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Mycological and histopathological findings of experimental disseminated candidiasis in dogs

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Summary

Disseminated candidiasis is an opportunistic infection in immunosuppressed animals by *Candida* species. The purposes of this study were to determine the predominant candidal forms in different tissues and tissue reactions. Sixteen dogs were selected in this study. The treatment dogs were immunosuppressed by intravenous administration of cyclophosphamide and after 5 days, they were challenged with 1×10^5 blastospores of *C. albicans* by intravenous injection. Both mycological and histopathological examinations were performed for detection of *Candida* in various tissues. The results showed that the highest counts of *C. albicans* were recovered from the lungs, followed by the kidneys, heart and liver on day 2 after challenge. The presence of yeast mixed with hyphal forms of *C. albicans* was confirmed in all tissues. In most tissues, the yeast cells of *Candida* were predominant, whereas hyphal forms, particularly true hyphae, were mostly found in the brain and eyes.

Key words: Disseminated candidiasis, Dog, *Candida albicans*, Mycopathology

Introduction

Candida species are natural inhabitants of the alimentary, genital and upper respiratory tracts of mammals (Greene and Chandler, 1998). These yeast-like fungi can cause opportunistic infections in animals treated with antibiotics, corticosteroids, cytotoxic agents and immunosuppressive drugs (Macphail *et al*., 2002; Tunca and Haziroglu, 2004). In the veterinary literature, occasional skin and intestinal infections caused by *Candida* species have been reported (Carrasco *et al*., 1993; Ochiai *et al*., 2000). Of the *Candida* species, *C. albicans* is most commonly isolated in animals (Barker *et al*., 1992). *Candida albicans* is a dimorphic fungus that has a distinct predilection for mucosal surfaces and areas of mucocutaneous junctions of warm-blooded animals where it resides as commensal. Its biotope is the digestive system where it is present as a minor member of the microbial flora, but under particular conditions, *C. albicans* becomes an opportunistic pathogenic microorganism, which may produce serious local infection in the external ear, perineum, nail folds, oral mucosa, cornea and the urinary tract and/or systemic invasion of the internal organs such as the kidney, liver, lungs, meninges and heart (Holoymoen *et al*., 1982; Gheorghiu *et al*., 1996; Kim *et al*., 1998; Kuwamura *et al*., 2006). An interesting feature of *C. albicans* is its ability to grow in two different ways; reproduction by budding, forming ellipsoid yeasts (blastospores), and in a hyphal form, which can periodically fragment and give rise to new mycelia, or in yeast-like forms. In addition to the intrinsic biological interest of this dimorphism, its ability to switch between the yeast and the hyphal forms of growth has been implicated in its pathogenicity (Leberer *et al*., 1997;

Molero *et al*., 1998; Whiteway and Oberholzer, 2004). Whether the yeast, the hyphal, or both forms of *C. albicans* are pathogenic has been a question of considerable debate for some time (Anaissie *et al*., 2002). Although focal infections with *Candida* species are common, there are only a limited number of reports describing disseminated candidiasis (Greene and Chandler, 1998; Rodriguez *et al*., 1998; Ochiai *et al*., 2000; Heseltine *et al*., 2003). The objectives of this study included determining the candidal infection rate in different tissues; determining the predominant candidal forms in different tissues and characterizing the colonization regions and predilection of *Candida* into the tissue structures.

Materials and Methods

Animals

Sixteen healthy mixed breed dogs (10 male and 6 female; weight, 10-22 kg; age, 4-12 months) were obtained from the Animal Research Division (Tehran, Iran). All 16 dogs received the routine vaccines and anti-helminthic drugs. The animals were randomly divided into two groups, treatment and control, comprised of 8 animals in each group. They were maintained for a period of 10 days before the initiation of the experiment and monitored for hematological parameters.

Organism and inoculation

The strain of *C. albicans* used was isolated from the blood of a dog with disseminated candidiasis and stored at -70°C in skim milk suspension. To prepare the inoculum, fresh cultures were grown on Sabouraud glucose agar (Merck, Darmstadt, Germany) for 24 h at 37°C. Yeast suspensions were prepared in sterile distilled water containing 0.01% Tween 80. After 3 washings with normal saline, the inoculum was standardized by hemocytometer counts and colony forming unit (CFU) was verified by viable counts. The viable counts of the test strain were confirmed by serially diluting the cell suspension 10-fold and plating the inoculum onto Sabouraud glucose agar plates. The mean viable count obtained was 1×10^5 /ml.

