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Immunological Response of Albino Rats Immunized with UV-Killed *Candida albicans*

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Abstract: The treatments of systemic candidiasis in humans with the current antifungal drugs of choice such as azole derivatives and amphotericin B have raised issues of toxicity and resistance among others. The immunogenic property of killed *C. albicans* was explored to evoke immunological response in the possible prevention of candidemia. Rats were immunized after acclimatization on two occasions with 10^6 cells/ml of Ultraviolet radiation killed *C. albicans* and subsequently challenged with 10^6 viable *C. albicans*. Two other groups; positive and negative controls received same dose of viable *C. albicans* and normal saline respectively. Five rats each from the groups mentioned were bled for the determination of antibody titers and white blood cell differentials. Also, other rats from the three groups were observed for 28 days for survival after challenge to determine the protective effect of the ultraviolet killed *C. albicans*. The results revealed that Ultraviolet killed *C. albicans* stimulated a significant amount of antibodies and white blood cell differentials that were immunoprotective. We also found that all immunized rats survived challenge with 10^6 viable cells while the kidneys of the dead unimmunized rats showed a positive growth of *C. albicans*. Thus, Ultraviolet killed *C. albicans* could provide significant protection.

Keywords: *Candida Albicans*, Candidiasis, Immunoprotection, Radiation

Introduction

It is well known that vaccines are developed for the control or prevention of diseases. They are derived from cell surface molecules (proteins and carbohydrate moieties), engineered organisms that have lost their virulence and pathogenecity or even killed organism that are immunogenic or have the ability of stimulating immune responses against a specific disease causing micro-organisms (Evron, 1980).The cell wall of microbes acts as protective, structural shield and also contributes to interactive contacts with the human host during the initiation of infection and development (Tada *et al*., 2013).

Candida albicans is the most common candida species that causes candidiasis or yeast infection (CDC, 2016). It is reported to be present in 80% of human population as commensal but also has the ability of transiting to pathogenic forms when there is an imbalance of the normal microbiological flora, breakage of epithelial barriers or dysfunction of the immune system (Staib and Morschhäuser, 2007; Gow and Hube, 2012). These conditions may result from a wide-broad spectrum use of antibacterial, surgery, mutation or heredity among other factors (Spellberg *et al*., 2012). *Candida albicans* have been reported to cause two major types of infection; viz: superficial and disseminated (systemic) infection (Ortega *et al*., 2010). Superficial infection in candidiasis is usually localized around the skin, mouth and genitals whereas in systemic infection *C. albicans* gained access to the blood stream and invade the organs (Ortega *et al*., 2010). Symptoms associated with superficial candidiasis include discharge, itching and inflammation of the skin while in systemic infection; kidney or brain damage may occur (Leleu *et al*., 2002). According CDC, 75% of women with vulvo-vaginal candidiasis have been reported to have at least one episode of candidiasis while 40-45% has recurrent episodes (CDC, 2015). In addition, candidemia which is a life threatening infection accounts for 50-70% of all cases of invasive mycoses (Lam*et al*., 2016). Despite the achievements recorded in the treatment of systemic infection, approaches still depend on the use of chemotherapy (antifungal agents). Therefore, search for novel prophylactic alternatives that is effective and safe

with no side effects continues due to a number of limitations with existing antifungal drugs such as toxicity, high cost and most times not available (Wang *et al*., 2015). More disturbing is the emergence of resistance to most of the commonly used antifungal drugs (Wang *et al*., 2015). This has led to increasing interest in the search for alternative strategies with immunostimulatory properties from a killed organism, subunits vaccine and even attenuated organisms for the protection of the host. Researchers mostly from other countries have reported the immunostimulatory potentials of *C. albican* killed using heat method with a number of solvent or adjuvants in mice (Evron, 1980; Lopez-Ribota *et al*., 2004; Cárdenas-Freytag*et al*., 1999). However, past studies in Nigeria on development of prophylactic options for the prevention of systemic candidiasis is scanty. Owing to the prevalence of mortality and morbidity attributed to systemic infection, it has become necessary to initiate studies.Therefore, the objective of this study therefore is to explore the potential of Ultraviolet (UV) radiation killed *C. albicans* sensitized host immune cells for the prevention of *C. albicans* infection.

