

## Immunological Defense Role of Oral Mucosa against Candidiasis Isolated from Human and Domestic Cats

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### Abstract

**Background:** *Candida albicans* (*C. albicans*) is an opportunistic fungus that infects the mucosa of oral cavity. Oral candidiasis remains one of the most common forms of *Candida* infections.

**Objective:** To isolated *C. albicans* from oral cavity of human and cats and study which one was more virulence.

**Patients and Methods:** *C. albicans* was collected by swabbing samples from oral mucosa to human and domestic cats. Mice injected with *C. albicans* isolated from human and cat divided to groups to study the immunological role. For each groups were divided depends of the time subjected to blood serum and tissue biopsy collected (6, 24 hours and 10 days) after mice injected with *Candida*. Measured IL-17, HMGB1 and G-CSF level in blood samples and tissue biopsy were done.

**Results:** The concentration of IL-17 in a group of mice injected with isolated *Candida* from mouth of human showed a significant increase in the two times of draw ( 24h and 10 days), while the concentration of IL-17 in group of mice injected with isolated *Candida* from mouth of cat showed significant increase and this elevation was shown in the two times of tissue biopsy collection ( 6h and 24h). While the higher increase in the levels of serum HMGB-1 recorded in mice at 6h and 24h after injected with isolated *Candida* from mouths of cats. On another hand the result of the concentration of G-CSF in tissue biopsy showed decreased at 10 days compared with control group.

**Conclusion:** *Candida* isolated from human more virulence form *Candida* isolated from.

**Keywords:** *Candida albicans*, IL-17, HMGB1 and G-CSF.

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### Introduction

The oral cavity is a place where many better habit to grow different microorganisms lived in as viruses, bacteria, microorganisms. This microorganism could fungus, and protozoa. These areas provide a cause disease, when it has the opportunities

[1]. One of the microorganisms *C. albicans* lived normally in mouth, upper respiratory tract, female genital tract and gastrointestinal tract, do not cause disease [2]. Oral candidiasis is the disease that caused by opportunity *C. albicans*. The Oral candidiasis lesion differs in size, colour and shape in oral cavity. It can cause superficial and serious systemic disease in immune comprised patients [3]. Pathogenicity of *C. albicans* relies on successful production of virulence factors. Virulence factors are responsible for the transformation of *C. albicans* to causing a disease (pathogen) and makes it possible for *C. albicans* to survive, persist within the host and cause disease when the opportunity arises [4,5]. The skin surface is a unique position, harboring a distinct groups of bacteria and fungi. From important fungi, *C. albicans* causes cutaneous lesions with the thickening, hyperkeratosis, and erythema of the skin [6,7]. Presence of fungi on the skin lead to interact with keratinocytes, dendritic cells (DCs), and macrophages, as well as with myeloid cells [8]. *C. albicans* can caused a common mucosal infection in vagina worldwide, which called Vulvovaginal candidiasis (VVC) this disease characterized by itching, burning, pain, redness of the vulva and vaginal mucosa, and discharge [9]. Neutrophils play an essential role during VVC immunopathology. Also, studies have showed that neutrophils have an essential immunoregulatory role by releasing important cytokines, such as IL-10 and IL-12, to help antifungal T cell development. Aims of study was isolated *C. albicans* from

oral cavity for human and cats and comparative density of infection between infected mice with human *C. albicans* isolated and cats *C. albicans* isolated by examination the histopathological changing and the level of IL-17, HMGB-1 and G-CSF to evaluated which isolated more virulence.

## Patients and Methods

### Study Samples

*C. albicans* was collected from December 2018- February 2019 through swabbing samples from human oral mucosa and domestic cat's oral mucosa. The samples were obtained from the archives of the Department of Oral Diagnosis / College of Veterinary Medicine / Diyala University / private laboratories / Private dental center, Baquba.

### Sample collection and candida albicans isolation

These samples collected from human between (19-30) age old ,from the tongue and sub mucosa was been taken to the lab, to be cultured on Sabouraud agar (SAD) incubation for 24 hr. at 37c for Candida albicans isolation. And the samples collected from cats, from the tongue and sub mucosa was been taken to the lab, to be cultured on Sabouraud agar (SAD) incubation for 24 hr. at 37c for *C. albicans* isolation.