Disseminated candidal infection in dog

The treatment dogs were immuno-suppressed by the intravenous injection of cyclophosphamide (Acros Organics, Springfield, N.J.) at a dose of 200 mg/kg body weight 5 days before infection. Then, the immunosuppressed and control dogs were infected by cephalic vein injection of 1 ml of a saline suspension of *C. albicans* containing 1×10^5 CFU. All the dogs were euthanized by intravenous injection of nesdonal solution (Sankyo Co., Tokyo, Japan) at a dose of 20 mg/kg body weight and those that died spontaneously were subjected to detailed necropsy examinations under aseptic conditions. Tissue specimens from the brain, lungs, heart, liver, spleen, eyes, stomach, intestines, kidneys, skeletal muscles, gonads (testes/ovaries), lymph nodes, pancreas, thymus and adrenal glands of each animal were taken for histopathology and qualitative and quantitative cultures. All the experiments were performed based on veterinary research ethics and this study was approved by the Iranian Society for the Prevention of Cruelty to Animals (ISPCA).

Histopathology

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5-7 µm, and stained with haematoxylin and eosin (H&E). Selected sections were also stained with periodic acid-Schiff (PAS) and Gomori's methenamine silver stain (GMS). Lesion rates on each organ were calculated as follows: in every tissue the degree of lesions was assessed between zero and 4^+. When the tissue was negative for *Candida* particles it was considered as zero, less than 10 colonies as 1^+, between 11 and 20 colonies as 2^+, between 21 and 200 as 3^+, and more than 200 as 4^+. Since serial sections were considered to show the different *Candida* lesions, in order to obtain the lesion rate means we counted up the rates (0 to 4^+) and divided them to the number of sections studied.

Mycology

The visceral organs of the infected animals were removed, placed in glass vials containing 10 ml of sterile saline and

subsequently homogenized. One gram of the homogenates was made in sterile normal saline and cultured on Sabouraud glucose agar containing antibacterial antibiotics (20 IU penicillin and 40 µg/ml streptomycin). The plates were incubated both at 30 and at 37°C for 14 days, and the number of CFU per gram of tissue was determined.

Statistical analysis

Statistical analyses of these data were performed with Sigmastat software (SPSS Corp., Chicago, IL). The candidal lesion and colony counts are expressed as the log of mean CFU ± standard error from 16 animals. Student's t-test was used to determine statistical significance between control and treatment groups; $p<0.05$ was considered significant.

Results

Clinical signs including pyrexia, dyspnoea, and neurological signs along with restlessness were observed in the infected dogs. Treatment dogs died at 2-days post inoculation and control dogs were sacrificed at 10-days post inoculation. White blood cell (WBC) and neutrophil mean counts were recorded in the treatment group after cyclophosphamide injection; WBC: 3500/µl (before injection, 12550/µl) and neutrophil: 2315/µl (before injection, 8478/ µl).

In necropsy, congestion and petechial hemorrhages were observed in all the visceral organs of the treatment dogs. Small multiple necrotic foci and grayish-white nodular abscesses were seen in the lungs, liver, heart, brain, meninges, spleen and kidneys. In the control dogs, no lesions were observed in the visceral organs except for the lungs.

The microscopic results of *Candida* showed there were masses of branching, septate hyphae, pseudohyphae and round to oval budding yeast cells (blastospores) measuring 3-5 µm in diameter in different tissues. The yeast forms were predominant in most tissues, whereas filamentous forms, especially true hyphae of *Candida*, were predominant in the brain and eye. In addition, considerable true hyphae were found in the lungs, kidneys and liver. Radiate colonies of *Candida* were observed only in the liver, brain and skeletal muscles of 2 treatment dogs.

All tissue cultures of the control dogs were negative for *C. albicans*. Tissue cultures of different organs revealed that the lungs contained the highest burdens of *C. albicans* (4.6×10^4 CFU/g) in the present study. Tissue burdens followed in kidneys (2.4×10^4 CFU/g), heart (4.2×10^3 CFU/g), liver (2.6×10^3 CFU/g), brain (50 CFU/g), spleen (48 CFU/g), adrenal glands (17 CFU/g), lymph nodes (13 CFU/g), skeletal muscle (9 CFU/g), pancreas (8 CFU/g), eye and thymus (5 CFU/g), which was significant as compared to the control group ($P<0.05$).

