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Full Length Research Paper

Effect of glucose supplementation on *Candida albicans* virulence in immunosuppressed Swiss albino mice (*Mus musculus*)

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We investigated the effect of dietary carbohydrate supplementation on *Candida albicans* colonization using an animal model and a *Candida* strain with both high phospholipase and proteinase activity. Normal and immunosuppressed mice were inoculated intraperitonially with *C. albicans* and were allowed free access to drinking water supplemented or not with glucose. Blood and different organs including liver, spleen, lung and kidney were aseptically collected every 72 h post-infection. The presence and the growth of *C. albicans* in blood and each organ were investigated. We also performed histopathologic investigations on each organ to assess tissue structure, the presence of *C. albicans*, and its form (blastospore or hyphae). The results showed that on the third day post-inoculation, the cfu of *C. albicans* per organ was significantly higher in mice inoculated by *C. albicans* and receiving the glucose as supplement (433 cfu/liver) comparatively to the group receiving *C. albicans* only (140 cfu/liver). Histological analysis revealed the presence of *Candida* cells in blastospore and hyphal form, particularly in the *Candida*-infected glucose-supplemented mice whose livers displayed oedema and leukocyte infiltration with a high density of polymorphonuclear cells. Overall results indicate that dietary glucose supplementation leads to higher rates of *Candida* growth and invasion. This suggests that glucose restriction could be a possible way to control *C. albicans* pathogenesis *in vivo.*

Key words: *Candida albicans*, glucose, immunosuppressed mice, experimental infection.

INTRODUCTION

Oral candidiasis is the most common fungal infection in immunosuppressed patients caused by the proliferation of *Candida* species (Klein et al., 1984; Samaranayake and Holmstrup, 1989). *Candida albicans* is an opportunistic pathogen which normally resides in the oral cavity of humans, but which can take advantage of host disorders to cause infections in a variety of tissues. Physiological, mechanical, and iatrogenic factors have been shown to predispose the host to infection by *Candida* (Odds, 1979). Alterations in the balance between the commensal and the host, such as those that occur in the immunocompromised patients, may trigger infection of the mucosal epithelia, followed by dissemination via the bloodstream and colonization of internal organs (Barnett et al., 1990; Singh et al., 2001; Staib et al., 2002).

Several virulence factors have been proposed for *C. albicans* including extracellular enzymes such as phospholipases and proteinases production. Phospholipase activity has been considered an important factor for the infection process for acting on

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phospholipids hydrolysis, damaging epithelial cells (Neder, 1992). Secreted aspartic proteinases (SAP_S) are important virulence factors during mucous or disseminated infections caused by *C. albicans*. Extracellular proteolytic activity enables *C. albicans* to utilise exogenous protein as the sole nitrogen source. Although, it has been known that dietary glucose supplementation leads to higher rates of *Candida* growth and invasion (Sergio et al., 1993). Some studies have demonstrated that animal models of oral candidiasis are useful for investigating the pathogenesis of this infection (Samaranayake and Samaranayake, 2001)

To test the idea that *C. albicans* growth in different organs, especially in liver might be influenced by the dietary intake of glucose and in order to evaluate the virulence of *C. albicans* strain (*in vivo*) with high phospholipase and proteinase activities, we selected adult mice as an experimental model and glucose as dietary carbohydrate. The adult mouse model has been used successfully to study *Candida* colonization of the liver and invasion from that site. Infections in this host, including invasiveness under immunosuppression (Guentzel and Herrera, 1982), closely resemble those in humans (Walbaum and Dujardin, 1985).

The aims of the present work was to evaluate the virulence *in vivo* of *C. albicans* selected on the basis of its high phospholipase and proteinase activities and the effect of dietary carbohydrate supplementation on *C. albicans* colonization and invasion in immunosuppressed Swiss albino mice (*Mus musculus*).

MATERIALS AND METHODS

Animals and maintenance

Fourty female Swiss albino mice (*Mus musculus*) immunosuppressed by hydrocortisone were used. The animals were approximately eight weeks old and with a weight of 25 to 30 g. The mice were separated into four groups of 10 animals per covered plastic cage, and were supplied sterilized tap water and food *ad libitum*. The mice in group C (n = 10) and D (n = 10) were allowed free access to drinking water supplemented with 5% of glucose (w/v) (Sigma Chemical Co., St. Louis, MO, USA) for the duration of the experiment. The control group (Group A, n = 10) and Group B (n = 10) received water without glucose. Each procedure was performed in accordance with the standards for the humane handling, care, and treatment of research animals. The mice and each organ were weighed on an electronic scale (Kern 770, Germany) on Days 0 and at the time of sacrifice.

