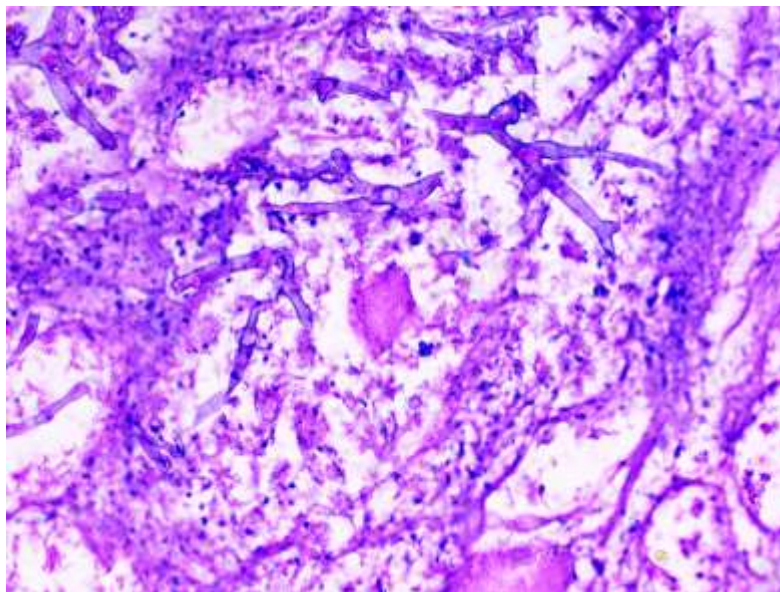


Fig 1 H&E 400X Broad aseptate ribbon like hyphae of mucor branching at right angles

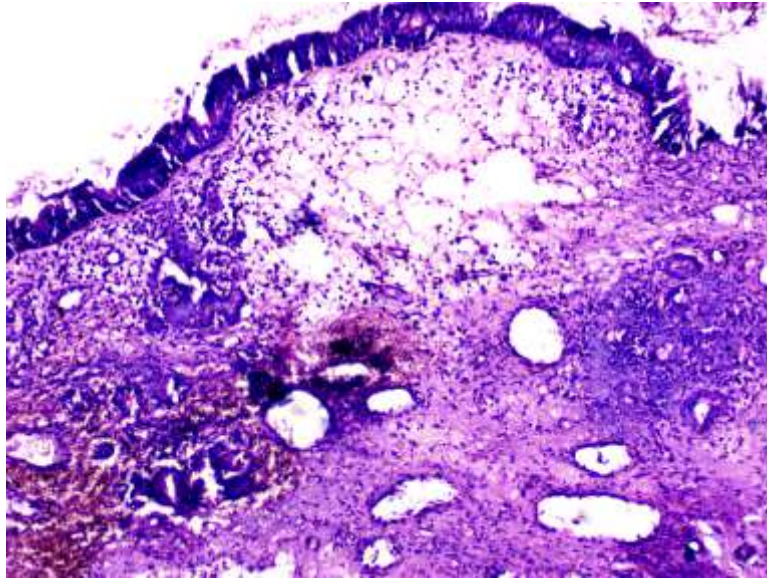


Fig 2 H&E 400X Inflammatory nasal polyp with edematous stroma, atrophied glands and metaplastic respiratory epithelium