Materials and Methods

Experimental Animals

Male albino rats of 6-7 weeks old were used in this study. The rats were provided with water and food ad libitum and allowed to acclimatize for 7 days prior to the commencement of the experiment. The use of animal for this study was approved by the Department of Pharmacology and Toxicology, National Institute of Pharmaceutical Research and Development on the standard practices of handling experimental animals in line with international best practice.

Vaccine Preparation

Candida albicans isolate was obtained from a clinical sample of a patient suspected with candida vaginitis. An informed consent was sought from the patient before the collection of the sample. The sample was cultured on Sabouraud Dextrose Agar (SDA) at 37°C for 2 h (Pereira *et al*., 2011). The creamy, white and pasty appearance of *C. albicans* produced on SDA was sub-cultured on a chromogenic agar (colour based selective medium) at 37°C for 48 h (Cooke *et al*., 2002). This was followed by a germ- tube test to differentiate albicans from non- albicans. Germ tube test was achieved by inoculation of a portion of *C. albicans* in serum and incubated at 37°C for 3 h. This preparation was viewed under the microscope for the presence of filament or outgrowth (Elmer *et al*., 1992). *Candida albicans* used for immunization was exposed directly to a source of UV (Vilber Lourmat, UV trans-illuminator) at a wavelength of 254 nm. Non-viability was confirmed every 10 minutes using methylene blue stain and sub-culturing on SDA for 30 min (Evron, 1980).

Immunization and Challenge

Three types of experiments were carried out in this study. Hence, rats were divided into fifteen each of three groups namely; negative control, positive control and UV inactivated group. Baseline was taken for all the rats 7 days after acclimatization. The UV inactivated group was immunized with 10^6 UV killed *C. albicans* subcutaneously on two occasions 7 days after acclimatization and 21 days after. Positive and negative controls received same concentration and route of viable cells and sterile normal saline respectively on the 21st day.This is followed by an intravenous (caudal) challenge with viable *C. albicans* after 14 days of booster immunization.

The first experiment was performed to determine variation or abnormalities of antibody titers and white blood cells differentials. Five rats each from the three groups were finally sacrificed after 3 days of challenge and their blood collected by cardiac puncture. The following parameters were determined using relevant assays as detailed below to check abnormalities.

Determination of WBC Differentials and Antibody Titers

Blood samples were collected in EDTA bottles and analyzed with the aid of an automated hematology analyzer (Abacus 380) to determine the frequency of abnormalities of white blood cell differentials.

Antibody titers were determined as described by Bin-Hafeez *et al*. (2001). Blood samples were collected in plane bottles and centrifuged at 2500 rpm for 10 minutes and sera obtained using a micropipette. As much as 100 µl of serum was heat- inactivated at 56°C in water bath for 30 minutes. About 50 µl of Phosphate Buffered Saline (PBS) was then added to all 12 tubes. The first tube was considered the control hence it only received 50 µl of PBS, while the second well received 50 µl of heat-inactivated serum to form a mixture of serum and PBS. From the second tube, 50 µl of the mixture was used to serially dilute by 2 - fold in the subsequent tubes. Finally, 50 µl of heat killed *C. albicans* with a cell density of 10^6 cells/ml was added to all the tubes and incubated at 37°C for 2 h. The values of antibody titer were assigned to the highest serum dilution showing at least 50% of visible agglutination.

Determination of Weekly Survival Analysis

The second experiment was carried out to determine survival of the rats after challenge. This was achieved according to Thomas *et al*. (2006) method. Another five rats each from the three groups that were immunized and challenged as described above were not sacrificed but monitored for a period of 28 days after challenge. Kidneys of dead rats during this period were harvested. The kidneys were sectioned, stained with crystal violet stain (microscopic stain BDH chemicals Ltd) and viewed

under × 500 digital microscope (Coolinttech, UK). Also homogenized portion were cultured on agar plate containing chloramphenicol. This is to check for the growth of *C. albicans*.

Compliance with Ethical Standards

There is no conflict of interest. The patient was also consented before the sample was taken for identification. The use of rats in this study is in accordance with the international best practices approved by the department of Pharmacology and Toxicology, National Institute of Pharmaceutical Research and Development, Abuja.