Fungi

One strain of *C. albicans* with high and very high phospholipase (Pz = 0.67) and proteinase (Pz = 0.38) activities and characterized previously (Noumi et al., 2010) was used in this study. The strain was originally isolated from the Dental Hospital of Monastir (Tunisia) from a patient with denture stomatitis and stored for 9 months. The strain was maintained on slopes of Sabouraud-chloramphenicol agar (Bio-rad, France) and subcultured routinely. Identification of the isolate was verified by using the ID 32 C yeast identification system (bioMérieux, Marcy l'Étoile, France).

Preparation of *Candida albicans* inoculum and administration

All mice were divided in four groups: control group (A: 10 mice), Group B (10 mice) was inoculated intraperitoneally with 2×10^7 cells (0.2 ml), Group D (10 mice) was also inoculated intraperitoneally with 2×10^7 cells (0.2 ml) and has a diet supplemented with glucose (10 mice) and the last Group C (10 mice) has a diet supplemented with glucose (Table 1).

A single colony from a 48 h growth on Sabouraud chloramphenicol agar plates was suspended in 10 ml of YEPD broth (1% yeast extract, 2% peptone, 2% dextrose). The inoculated broth was incubated overnight at 37°C in an orbital shaker (SI-600) at 200 rpm. The cultures were then washed three times with sterile phosphate-buffered saline (PBS: 0.2 g of KCl, 0.2 g of KH_2PO_4, 8 g of NaCl, and 1.15 g of Na_2HPO_4 per litre, pH 7.2). The inoculums were adjusted to an OD520 nm of 0.4 corresponding to 10^8 cells/ml (Mellado et al., 2000). During the experiment, we compared the effect of *C. albicans* alone on colonization versus that of *C. albicans* with dietary glucose.

Induction of immunosuppression

The animals of all groups were inoculated via intraperitoneum with 10 $mg.ml^{-1}$ of hydrocortisone succinate (Laboratoires Leurquin Mediolanum S.A., Hydrocortisone Leurquin®) according to Holzschu et al., (1979). Each animal received 0.1 ml of hydrocortisone succinate suspended in saline solution for 3 days before and 5 days after *C. albicans* inoculation (Figure 1).

The first sacrifice was done three days after of the intraperitoneal inoculation of adult mice with *C. albicans* and six days of the first injection of hydrocortisone (Figure 1). In fact, two mice were sacrificed from each group (one mouse was used for the determination of cfu number in the internal organs and the second one was used for the histological examination). The second sacrifice was done on the ninth day of the experiment and one day after the last injection of the hydrocortisone. After three days, the third sacrifice was done. In the 13th day, we noted a death of one mouse in Group B. The forth sacrifice was done in the 15th day and the last sacrifice (two mice from the Groups A,C, D and one mouse from the Group B) was done after 18 days of the beginning of the experiment. The unique mouse in the Group B was used for the determination of cfu number in the internal organs only.

Quantification of the colony-forming units in the organs

Every 72 h post-inoculation, the organs (liver, spleen, lungs and kidneys) were aseptically removed and homogenized with a tissue homogenizer (Seward, Stomacher® 400, LAB System) using 5 ml of sterile buffer (Tween 80 0.05% v/v in NaCl 150 mM). Each homogenate was serially diluted 10-fold and plated onto Sabouraud chloramphenicol agar. The blood (100 µl) was collected from the heart and transferred to sterilized eppendorf tubes containing heparin 10% (v/v) than plated onto Sabouraud chloramphenicol agar. After incubation at 32°C for 48 h, the number of colony-forming units (CFU) was determined. One isolated colony from each organ was transferred to Sabouraud dextrose agar slant and submitted to biochemical and physiological tests to confirm if the colony recovered was *C. albicans* (Sther et al., 2000).

Histopathologic examination

Histopathologic examinations were performed every 72 h following infection of the animals. The organs were perfused with saline solution, fixed in formaldehyde (4.0% v/v, prepared in PBS 0.01 M, pH 7.2) for 7 days, dehydrated in ethanol at concentrations of 70,

Table 1. Evolution of the weight of the internal organs in the different groups of mice.