### Isolation of Candida albicans

Inoculated the sample from human and cat in the Sabouraud dextrose agar, after that incubated by using incubator for 24hr in 37°C was to get *C. albicans* isolate.

Germ tube test: formed of germ tube was done by inoculation of five hundred micro

liters of human serum and incubation the inoculum two and half hours at 37°C. Then centrifuged the mixture, removed supernatant and reconstituted the cell precipitate with 500  $\mu$ L distilled water. Take a drop of suspension, put on the clean slide, covered with cover slide and examined under 10X and 40X power for detection of germ tube [10].

### **Induction of inflammation by *Candida albicans***

This experiment was performed by using laboratory mice from (Iraqi Center for Cancer and Medical Genetics Research/ Unit of Animals House). The mice were divided in to seven groups each group contains 6 mice. First group was normal mice without infection (control group), the second, third, and four groups were injected in oral cavity in a depth 1mm with solution of 1 ml/10- cell of *C. albicans* isolated from human. While the fifth, sixth, and seventh groups were injected in oral cavity in a depth of 1mm with solution 1 ml/ 10- cell of *C. albicans* isolated from cat. The mice in the second and fifth groups were anesthesia by diethyl ether after 6 hours from injection third , sixth groups were anesthesia after 24 hours after injection and fourth and seventh groups were anesthesia after 7 days from injection to collected oral biopsy each mouse are stored in phosphate buffer solution (PBS). Blood sample also collected from each mouse from heart puncture Was collected in tube centrifuged for 20 minutes at( 2000 – 3000 rpm) then aspirated serum in new tube and preserved at -20°C till used.

### **Tissue samples and Homogenization**

After cutting tissue samples from the oral mucosa and lip of the mice, checked the weight (1g) added to 10mL for PBS, added 0.5 mL trypsin, maintained samples, homogenized by mortar and pestle. Centrifuged the homogenization for 20-min at the speed of 2000-3000 rpm removed supernatant and preserved in -20c for detection of cytokines.

### **Mouse (IL-17) ELISA Kit**

The detection of mouse IL-17 by using ELISA kit from SunRed Company (Catalogue No.201-02-0042) the methods was done depend on manufacture instruction.

### **Mouse (HMGB-1) ELISA Kit**

The detection of mouse HMGB-1 by using ELISA kit from Sunred Company (Catalogue no.201-02-0524) the methods was done depend on manufacture instruction.

### **Mouse (G-CSF) ELISA kit**

The detection of mouse (G-CSF) ELISA kit from Sunred Company (Catalogue no.201-02-0642) the methods was done depend on manufacture instruction.

### **Tissue preparation**

Mice were euthanized at various time (6hr. ,24 hr. ,10days) during the course of the experiment for pathological observation of tissues. After that the heads of animals were dissected and tissue samples (4-6mm thick) from the oral mucosa and lips were extracted and immediately immersed in the 10% fixative formalin solution for 24hr at room temperature to prepare slide for stain by hematoxylin and eosin (H&E). The sections were examined by light microscopy and

photomicrographs were taken of each section.

## Results

### Estimating the concentration of interleukin-17 in studied groups relative to the times of blood collection

The results of IL-17 showed a significant elevation at ( $P \leq 0.05$ ) in all three times of the draw (6h, 24h and 10 days) in a group of mice injected with isolated Candida from mouth of human compared with the control group at (mean  $\pm$  SD): (458.49 $\pm$ 112.61, 543.35 $\pm$ 65.12 and 391.86 $\pm$ 168.52) pg/mL respectively. While in control group it was

(30.5  $\pm$  5.53 ) pg/ml as shown in Table (1). The concentration of IL-17 in a group of mice injected with isolated Candida from mouth of human (G2) showed a significant increase at ( $P \leq 0.05$ ) and this elevation was shown in the two times of draw ( 24h and 10 days), while there are statistical differences but non significant at 6h of draw compared with the control group at (Mean  $\pm$  SD), (511.59 $\pm$  30.74, 493.29 $\pm$ 70.69 and 458.49 $\pm$ 112.61) pg/ml respectively as shown in Table (1).