Statistical Analysis

The data collected from this study were subjected to statistical analysis. Analysis of Variance (ANOVA), student t-test and simple percentage were used to analyze these parameters. Analyses were performed using VassarStat Software (USA). The level of significance was set at a *P* value of <0.05.

Results

Identification of Candida Albicans

Candida albicans isolates from the clinical sample obtained produced green coloration on the selective medium of Brilliance Candida Agar™, germ tubes (small, sprouting tube-like outgrowths filaments projecting from the cell surface) were formed in serum on incubation at 37° and also a white to cream in color with smooth border appearance was formed on SDA plate incubated at 37°C to confirmed *C. albicans*. Cells exposed to UV radiation became stained in methylene blue and showed no evidence of growth on SDA plates. This confirms non- viability of the cells.

Antibody Titers of Immunized Rats and Controls

Result of antibody titers of rats showed that vaccination with UV killed *C. albicans* stimulated significant (P<0.05) amount of antibodies compared to controls (Fig. 1). The mean baseline antibody titer of the rats taken after 7 days of acclimatization was 25.6±10.55 for all the groups. After two occasions of vaccinations (first and booster vaccinations) and challenge, mean antibody titers recorded for the negative control group increases slightly to 32±8.76 from the baseline titers of 25.6±10.55.

The difference between antibody titers of rats in the negative control when compared to the baseline titers did not show any statistically significant difference (P>0.05). However, in the UV treated group, there was a statistically significant (P<0.05) increase in antibody titers (972.8±307) when compared with both control groups after first and booster immunizations with UV killed *C. albicans* and subsequent challenge with viable cells. The positive control which was not immunized but infected during the same period of the study showed a statistically significant (P<0.05) lower mean antibody titers (256±70.11) when compared to the immunized group.

WBC of Immunized Rats and Controls

The WBC count of albino rats vaccinated and challenged with *C. albicans* is presented in Fig. 2. The mean WBC count of the rats after acclimatization (baseline) was 7.6±0.3195. At the end of the experiment, the mean WBC counts of rats in the negative control group increased slightly to 8.8±0.3458 from the baseline. In addition, immunizations and challenge of rats in the UV inactivated group resulted in a statistically significant (p<0.05) increase in mean white blood cell counts compared to both baseline counts and the positive control group. This is because the immunized rats recorded relatively higher mean WBC counts of 10.58 ± 0.7702. However, rats in the positive control group had decreased WBC counts of 5.12±0.3385 which is the lowest when compared to all groups. Statistical analysis shows a significant difference (P<0.05) in WBC counts of rats in the immunized and positive control groups.

Lymphocytes Profile of Rats Immunized and Controls

Albino rats in all the three groups recorded an increase in mean lymphocyte counts after 38 days as follows: UV inactivated (73.18%), Negative control (66.32%) and Positive control (57.06%) (Fig. 3). Although the increase in lymphocyte counts relative to the baseline counts (67.42%) in all the groups was not significant (P>0.05), the mean lymphocyte counts of rats in the vaccinated group shows a significant difference (P<0.05) when compared to rats in the control groups after 38 days. Similarly, there was a significant difference (P<0.05) in lymphocyte counts when negative and positive control groups were compared. However, mean lymphocyte counts of rats in positive control group at the end of the experiment (57.06%) shows a significant decrease when compared with the baseline counts.

Granulocytes Profile of Immunized Rats and Controls

The mean granulocytes counts of immunized and control groups is presented in Fig. 4. The mean percentage granulocyte counts of rats after acclimatization (baseline) in all the groups was 25.94±1.061. At day 38, rats in the negative control group recorded a statistically insignificant slight increase (P>0.05) in the level of granulocytes to 25.94±1.061. However, rats in UV inactivated and positive control groups recorded significantly lower (P>0.05) granulocyte counts of 19.88% and 15.7% respectively as compared to the baseline counts.