Groups	Day	Weight of the internal organs (g)			
		Liver	Spleen	Kidneys	Lungs
Group A (control)*		1.585±0.183	0.230±0.025	0.366±0.021	0.331±0.015
	6	1.725	0.194	0.425	0.31
	9	1.884	0.313	0.515	0.373
Group B (*C. albicans*)	12	2.584	0.373	0.668	0.570
	15	1.845	0.272	0.6	0.299
	18	1.57	0.175	0.42	0.268
	6	2.058	0.175	0.44	0.237
	9	2.232	0.372	0.451	0.271
Group C (Glucose)	12	1.093	0.112	0.337	0.277
	15	1.162	0.074	0.336	0.317
	6	2.027	0.188	0.516	0.386
	9	1.876	0.223	0.458	0.382
	12	1.788	0.241	0.463	0.401
Group D(*C. albicans*+ glucose)	15	2.07	0.314	0.425	0.291
	18	1.702	0.299	0.359	0.285

* : These values represent the mean of five experiments corresponding to results obtained for the five sacrifices.

80, 90, and 100% v/v, diaphanized with xylol, and finally embedded in paraffin for routine histology (Guilherme et al., 2004). Following microtomy (4 µm), the sections were stained with hematoxylin-eosin and Periodic acid Schiff (PAS).

RESULTS

Virulence of *C. albicans* in the mice following intraperitoneal inoculation

Mortality attributed to the inoculation procedure was approximately 5% (1/20) of all of the infected mice with *C. albicans*. This mortality rate was observed in the *Candida*-inoculated mice that had access to the glucose regime (Group B) in the Day 13, which may suggest the virulence of *Candida* under glucose conditions.

Evolution of the organs weight

The weight of each group of mice decreased during the experimental period. The average ± standard error of weight for the *Candida* strain-inoculated animals (Group B) was 26.38±2.30 g, comparatively to the control animals (Group A) was 22.10±3.59 g. For the other two groups, the weight of the animals was 22.04 ±1.32 g (Group C) and 24.57± 2.98 g (Group D). During the first days post-inoculation with the tested *C. albicans* strain, the weight of the four organs increased in Groups B (*C. albicans*) and D (*C. albicans* + glucose) and especially liver, kidneys and lungs (Table 1).

In the end of the experiment, the weight of the internal organs in Groups B, C and D were strictly related to those of the control group. Figure 2 shows the evolution of the liver weight in the four groups (CTRL: control Group, Ca: group inoculated with *C. albicans*, G+: group receiving glucose, CaG: group inoculated with *C. albicans* and receiving glucose) after six days (A), nine days (B), twelve days (C) and fifteen days (D) of the beginning of the experiment.

Quantification of the colony-forming units in the organs

The liver was the first organ where capillary filtration of *C. albicans* occurred, followed by the kidneys. The lungs and spleen were less positive for *C. albicans* at all of the recorded time periods.

In Group B, the liver showed the highest burden of *C. albicans* throughout the observation period, with initial counts of 4 cfu/organ at 3 days post-infection (Day 6) to 130 cfu/organ at 6 days post-infection (Day 9) as shown in the Figure 3. Tissue burdens in the kidneys, lungs, and spleen also followed similar patterns with *C. albicans*. On Days 12 and 15, the infected mice displayed low, yet detectable *C. albicans* counts in the 4 organs studied. *Candida* cultures were recovered from the liver, kidneys, spleen, and lungs of the animals in Group D (*C. albicans* + glucose). In Group B (*C. albicans*), positive cultures were obtained between 6 and 18 days of infection, with a remarkably high numbers of cfu found in the spleen at