**Table (1):** The level of IL-17 in serum of mice injected with Candida isolated from human and cats

Groups	Time	No.	IL-17 pg/ml
Control	IL-17 serum	6	26.37 $\pm$ 28.88
Candida isolated from Human	6 hours	6	458.49 $\pm$ 112.61 *
	24 hours	6	543.35 $\pm$ 65.12 *
	10 days	6	391.8 $\pm$ 168.52 *
Candida isolated from Cat	6 hours	6	182.6 $\pm$ 186.61
	24 hours	6	511.5 $\pm$ 30.74 *
	10 days	6	493.29 $\pm$ 70.69 *

\*The data was presented at (mean  $\pm$  SD), the result was

\*\*significant at ( $P \leq 0.05$ ) compared with control group

### Estimating the concentration of interleukin-17 in studied groups relative to the times of tissue biopsy collection:

The results of IL-17 in tissue showed a significant elevation at ( $P \leq 0.05$ ) in all three times of draw (6h, 24h and 10 days) in group of mice injected with isolated Candida from mouth of human compared with control group at (mean  $\pm$  SD): (402.17 $\pm$ 134.86, 443.52 $\pm$ 110.23 and 463.65 $\pm$ 150.83) pg/mL respectively. While in control group it was (138.2 $\pm$ 134.15)pg/mL as shown in Table (2).

The concentration of IL-17 in group of mice injected with isolated Candida from mouth of human (G2) showed significant increase at ( $P \leq 0.05$ ) and this elevation was shown in the two times of tissue biopsy collection ( 6h and 24h), while there are statistical differences but non significant ( $P \leq 0.05$ ) at 10 days of draw compared with control group at (Mean  $\pm$  SD), (457.84 $\pm$ 82.16, 429.73 $\pm$ 215.22 and 48.43 $\pm$ 75.29) pg/ml respectively Table(2).

**Table (2):** The level of IL-17 in tissue of mice injected with Candida isolated from human and cats

Groups	Time	No.	IL-17 pg/ml
Control	IL-17 tissue	6	138.2±134.15
Candida isolated from Human	6 hours	6	402.17±134.86 *
	24 hours	6	443.52±110.23 *
	10 days	6	463.65±150.83 *
Candida isolated from Cats	6 hours	6	457.84±82.16 *
	24 hours	6	429.73±215.22 *
	10 days	6	48.43±75.29

\*The data was presented at (mean ± SD), the result was \*significant at (P<0.05) compared with control group

**Estimating the concentration of HMGB-1 in studied groups relative to the times of blood collection:** The results showed that the levels of serum HMGB-1 increased significantly (P<0.05) in all three times of draw (6h, 24h and 10 days) in groups of mice injected with isolated Candida from mouths of both human and cats compared with

control group at (mean ± SD):( 519.72±160.17, 490.97±247.75 and 546.02±166.08) ng/mL respectively in first group. While the higher increase in the levels of serum HMGB-1 recorded in mice at 6h and 24h (636.41±321.04 and 616.69±240.26 )ng/mL after injected with isolated Candida from mouths of cats as shown in Table (3).

**Table (3):** The level of HMGB1 in serum of mice injected with Candida isolated from human and cats

Groups	Time	No.	HMGB1 ng/ml
Control	HMGB1 serum	6	371.81±162.27
Candida isolated from Human	6 hours	6	519.72±160.17 *
	24 hours	6	490.97±247.75 *
	10 days	6	546.02±166.08 *
Candida isolated from Cats	6 hours	6	636.41±321.04 *
	24 hours	6	616.69±240.26 *
	10 days	6	444.13±201.70 *

\*The data was presented at (mean ± SD), the result was

\*\*significant at (P<0.05) compared with control group

**Estimating the concentration of HMGB-1 in studied groups relative to the times of tissue biopsy collection:** The results showed that the levels of HMGB-1 in tissue increased significantly (P<0.05) in all three times of tissue biopsy collection (6h, 24h and 10 days) in groups of mice injected with isolated Candida from mouths of both human and cats

compared with control group at (mean ± SD) and the higher increase in the levels of serum HMGB-1 recorded in mice at 6h (624.35±69.89)ng/ml after injected with isolated Candida from mouths of human as shown in Table (4). While In the control group was( 303.35±153.25) ng/ml.