Histopathology

The lungs revealed sub-pleural hemorrhagic lesions extending deep into the parenchyma, accumulation of serofibrinous exudate into the alveolar spaces, emphysema and a marked infiltration of neutrophils, mononuclear cells and macrophages in alveolar walls. The presence of fungal organisms into the blood vessels of the lung and vascular invasion were the most predominant microscopic characteristic of hematogenous candidiasis. The most predominant forms of *C. albicans* in different regions of the lung were as follows: in fibro-thrombotic mass into the blood vessels of the lung as yeast, pseudo- and true hyphae; in intra-alveolar space as true hyphae and yeast; in interstitial tissue, alveolar wall and intra-vascular space as yeast.

The kidneys showed congestion, hemorrhages, tubular degeneration and neutrophil infiltration. The cortex and to a lesser extent medulla, revealed multiple necrotic foci and an inflammatory reaction initially predominated by neutrophils and later with macrophages with PAS-positive fungal elements. Interestingly, fungal elements were distributed much higher in the cortex, especially into the proximal and distal tubules than the medulla. The most predominant forms of *C. albicans* in different regions of the kidneys were reported as follows: in cortex, medulla, urinary tubules, interstitial tissue and glomeruli as yeast mixed with a few pseudohyphae; in addition, germ tubes were

present remarkably in the glomerular vessel that penetrated the urinary tubules (Fig. 1).

The heart showed hemorrhages separating the muscle fibers, degeneration, and multiple Zenker's necrosis in myocardium. In all samples, fungal elements were observed only in the myocardial layer, whereas no organisms were found in either the endocardium or epicardium. Fungal colonies and PAS-positive fungal structures were seen within the necrotic foci. Yeast mixed with a few pseudohyphae was the most predominant form of *C. albicans* in the muscular cells of the myocardium (Fig. 2).

Severe congestion, sinusoidal dilatation and fatty change as well as multiple necrotic foci with marked mononuclear cellular infiltrations such as macrophages in the hepatic lobules were seen in the liver. Besides macrophages, a giant-cell reaction

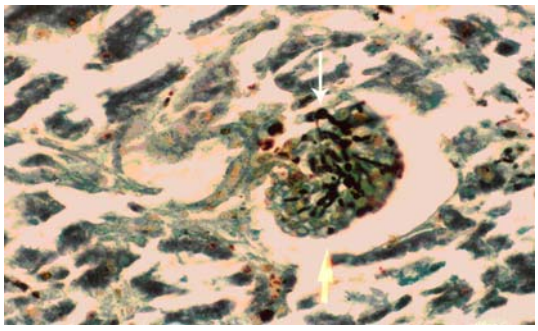

Fig. 1: The colonization of numerous yeast cells along with pseudohyphae in glomerular tufts of kidney (GMS, ×528)

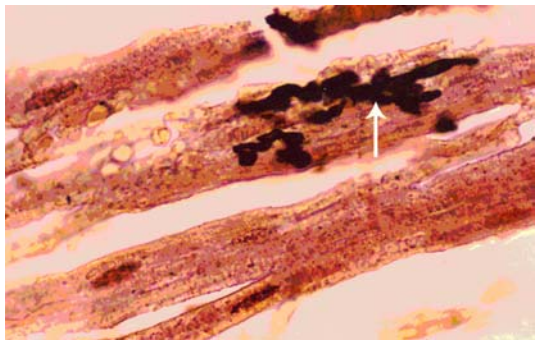

Fig. 2: The presence of candidal yeast cells and pseudohyphae in muscular fibers of the myocardium (GMS, ×1320)

and bile duct hyperplasia were also detected. The most predominant forms of *C. albicans* in different parts of the liver such as hepatocytes and portal space were yeast mixed with hyphal forms. In one case, true hyphae were developed in hepatic parenchyma and had invaded the vessels of the liver.

The spleen was congested and showed extensive haemosiderosis along with varying degrees of a histiocytic proliferation. The yeast forms were observed in sinusoids of red pulp and to a lesser extent in white pulp (Table 1).