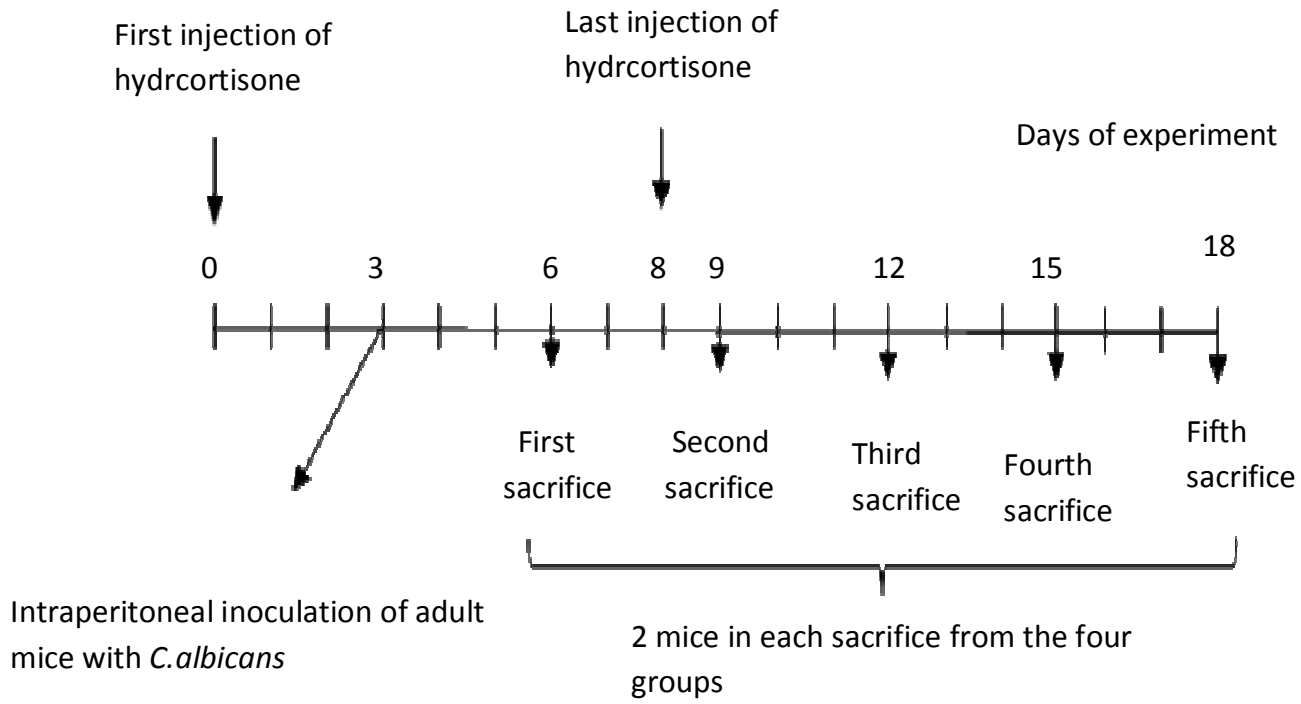

Figure 1. Diagram of experimental protocol: Day 0, immunosuppression: 0.1 ml of hydrocortisone succinate; Day 3, inoculation; Day 6, first sacrifice and culture of tissue homogenates from the liver, kidneys, lungs, spleen, and heart blood; Day 8, final injection of hydrocortisone succinate; Day 9, second sacrifice; Day 12, third sacrifice; Day 15, final sacrifice

Day 18 (Figure 3).

In the lungs, positive cultures were observed in Groups B and D. In Group B, *Candida* recovery was performed on Days 6, 9, 12 and 18, while in Group D, the recovery was done on Days 6,12 and 18 (Figure 3). The kidney was also targeted by the *Candida*, as yeast was recovered from Group D kidneys on Days 6 and 12 (70 and 10 cfu, respectively), and Group B on Days 9 and 18 (75 and 10 cfu, respectively) (Figure 3).

Of interest was that as early as 3 days post-infection (Day 6), the *C. albicans* concentration in the organs of glucose-supplemented Group D had risen to 433 cfu (liver), 70 cfu (kidneys), 49 cfu (lungs), and 19 cfu (spleen), compared to Group B and the controls. Under hydrocortisone succinate-induced immunosuppression, the number of *Candida* in the liver, kidneys, and lungs continued to increase in both Groups B and D on Days 6 and 9, but was relatively stable on Days 12 and 15. *C. albicans* was detected in the heart blood of the glucose-supplemented animals (Group D) on Days 9 (5 cfu/100 µl of blood) and 18 (96 cfu/100 µl of blood). The heart blood of the non-glucose-supplemented animals (Group B) was shown to be uncontaminated with the *C. albicans* strain.

Invasion of the hepatic wall

Results obtained with the hepatic wall homogenates were consistent with the histological findings. The glucose-supplemented mice (Group D) showed histological evidence of *C. albicans* invasion of the hepatic wall, ranging from numerous yeast cells to hyphae (Figure 4). The extent of mucosal invasion correlated well with the colonization of the hepatic surface.

Liver structure following *C. albicans* infection

Lesions in the liver were observed only in Groups B and D (non-glucose-supplemented and glucose-supplemented animals, respectively) that had been inoculated with the same strain of *C. albicans*. The liver showed multiple abscesses composed of polymorphs and monocytes during the first 3 days as well as a few areas of focal necrosis. No lesions were observed after day 3 post-infection. Multiple abscesses were found to have developed more on the Group D livers than on the Group B ones.