**Table (4):** The level of HMGB1 in tissue of mice injected with Candida isolated from human and cats

Groups	Time	No.	HMGB1 ng/ml
Control	HMGB1 tissue	6	303.35±153.25
Candida isolated from Human	6 hours	6	624.35±69.89 *
	24 hours	6	447.14±146.28 *
	10 days	6	366.34±32.73 *
Candida isolated from Cats	6 hours	6	484.12±88.90 *
	24 hours	6	432.08±100.59 *
	10 days	6	566.29±89.20 *

\*The data was presented at (mean ± SD), the result was

\*\*significant at (P≤0.05) compared with control group

**Estimating the concentration of G-CSF in studied groups relative to the times of blood collection:** Concentration of G-CSF in serum showed significant increasing at (P ≤ 0.05) in all three times of draw (6h, 24h and 10 days) and this increasing was more reliable at 6h (1359.29 ± 1031.9) ng/L after

injected with isolated Candida from mouths of human, also at 24h (920.09 ± 545.49) ng/L after injected with isolated Candida from mouths of cats compared with control group (84.77±92.86) ng/L as shown in Table (5).

**Table (5):** The level of G-CSF in serum of mice injected with Candida isolated from human and cats

Groups	Time	No.	G-CSF ng/L
Control	G-CSF in serum	6	84.77±92.86
Candida isolated from Human	6 hours	6	1359.29±1031.9 *
	24 hours	6	878.10±435.82 *
	10 days	6	456.98±411.20 *
Candida isolated from Cats	6 hours	6	877.84±442.32 *
	24 hours	6	920.09±545.49 *
	10 days	6	657.90±481.35 *

\*The data was presented at (mean ± SD), the result was

\*\*significant at (P≤0.05) compared with control group

Estimating the concentration of G-CSF in studied groups relative to the times of tissue biopsy collection As shown in table (6), The results referred to insignificant variation (P ≤ 0.05) in the concentration of G-CSF at all three times of tissue biopsy collection (6h, 24h and 10 days) (39.07±85.32, 130.46±319.56 and 39.07±72.60) ng/L respectively, in group of mice injected with isolated Candida from mouths of cats. As

well as inverse significant variation (P ≤ 0.05) in the concentration of G-CSF at two times tissue biopsy collection (6h and 24h) (844.67±56 and 777.48±577.23) ng/L respectively in group of mice injected with isolated Candida from mouths of human. Whereas the differences were decreased at 10 days (192.26±317.59) ng/L after injection with Candida compared with control group (53.23±95.76)ng/L.



**Table (6):** The level of G-CSF in tissue of mice injected with Candida isolated from human and cats

Groups	Time	No.	G-CSF ng/L
Control	G-CSF in tissue	6	53.23±95.76
Candida isolated from Human	6 hours	6	844.67±56 *
	24 hours	6	777.48±577.23 *
	10 days	6	192.26±317.59
Candida isolated from Cats	6 hours	6	39.07±85.32
	24 hours	6	130.46±319.56
	10 days	6	39.07±72.60

\*The data was presented at (mean ± SD), the result was

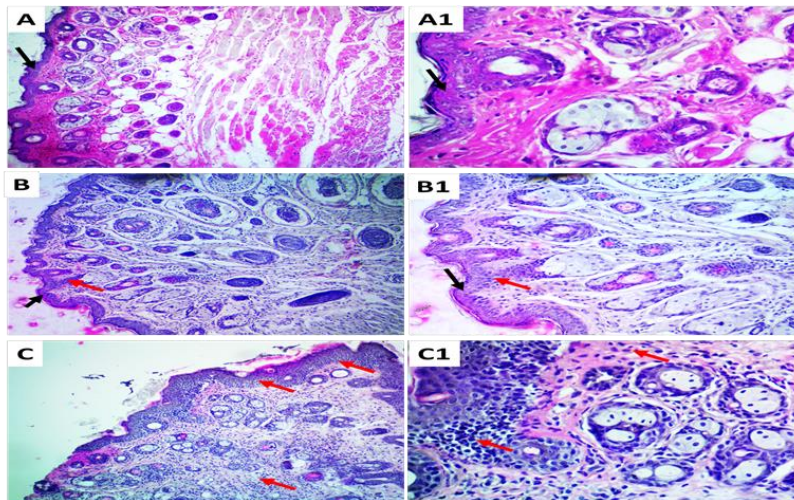
\*\*significant at ( $P \leq 0.05$ ) compared with control group