The stomach and intestines revealed congestion of the mucosal and submucosal blood vessels, and focal hemorrhages along with an increased goblet-cell activity. Besides the focal hemorrhages, the gastric mucosa revealed small necrotic foci

Table 1: Comparison of mean infection rates of various tissues according to H&E staining, GMS/PAS staining and fungal culture and predominant *Candida* forms in different tissues

Organ	Diagnosis method			Candida form
	H&E (LRM ± SE)*	GMS/PAS (LRM ± SE)*	Fungal culture (Log CFU/g Mean)	
Lung	2.42 ± 1.46	3.23 ± 0.72	4.66	Yeast, Hyphae
Heart	3 ± 0	3 ± 0	3.64	Yeast
Kidney	2.35 ± 0.81	2.98 ± 0.03	4.38	Yeast, Hyphae
Liver	1.26 ± 0.58	1.76 ± 0.55	3.41	Yeast, Hyphae
Spleen	0.55 ± 0.36	1.1 ± 0.22	1.67	Yeast
Pancreas	1 ± 0	2.83 ± 0.28	0.9	Yeast
Gastrointestinal tract	1.4 ± 0.89	1.6 ± 0.54	0.92	Yeast
Brain	0.89 ± 0.63	1.54 ± 0.85	1.69	Yeast, Hyphae
Eye	0.58 ± 0.57	0.86 ± 0.27	0.72	True hyphae, Yeast
Adrenal glands	0.33 ± 0.02	0.33 ± 0.02	1.22	Yeast
Thymus	0.33 ± 0.02	0.41 ± 0.1	0.74	Yeast
Skeletal muscle	2.25 ± 0.38	2.90 ± 0.19	0.9	Yeast
Lymph node	0.3 ± 0.01	0.35 ± 0.02	1.12	Pseudohyphae, Yeast

*LRM: Lesion rate mean ± SE

surrounded by inflammatory cells, including neutrophils and mononuclear cells along with macrophages. The yeasts were the most predominant form of *Candida* in different regions of the gastrointestinal tracts (Table 1).

There was a mild meningeal congestion of the brain. In addition, microabscesses comprised of neutrophils, mononuclear cells and gitter cells were also detected in the parenchyma. Both true hyphae and yeast were distributed in the white and gray matters of the brain and cerebellum except for the brain stem (Table 1).

In the eyes, fungal elements were seen only in the pupil and retina layers as true hyphae (Fig. 3), whereas yeast cells were found in the external muscle of the eye ball. No cellular responses were observed in the two above layers.

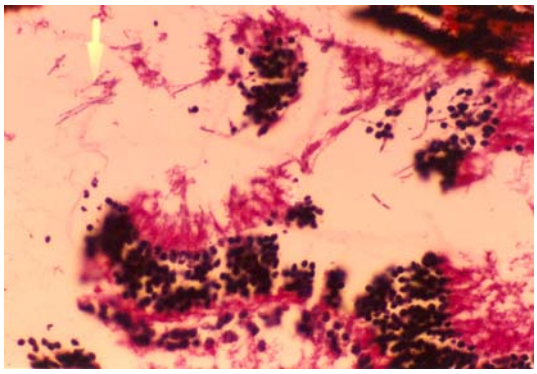

Fig. 3: Numerous true hyphae of *C. albicans* in retina of the eye (H&E, ×528)

The testes, ovaries, pancreas, adrenal glands, thymus, skeletal muscle and lymph nodes revealed mild to moderate congestion and the predominant form of fungal element in all of the above organs was found to be yeast (Table 1).

Discussion

In the present study, a histo-invasive disseminated candidiasis was induced in 8 immunosuppressed dogs following intravenous inoculation of *C. albicans*, 1×10^5 yeast cells/ml, and observations were recorded until death. Clinical signs observed in the infected dogs were initially characterized by pyrexia and dyspnoea, and neurological signs along with restlessness in the later stages. These findings can be attributed to severe stress due to systemic fungal infections that are in agreement with the earlier observations with experimental candidiasis in other animal models (Fransen *et al*., 1984; Chattopadhyay, 1989). The histopathological findings were initially characterized by congestion and hemorrhages in various organs along with focal areas of necrosis. These were followed by the development of multiple abscesses in the lungs, kidneys, heart, brain and gastrointestinal tract, in order of severity. Following blood distribution, the yeast forms are phagocytosed by the macrophages or neutrophils and grow out of the cells into mycelial filaments which penetrate the neighboring cells leading to widespread tissue necrosis and abscess formation in various organs (Chattopadhyay, 1989).