The histology of the liver during infection was examined to determine the level of inflammation and the invasiveness of the disease. Figure 4 shows a mouse liver section stained with PAS. The infection is well defined and superficial. The hyphae did not penetrate across the epithelium, and much of the yeast is in the blastoconidial phase in the lumen. There is significant inflammation, edema, and neutrophilic penetration in response to the

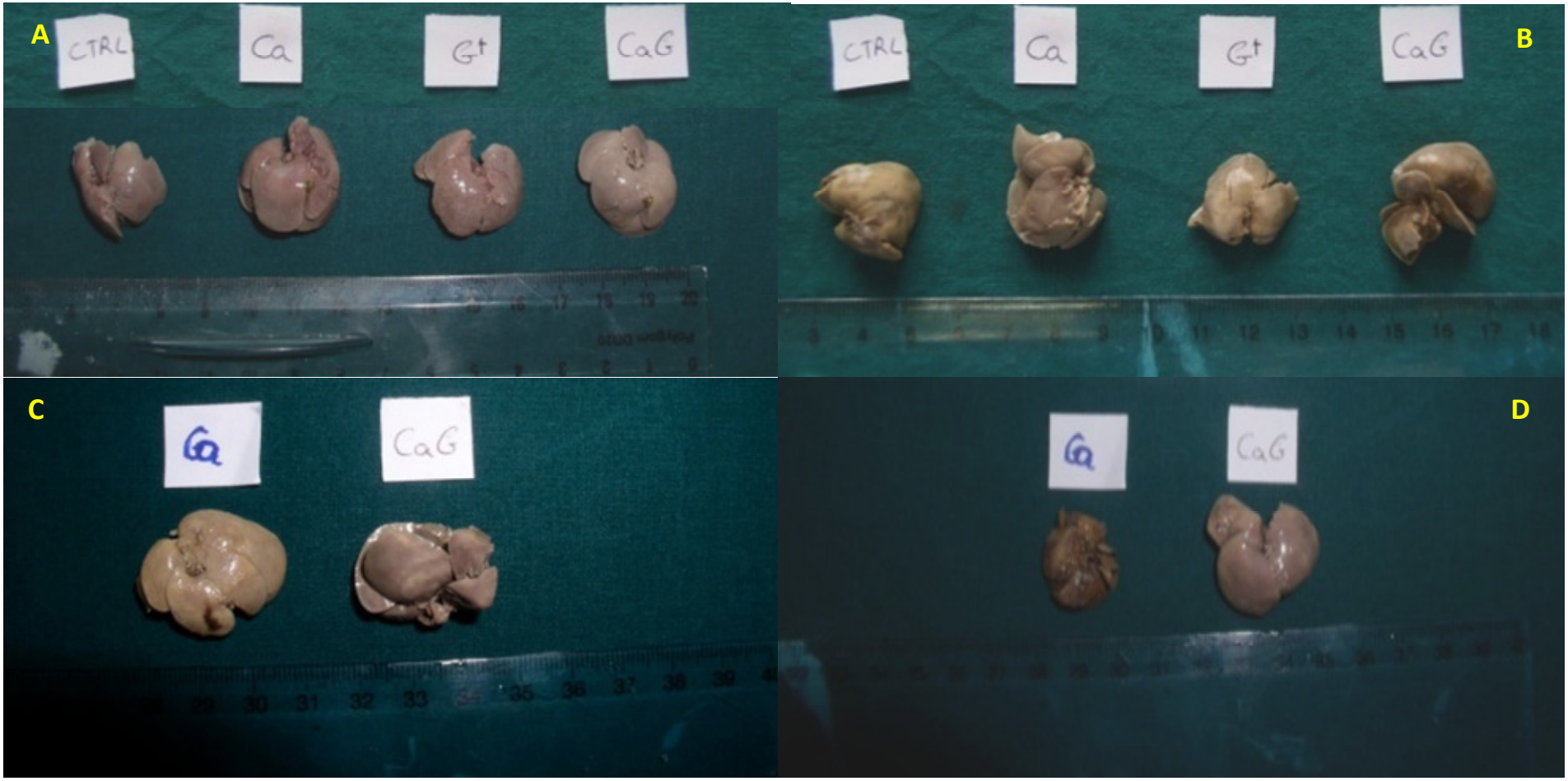

Figure 2. Variation in cfu counts of *C. albicans* recovered from several organs of Swiss albino mice (*M. musculus*) throughout the infection period.

yeast. However, very few leucocytes and histiocytes are seen either in the lumen or the submucosa.

Histological assessment of the stained liver tissue shown in Figure 4 revealed significant differences between Groups B and D. In fact, Group D displayed a significantly greater fungal burden than did Group B. When the extent of infiltrating neutrophils was assessed, Group D had markedly higher numbers of polymorphonuclear cells (PMNs) in the liver compared to the other groups. The inflammatory scores and PMN cell infiltration indicate that T cells may have exacerbated inflammation during the infection with *C. albicans*.