### Histopathological results

#### The source of *Candida albicans* from human smear

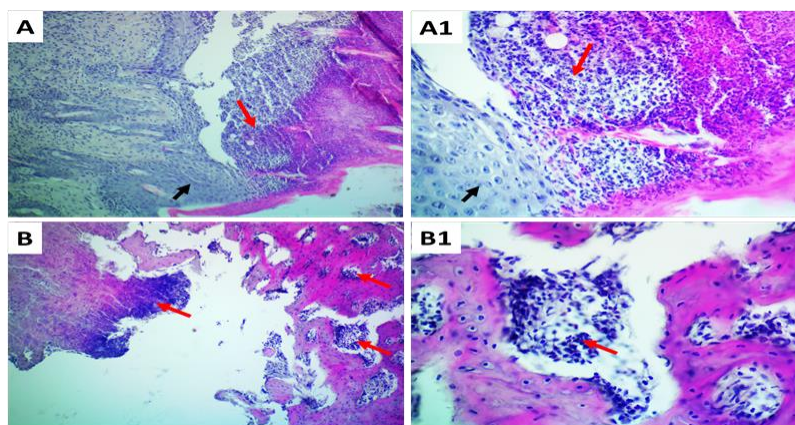
The current study revealed that there was a slight histological changes observed after 6 hours of induced infection with *C. albicans* obtained from human smear Figure (1); B and B1). These pathological changes manifested by distribution of inflammatory cells at the epidermal layers and mostly observed at the basal layer. The same results were observed after 24 hours but the inflammatory cells were more obvious than the previous one Figure (1); B and B1). However, at day 10, the results of histopathological changes in oral mucosa of the infected mice indicated there was a

visible localization of inflammatory cells (mononuclear cells) were associated with granulomatous inflammation of the epithelium Figure (2); A and A1). This granulomatous lesion and many mononuclear infiltration cells were also observed at the deepest layer of connective tissue attached with hard plate. Additionally, the damaged for the muscle layers were also extended, and many mononuclear cells were observed around these layers Figure (2); B and B1). The results also indicated that the inflammatory cells were also distributed throughout different layers of the degenerated tissue showed a clear damaged for the underlying tissue and separation of the epidermal tissue Figure (2) ; A and A1).



**Figure (1):** Photomicrographs shows the histopathological observations of oral mucosa induced infection with *Candida albicans* after 6 hours (B, B1), 24 hours (C, C1), and in normal tissue (A, A1).

(B, B1) sections shows mild inflammatory cells indicated by red arrows, while many of these inflammatory cells were observed in (C, C1) indicated with same red arrows, black arrows shows epidermal tissue were clearly seen in normal tissue (A, A1) did not show any inflammatory cells. (A, B, C, at 10X, while A1, B1, C1 at 40X) (H&E).



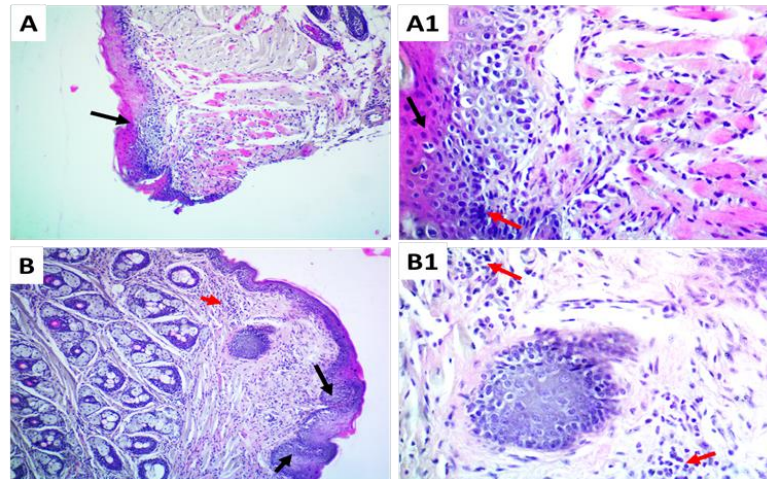
**Figure (2):** Photomicrographs shows the histopathological observations of oral mucosa induced infection with *Candida albicans* after 10 days (A, A1), and the inflammation was observed deeply to underlying tissue and close to hard palate (B, B1). (A, A1) sections shows granulomatous inflammation contained many inflammatory cells indicated by red arrows, while many of these inflammatory were observed in deepest tissue attached to the hard palate (B, B1) indicated with same red arrows, black arrows shows epidermal tissue. (A, B, at 10X, while A1, B1, at 40X) (H&E).