In this study, the presence of fungal elements in the blood vessels, especially in the lungs and some of the other organs, and vascular invasion were the most predominant microscopic characteristic of disseminated candidiasis. Sundstrom (2002) suggested that the transformation of yeast to the hyphal form occurs rapidly during an active infection and invades the alveolar septa, bronchial wall and small blood vessels resulting in thrombosis and focal areas of necrosis. It can be attributed to hydrolytic enzymes, namely aspartyl proteases produced by *C. albicans* following adhesion to epithelial and endothelial cells, facilitating invasion through the degradation of elastin and collagen of the basement membrane of the vascular endothelium (Ruchel *et al*., 1992).

The mean infection rate was higher in the lungs (4.6×10^4 CFU/g), kidneys (2.4×10^4 CFU/g), heart (4.2×10^3 CFU/g) and liver (2.6×10^3 CFU/g) than those of the other organs. Papdimitriou and Ashman (1986) reported that though significant yeast deposition of *C. albicans* occurred in the lungs, kidneys, spleen and liver, the only histological evidence of colonization by the fungus, with a mild transient infection, was observed in the liver. In the same study, animals that received a lower inoculum developed moderate to severe myocarditis with foci of fungal replication being in the heart rather than the spleen and liver. These lesions gradually subsided while the lesions

in the kidneys continued to develop into abscesses (Papdimitriou and Ashman, 1986). Brown *et al*. (2005) also reported that *Candida* organisms multiplied to a greater extent in the kidneys than in the liver, spleen and lungs of rats and mice. As mentioned, discrepancy in *Candida* distribution is dependent on the animal species challenged, the growth conditions used for the challenge inoculum, and differences between fungal strains as well as route of *C. albicans* inoculation. When *Candida* is injected into a mesenteric vein, the liver takes up approximately twice as many organisms as the lungs. However, with injection into a peripheral vein, the lungs take up was predominate over that in the liver, which is in agreement with our results. The transient infection in the spleen, lymph nodes and thymus with minimal inflammatory reaction towards the terminal stage of the experiment and the failure to produce chronic lesions is due to the larger population of tissue phagocytes (Sundstrom, 2002).

The morphological forms of *C. albicans* recovered from infected tissues differed at the level of the host tissues. In this study, the yeast mixed with hyphal forms predominated in the lesions of the most affected organs such as the lungs, kidneys, spleen and liver. The observations of Romani *et al*. (1996) suggest that the enzyme phospholipase, which is concentrated at the hyphal tip, may be related to the greater invasiveness of this form compared to the yeast form and thus the morphological change may contribute to the increased pathogenic potential of the fungus. Also, *C. albicans* produces a variety of endo- and exotoxins (Shah and Larsen, 1991; Hube, 1998), including a toxic epipolythiodioxopiperazine metabolite called gliotoxin, which has been shown to possess antiphagocytic property (Sutton *et al*., 1994; Waring and Beaver, 1996). Based on the property of hyphae to penetrate tissues more readily than yeast and its possible resistance to phagocytosis, it is possible to claim a relationship between the hyphal formation and infection severity rate by *C. albicans* (Ochiai *et al*., 2000). On the other hand, the greater virulence of the yeast forms of *C. albicans* over the hyphal forms has also been reported earlier (Evans, 1980; Liu, 2002; Romani *et al*., 2003). Shepherd (1985) suggested that both the yeast and hyphal forms of *C. albicans* are capable of invading soft tissues and the ability of tissue fluids to interconvert the two morphological forms means, however, that experiments with either yeast or hyphal forms will inevitably give equivocal results. Those observations and the results of the present study indicate that morphogenesis of *C. albicans* is a highly complex response of the fungal cells to their micro-environment and could be related to infecting fungal strain and the animal host (Odds *et al*., 2000).

In conclusion, the results showed that the highest counts of *C. albicans* were recovered from the lungs, followed by the kidneys, heart and liver on day 2 after challenge. In most tissues, the yeast cells of *Candida* were predominant, whereas hyphal forms, particularly true hyphae, were mostly found in the brain and eyes.

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