In Group B, inflammation, abscesses, and necrosis were all observed, as well as yeast presenting blastospores, pseudohyphae, and true

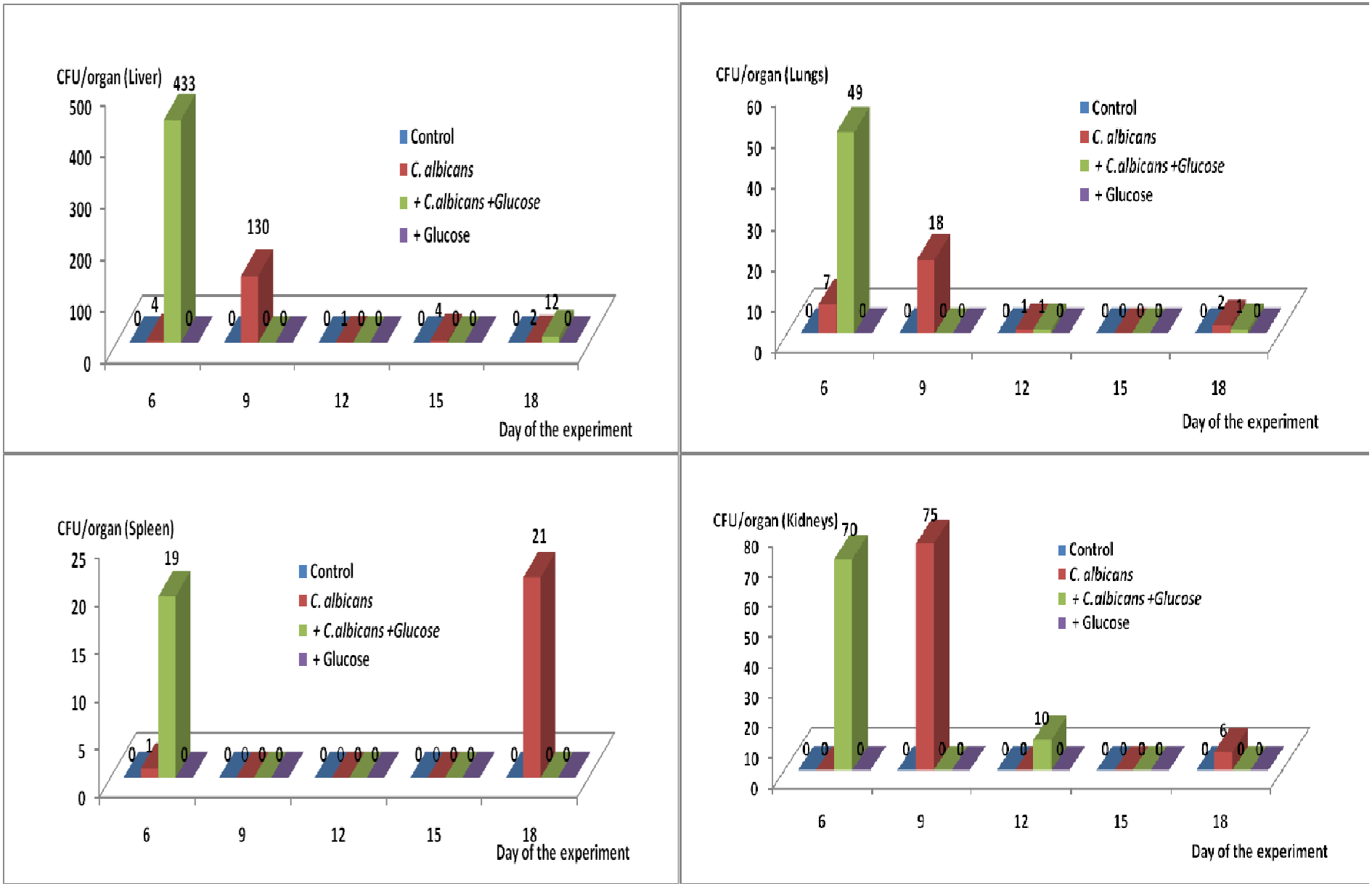

Figure 3. Evolution of the liver weight in the four groups (CTRL: control Group, Ca: group inoculated with *C. albicans*, G+: group receiving glucose, CaG: group inoculated with *C. albicans* and receiving glucose) after six days (A), nine days (B), twelve days (C) and Fifteen days (D) of the beginning of the experiment.