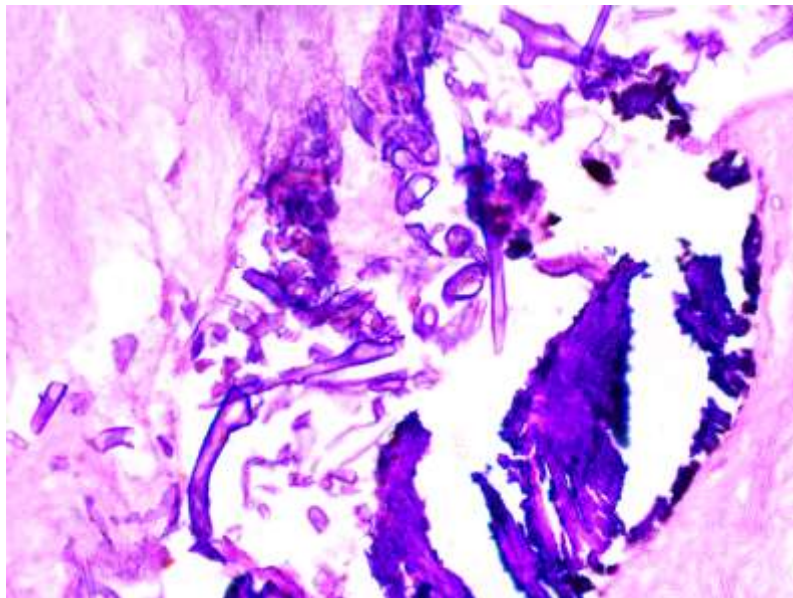


Fig 3 (a) H&E 400X Hyphae invading bone tissue

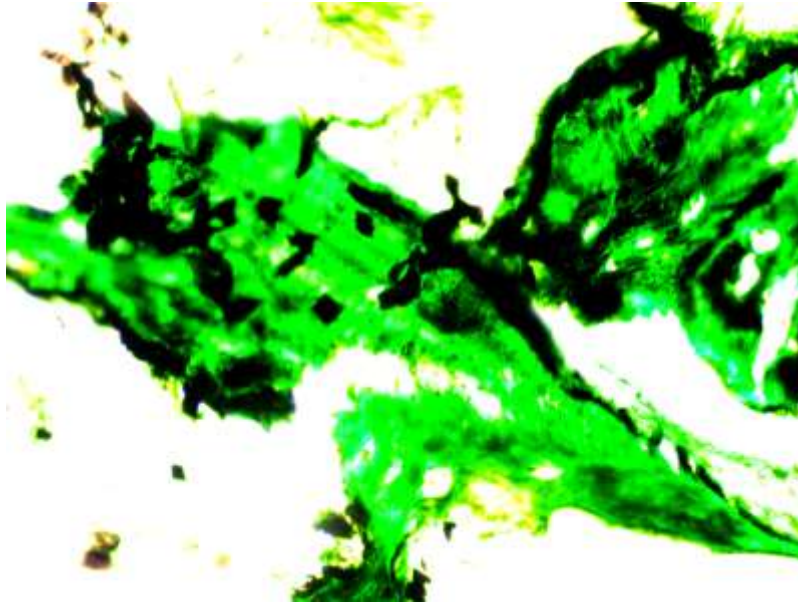


Fig 3 (b) GMS 400X Hyphae invading bone tissue

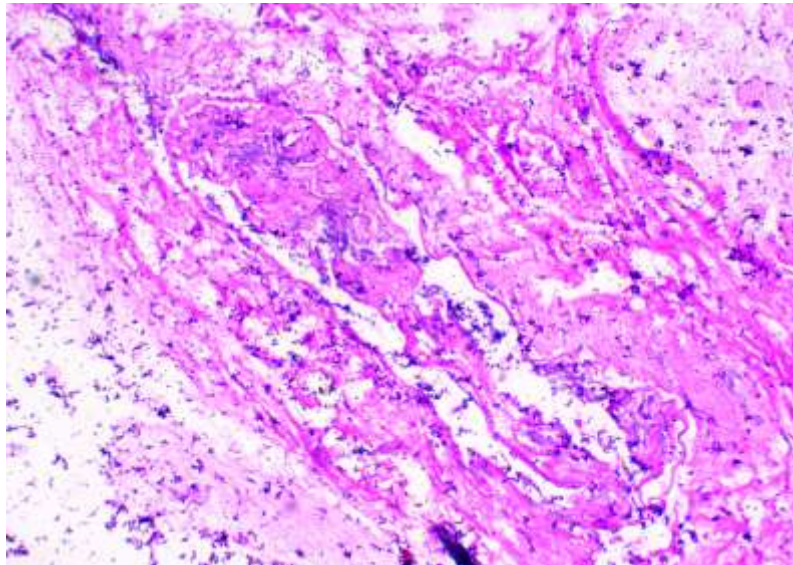