Mid-Range Cells (MEB) Profile of Immunized Rats and Controls

The result of percentage Mid-range cells (monocytes, eosinophils and basophols) for immunized and control albino rats after acclimatization and subsequent treatment is presented in Fig. 5. The mean percentage of MEB for rats in all groups after 7 days of acclimatization (baseline) was 3.66±0.16. With the exception of rats in the positive control group that recorded a statistically significant ($P<0.05$) MEB of 27.28% at day 38, rats in UV inactivated and negative control groups recorded lower MEB of 5.82 and 4.32% respectively. Although the slight increase in MEB at day 38 in the two groups (negative control and UV inactivated groups) were not statistically significant ($P<0.05$) from the baseline counts and between UV inactivated and negative control groups at day 38, there was a statistically significant difference ($P<0.05$) in MEB recorded when compared to positive control group.

Survival Analysis

The survival result of the rats immunized with UV killed *C. albicans* and the controls after 28 days is presented in Table 1 and Fig. 6. No death was recorded in rats from negative control and Heat inactivated groups during the four weeks of observation. However, 75 and 25 percent of rats in the positive control group died in the first and second weeks respectively.

Table 1: Percentage mortality rate of rats immunized with Heat inactivated and control groups

Vaccination Status	--- % Survival -- Week 1	Week 2	Week 3	Week 4
Heat group	0.0	0.0	0.0	0.0
Negative control	0.0	0.0	0.0	0.0
Positive control	75.0	25.0	0.0	0.0

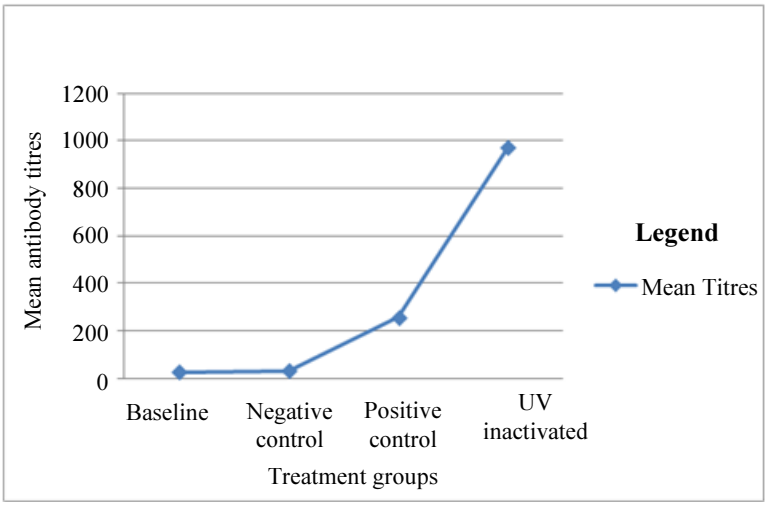

Fig. 1: Antibody titers of rats vaccinated with inactivated *C. albicans* and controls

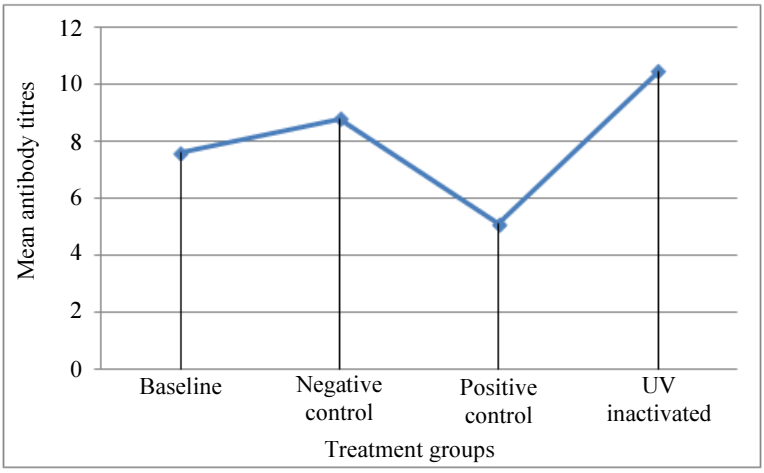

Fig. 2: White blood cell count of rats vaccinated with inactivated *C. albicans* and controls