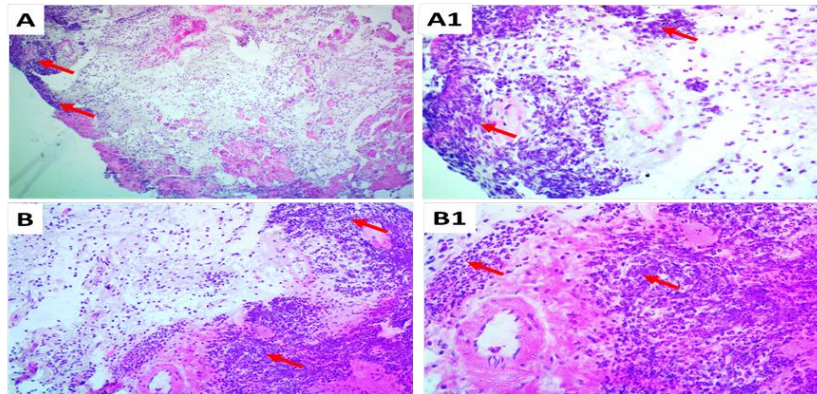
### The source of *Candida albicans* from cat smear

The second group infected with *Candida albicans* obtained from cat smear were indicated the same histopathological results observed after 6 hours of induced infection Figure (3); A and A1). These histopathological changes showed mild inflammatory cells at the epidermal layers and mainly observed at the basal and suprabasal layer. The same results were detected after 24 hours but the inflammatory cells were more obvious than the previous one Figure (4). However, at day 10, the results of histopathological changes in oral mucosa of the infected mice indicated there

was a visible localization of inflammatory cells (mononuclear cells) were associated with granulomatous inflammation of the epithelium. This granulomatous lesion and many mononuclear infiltration cells were also observed at deepest layer of connective tissue and reached to the attachment with hard palate. Additionally, the damaged for the muscle layers were also extended, and many mononuclear cells were observed around these layers. The inflammatory cells were also distributed throughout different layers of the tissue causes a clear degeneration and separation of the epithelial tissue Figure (4); B and B1).



**Figure (3):** Photomicrographs shows the histopathological observations of oral mucosa induced infection with *Candida albicans* after 6 hours (A, A1), 24 hours (B, B1). (A, A1) sections shows mild inflammatory cells indicated by red arrows, while many of these inflammatory were observed in (B, B1) indicated with same red arrows, black arrows shows epidermal tissue. (A, B at 10X, while A1, B1 at 40X) (H&E)



**Figure (4):** Photomicrographs shows the histopathological observations of oral mucosa induced infection with *Candida albicans* after 10 days (A, A1), and the inflammation was observed distributed throughout the tissue (B, B1). (A, A1) sections shows granulomatous inflammation contained many inflammatory cells indicated by red arrows, while many of these inflammatory were observed distributed throughout the muscle (B, B1), these cells were indicated with red arrows. (A, B at 10X, while A1, B1 at 40X) (H&E)