Fig 4a

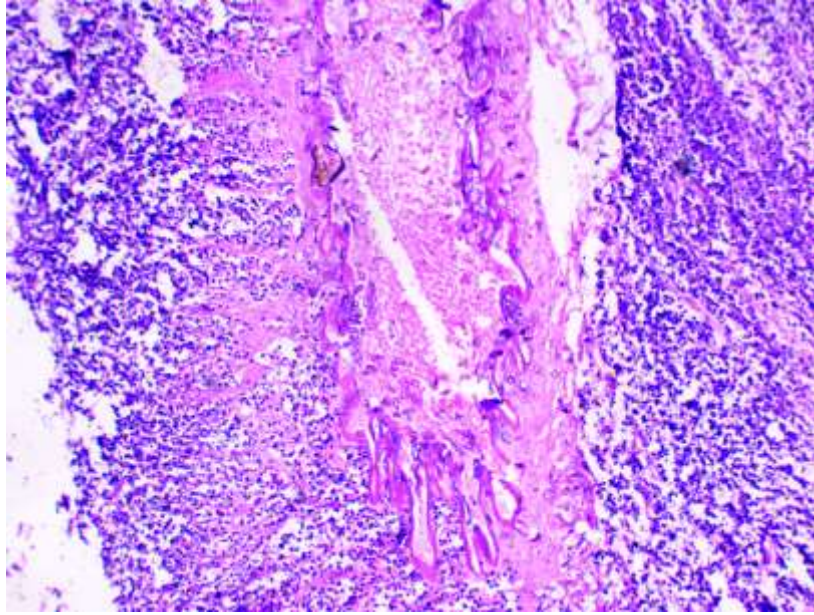


Fig 4b

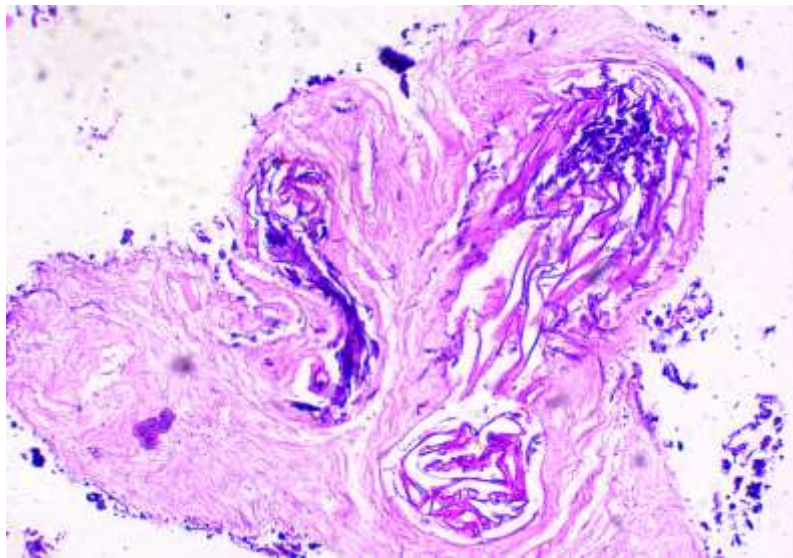


Fig 4c

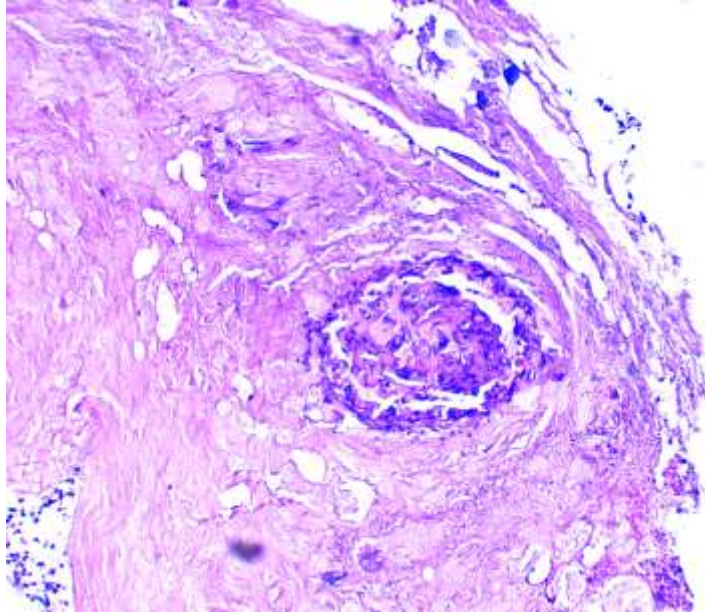


Fig 4 d
Fig 4 (a-d) H&E 400X Hyphae invading blood vessel (angio invasion)