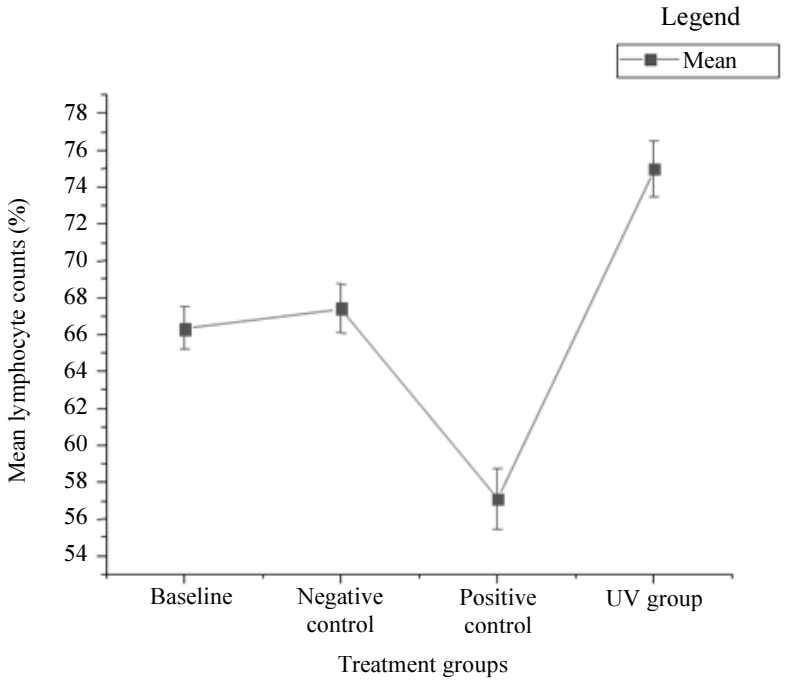

Fig. 3: Lymphocytes Profile of Albino Rats Vaccinated with UV killed *C. albicans*

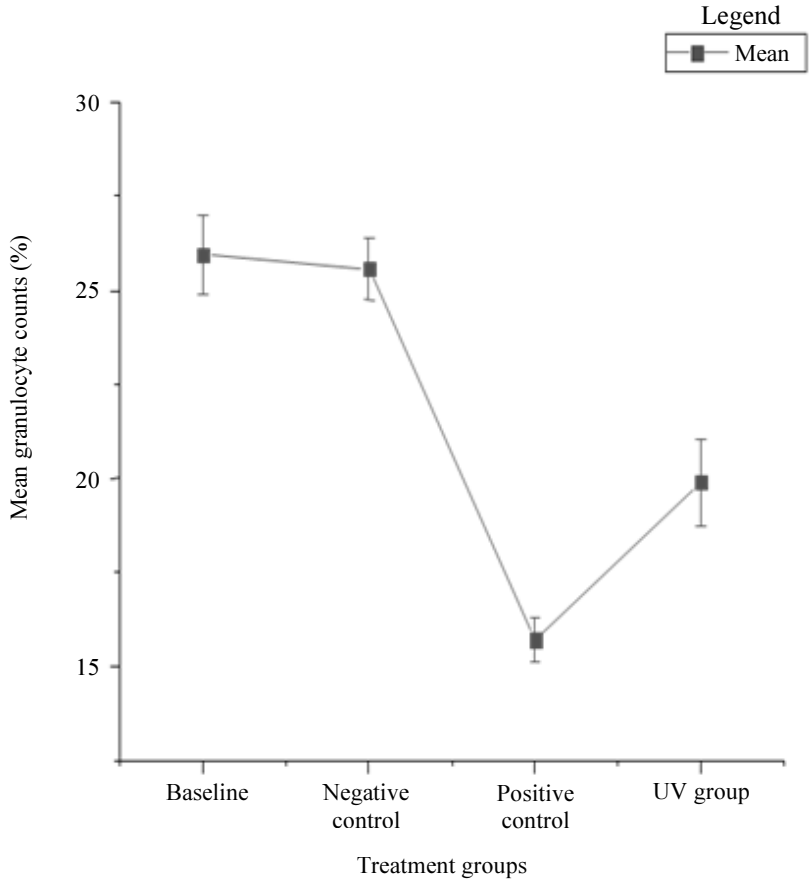

Fig. 4: Granulocytes Profile of Rats Vaccinated with killed *C. albicans* and Control