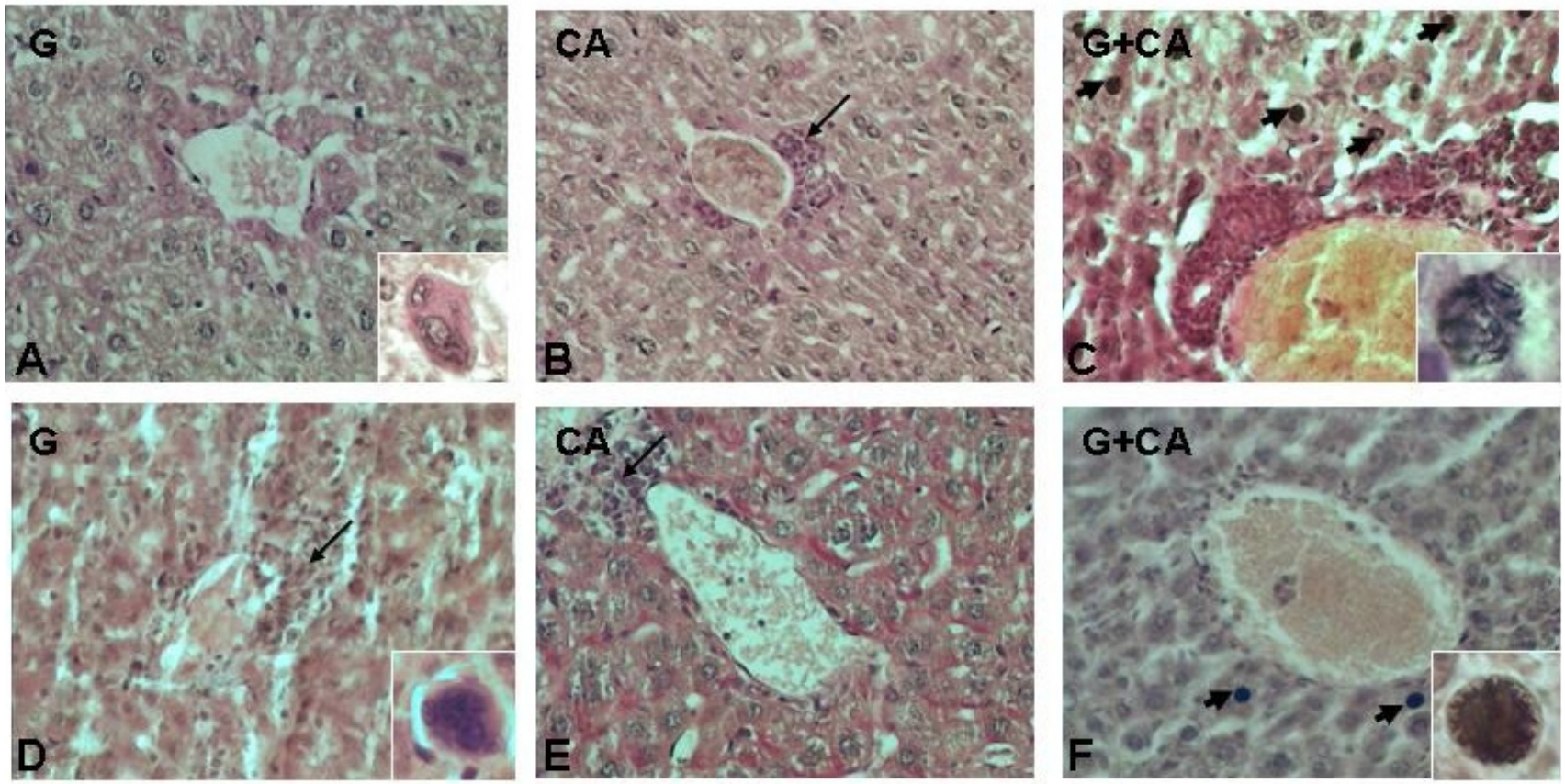

Figure 4. Histopathological appearance of the liver of immunosuppressed mice after 3 days (Figures A, B, and C) and six days (Figures D, E, and F) following intraperitoneal inoculation with *Candida albicans* strain. Arrows show hepatic abscesses with a destruction of hepatic acini. The central necrosis is surrounded by peripheral hyphae destroying hepatocytes. Periodic acid-Schiff (PAS) stained. Figures A, B, C, D, E, and F: × 100. G: Glucose; CA: *C. albicans.*

Hyphae.