Fig 5 (a) GMS 400X fungi inside blood vessels

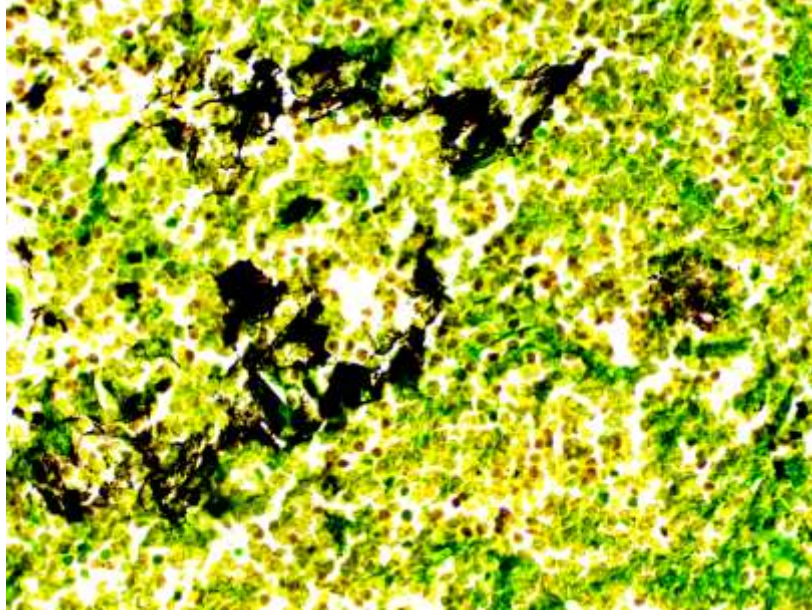


Fig 5 (b) GMS 200X fungi with spores in a necrotic background

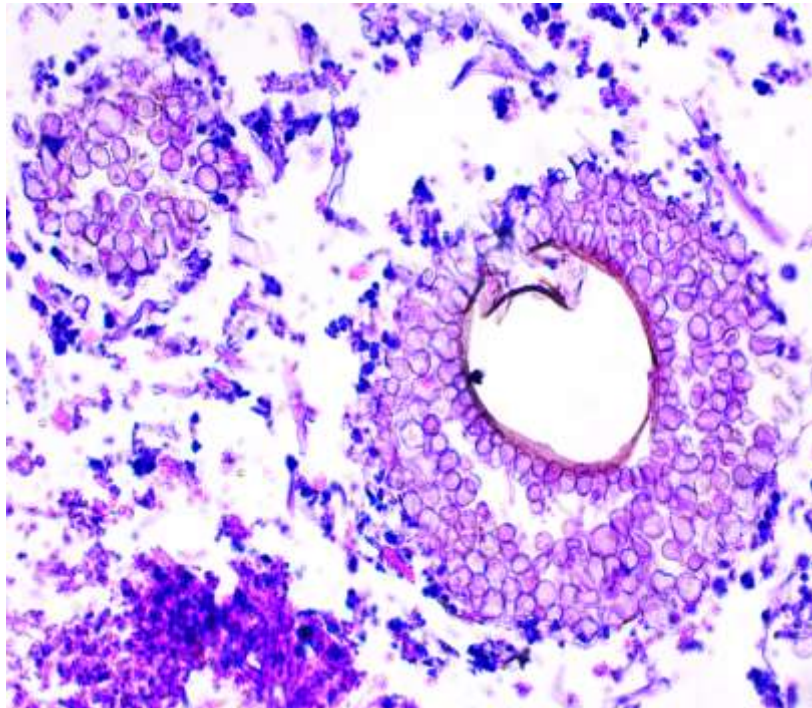


Fig 6 (a) H&E 400X mucor with fruiting bodies of aspergillus and mucor hyphae

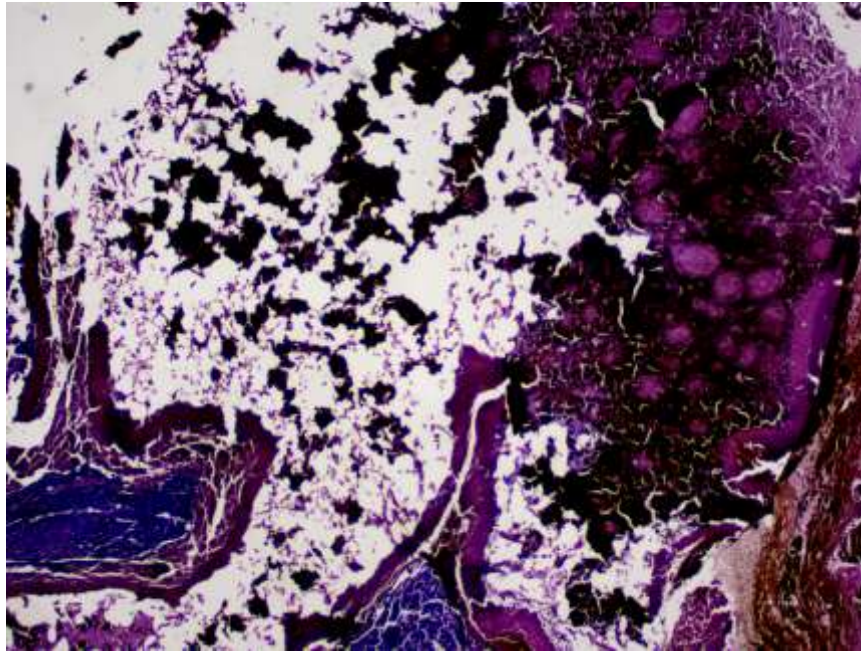


Fig 6 (b) H&E 400X mucor with lymphoid aggregate, destroyed soft tissue and aspergillus fruiting bodies