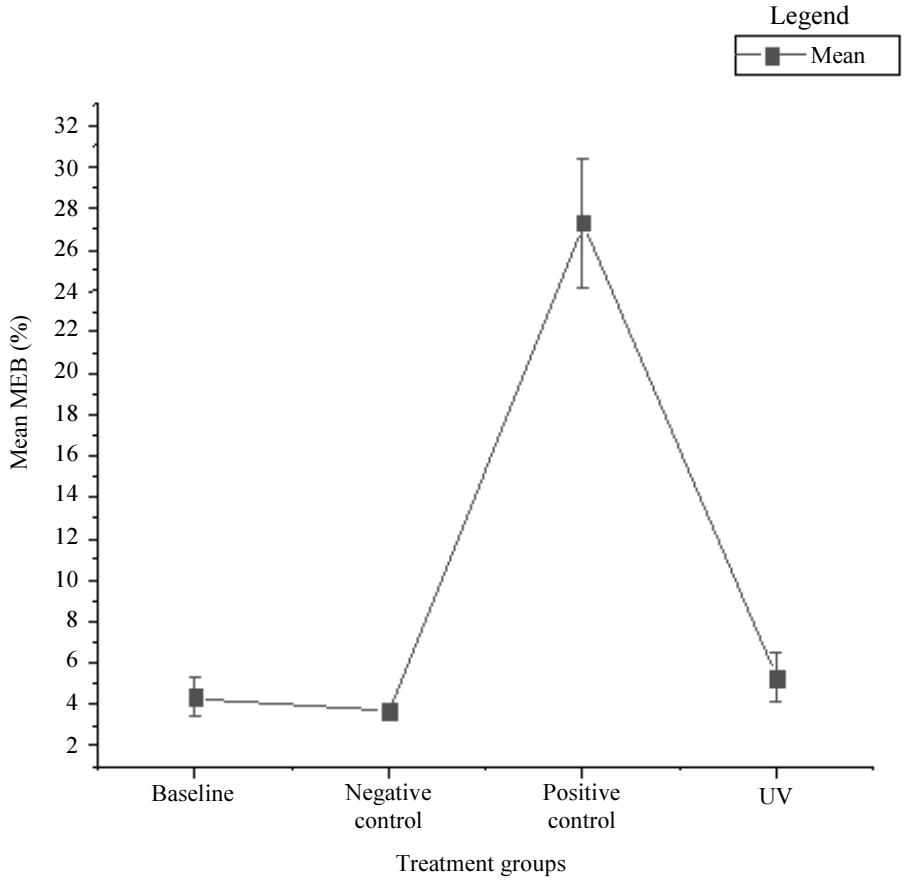

Fig. 5: Mid-Range cells (MEB) profile of rats vaccinated with inactivated *C. albicans* and controls

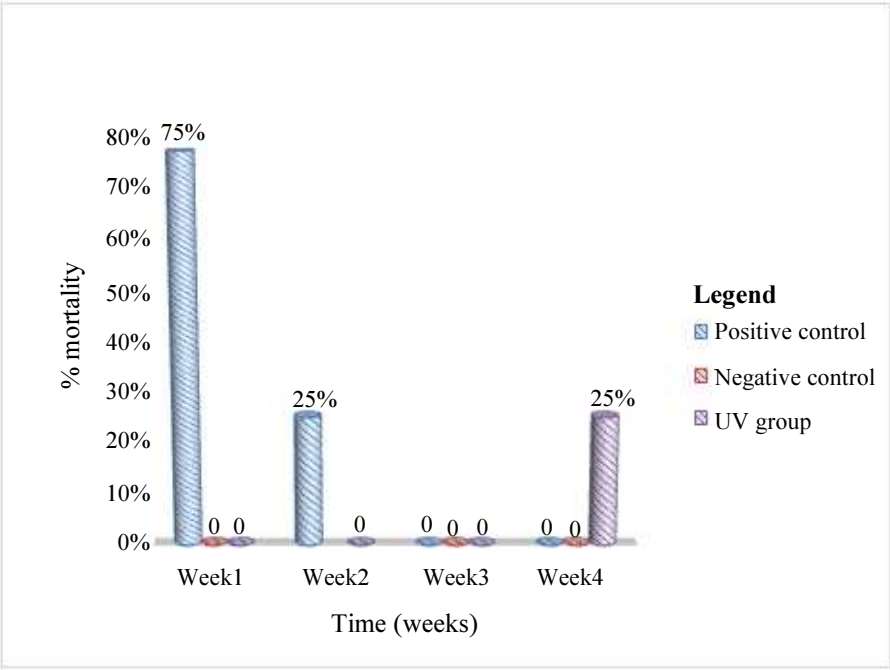

Fig. 6: Percentage mortality of rats immunized with UV inactivated *C. albicans* and Control groups