DISCUSSION

In this study, glucose added to the drinking water of adult mice stimulated the growth of *C. albicans* in different organs such as the liver, kidneys, spleen, and lungs. This suggests that the *Candida* used the glucose as a preferred growth substrate (Winblad, 1975; Freter et al., 1983; Pasqualotto, 2009; Rodaki et al., 2009). This is supported by the high number of cfu isolated from the mice in Groups B and D, and correlates with the inoculum and the immunosuppression regimes. Our results confirm those previously reported by Holtschu et al. (1979) showing the presence of 50% more positive cultures in immunosuppressed infected animals.

Histological examination showed numerous *Candida* cells (blastospores and hyphae) in the Liver of infected Group B. The high number of positive cultures concurs with previously reported data showing that this organ is the least

effective in clearing the microorganisms. *Candida* infection led to the formation of multiple abscesses in the liver resulting in tissue necrosis. Indeed, the liver showed exudate containing polymorphs and monocytes. While abscesses were in fact observed 24 h post-infection, they did begin to heal on Day 3 and were completely healed by Day 7 post-infection. This confirms the absence of *Candida* growth in the livers harvested at a later infection period (over 6 days). In addition, our findings support those reported by Louria et al. (1964) and Kuttin et al. (1983).

The spleen plays an important role in clearing *Candida* cells from the bloodstream (Walbaum and Dujardin, 1985). Our study confirms this by the presence and growth of *Candida* in the spleen of the mice in Group D. The lungs are also effective against, and a target of, *Candida* infection. Indeed, Group D showed high cfu counts in the lungs compared to other organs. Our results confirm those previously reported that emphasize the efficiency of the lungs in retaining and eliminating viable *Candida* (blastospores and pseudohyphae) in *in-vivo* experimental infection models (Walbaum and Dujardin, 1985; Damodaran et al., 1973). This also supports the absence of pneumonitis in our experimental model.

The kidneys have been identified as the most affected organ following *Candida* infection in experimental models (Mariné et al., 2006; MacCallum, 2009) and also in human (Karlowicz, 2003) disseminated candidiasis due to the ability of the yeast to produce pseudohyphae in the tubular renal lumen and to penetrate into the renal parenchyma (Winblad, 1975). This is confirmed by our study which demonstrates the disorganization of the tissue and its invasion by the *Candida* strain. In addition, our results highlight the renal susceptibility to *Candida* infection that leads to tissue necrosis basically in the parenchyma, as observed in the Group B mice.

These findings also support those previously reported showing the severe invasion of the kidneys by *C. albicans*, *C. tropicalis* (Hasenclever and Mitchel, 1962; Majithiya et al., 2009), and *Candida parapsilosis* (Arendrup et al., 2002). The histologic analyses showed the presence of blastospores and pseudohyphal forms of *Candida* in the kidneys, with severe inflammation, as ascertained by leukocyte infiltration, abscesses, necrosis, and varying degrees of hydronephrosis. Thus, the most effective organ in clearing *Candida*, the kidneys are also the most affected.

Candida recovery from the blood was obtained solely in Group D after 6 days of infection with the *Candida* strain; the absence of *Candida* from the blood at later periods may in fact be due to the clearance of *Candida*. Indeed, it has been reported that yeast is generally recovered from the blood after only a few hours of inoculation (Trnovec et al., 1978). Further studies must be performed at shorter infection periods to follow the effects of *Candida* on the host as well as the defence mechanisms deployed by the host against *Candida* infection.

Host immunity is important in modulating the status of *C. albicans* in the organism (Klein et al., 1984, Rouabhia 2002; Bahri et al., 2010). In this study, inducing the immunosuppression status promoted *Candida* infection, which concurs with previously reported studies (Lass-Flörl, 2010; Murchan et al., 2010). Although our study demonstrates the effect of *Candida* infection on various host tissues, further investigations will undoubtedly shed light on the interaction pathways between *Candida* and the host cells, which may help to identify key mechanisms to improve the host's innate immunity against *Candida* infection. Our overall results confirm that dietary glucose supplementation leads to higher rates of *Candida* growth and invasion. It may thus be possible to control this organism in immunocompromised patients by restricting the availability of this carbohydrate.

Based on the various patterns with which *Candida* cells use carbohydrates, two strategies emerge. One would be to develop drugs capable of blocking the pathway for glucose utilization by *Candida*, while the other would be to limit the patient's intake of dietary glucose by substituting carbohydrates with xylitol, for example (Larmas et al., 1974; Krishnan et al., 1980). Xylitol is naturally present in many vegetables, and its palatability, safety, and oral use are well documented (Wang and van Eys, 1981; Mellado et al., 2000; Sreenath and Venkatesh, 2010). It may therefore constitute a suitable carbohydrate for use in immunocompromised patients during periods of high risk for mucositis and candidemia.

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