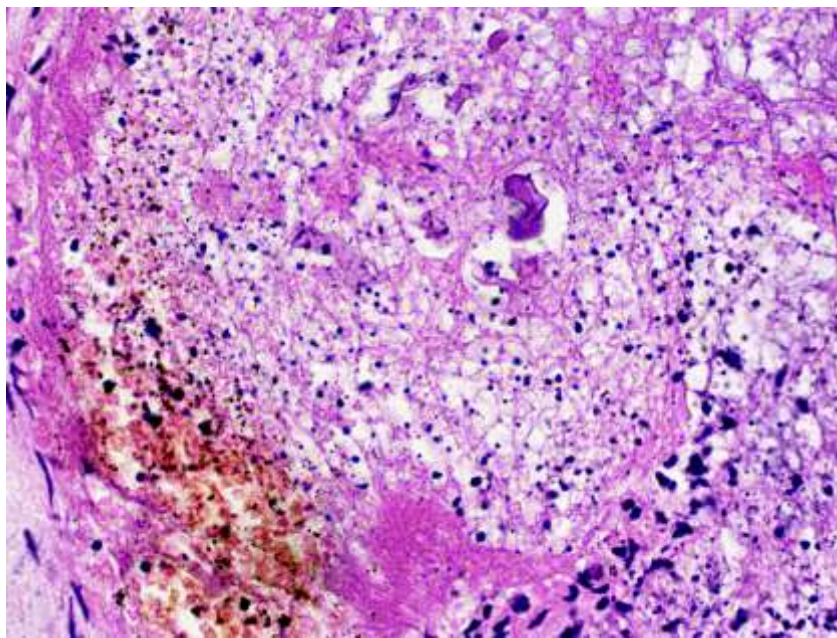


Fig 7 (a) H&E 400X mucor with aspergillus and surrounding granuloma

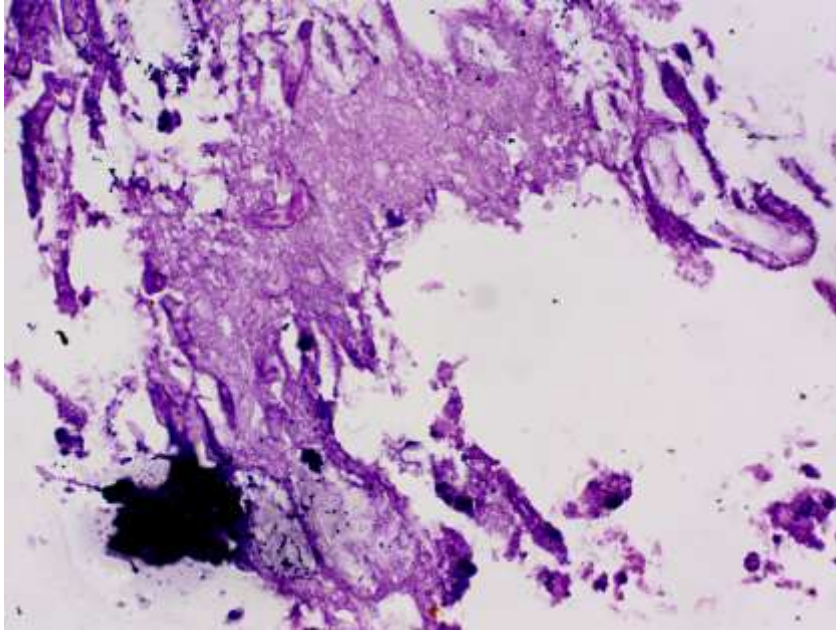


Fig 7 (b) H&E 400X mucor with actinomycotic colonies