## Discussion

There are significant increases in amount of IL-17 in both, collected blood samples and tissue biopsies, and specially 6h and 24 h Post infection. Many studies have mentioned that many defense mechanisms were included in response to candidiasis, and IL-17 is the most strongly recommended in such immune response [11]. It was reported that IL-17 and  $\text{INF-}\gamma$  play an important roles in protection against fungal infections, when they effectively enhanced neutrophils and macrophages in killing of fungi during the innate responses of the host. The importance of IL-17 extended to candidiasis in systemic infection, and this was attributed to the fact that laboratory mice are more susceptible to candidiasis when compared to the mice of wild type. The laboratory mice showed significant increases of IL-17 A2/2 and IL-RA2/2 [12,13,14]. Using of *C. albicans* vaccines showed protective responses

experimentally and produced both Th17 and Th1 the most effective cells against candidiasis [16]. High mobility group box-1 (HMGB-1) protein showed significant increase during the three selected times (6h, 24h, and 10 days) PI when samples were collected from blood or tissue biopsies. These findings come in agreement with many studies worldwide. Many immune cells were reported to secrete HMGB-1, including dendritic cells, macrophages and monocytes as a cytokine for the mediation of inflammation [17,18]. Binding of HMGB-1 to TLR2 and TLR4 will mediate the activation of macrophages to release cytokines during inflammatory responses [19,20]. HMGB-1 can bind to receptor for advanced glycation end products (RAGE) when released from cells and mediate the inflammation process [21]. Neutralization of HMGB-1 by circulating antibodies aided in relief of damaged tissues. Upregulation of

NF- $\kappa$ B was mediated by the interaction between TLR4 and HMGB-1. This upregulation increased cytokines secretion by many immune cells and also stimulate neutrophils to produce NADPH oxidase that was essential for the production of reactive oxygen species[22].

Granulocyte colony-stimulating factor in present study showed significant increase in both blood and biopsy tissue samples when compared to that of control. Such a relationship would suggest that G-CSF functions in a feedback relationship similar to erythropoietin and the endocrine hormones. It can be inferred on the basis of other studies that such a relationship exists. The repeated injection of dogs with G-CSF from human resulted in formation of antibodies that reacted crossly with G-CSF from dogs [23], the same dogs showed blood pictures of neutropenia lasted for long time and declined with decreased levels of anti G-CSF antibodies in blood circulation. The same results were obtained in dogs injected with plasma with anti G-CSF antibodies. Targeting of G-CSF gene in mice resulted in chronic neutropenia with 20% to 30% less than normal mice and lacking G-CSF [24]. It was hypothesized that G-CSF cytokine is essential and critical for the regulation of the neutrophil levels in blood circulation, but acute tissue injury, endotoxin administration and acute infections increased the levels of G-CSF. There is a parallel increase in blood neutrophils, consistent with the hypothesis that this cytokine serves as a long-range stimulus for marrow neutrophil release and

increased neutrophil production. Further understanding of the physiologic role of G-CSF will require more sensitive immunoassays and more detailed studies of cytokine responses during infectious and inflammatory diseases.

In the current experimental study indicated that the virulence of *C. albicans* induced a clear damaged to underlying tissue and it was observed extended deeply to reach to the muscle and bone. A possible explanation for these findings may be attributed to the virulence of *C. albicans* that is represented by having many factors such as, production extracellular hydrolytic enzyme [25], hyphal formation and surface recognition molecules [26]. The present study was also showed a clear differences in histopathological changes throughout the course of the experiment, as it was observed that the degree of damage of oral mucosa in inoculated mice was found to be further severe at day 10 than to other at 1 and 6 days for both isolation from human and cat. These differences can be clarified in part by the formation of granulomatous inflammation on the epithelial tissue after prolonged survival of *C. albicans*. Comparison these results with those of other studies confirms the formation of granulomatous papillary tissues infiltrate with inflammatory cells [27]. It seems possible that the granulomatous inflammation and thickening in the epithelium are due to increasing the fungal infection over time in most of the epithelial surfaces and induces bacterial infection [25]. Overall, this study compared the virulence among the different

isolations and may in part be attributed to the local immune responses induced by different isolations.

## Conclusions

The findings of this study was approved that *C. albicans* occurs as a commensal in the oral cavity of healthy humans and animals. IL-17 is the most strongly recommended in such immune response. High mobility group box-1 (HMGB-1) protein showed significant increase during the three selected times (6h, 24h, and 10 days) post infection when samples were collected from blood or tissue biopsies. The *C. albicans* that isolated from human more virulence than *C. albicans* isolated from cat.

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