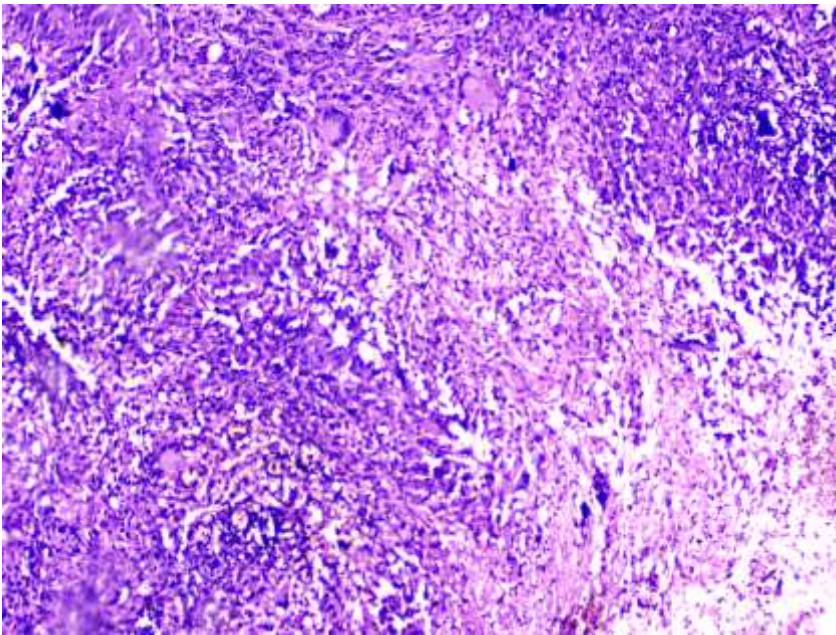


Fig 8 (a) H&E 400X granuloma with giant cell reaction

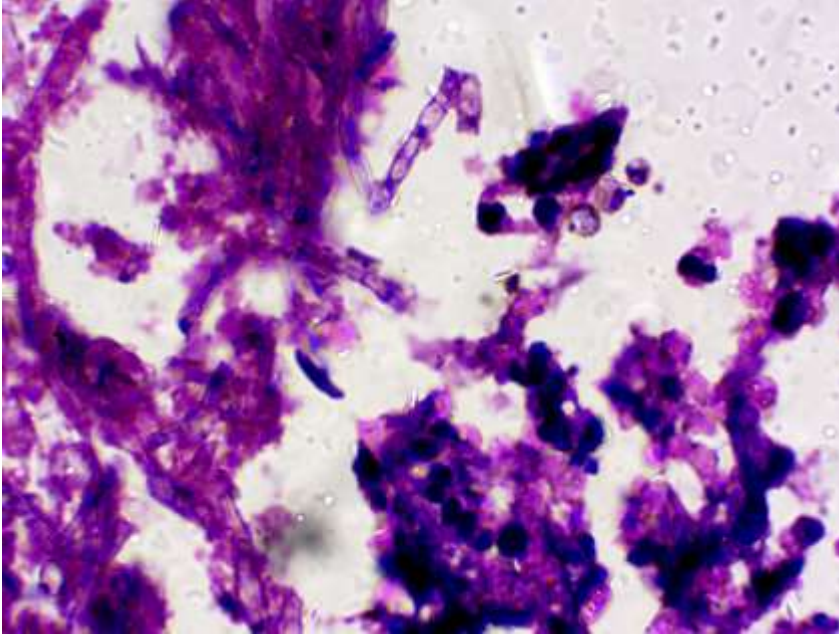


Fig 8 (b) H&E 400X septate acute angled aspergillus hyphae