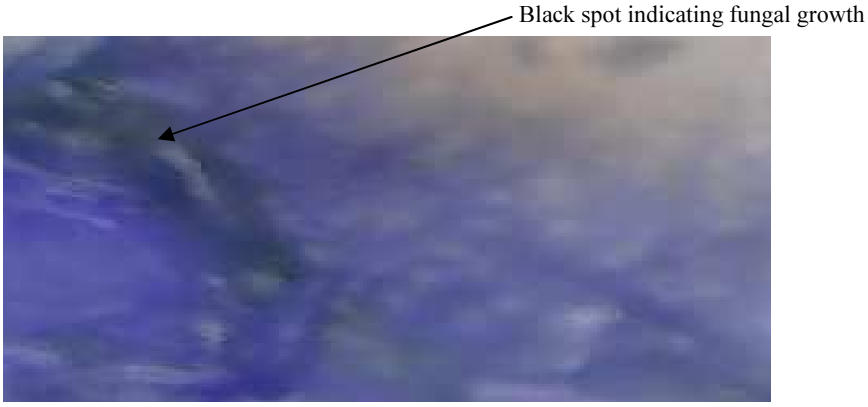

Fig. 7: Sectioned and stained kidney of fungal infected albino rat (X500 magnification)

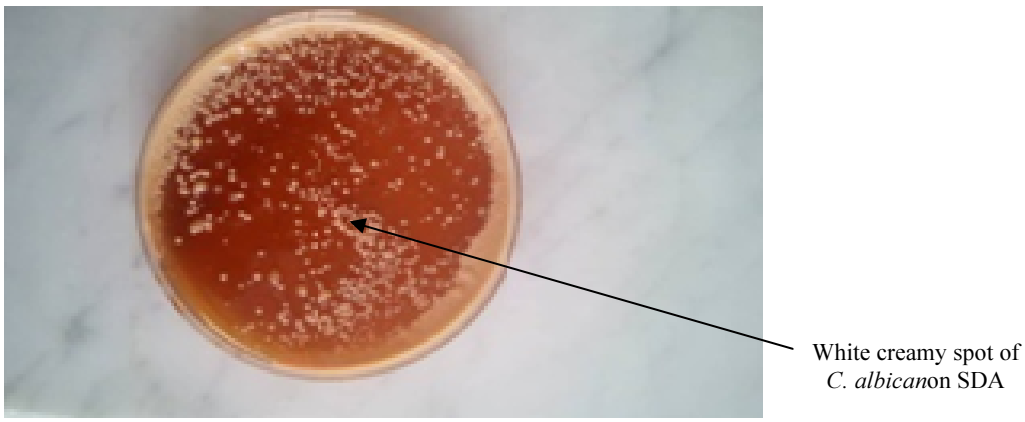

Fig. 8: Fungal growth of homogenized and cultured kidney

Kidney Fungal Burden

The fungal burden in kidney of dead rats is presented in Fig. 7, as black spots in the sectioned and stained kidneys of rats. In addition, fungal (*C. albicans*) growth was observed when homogenized kidneys of dead rats were cultured on SDA plates (Fig. 8).

Discussion

The significant increase of mean percentage granulocytes in the immunized groups in response to the challenge shows the roles of white blood cells in the fight against *Candida albicans* infections. This is because granulocytes, macrophages and monocytes are the first line defense mechanism recruited in the first few days of infection to engulf, digest and present pathogens for specific immune response (Aderem, 2003). This increase in granulocytes for immunized albino rats is in line with the findings of Gow *et al*.(2007) that reported significant production of cytokines and chemokines which have phagocyte stimulatory properties.

However, the observable decrease of granulocytes in the infected group (positive control) is an indication of immunosuppression. This could be as a result of the ability of *C. albicans* to undergo switching or morphogenesis. These properties allow *C. albicans* to escape phagocytosis by piercing and subsequent killing of phagocytic cells leading to a decrease in circulating granulocytes (Uwamahoro *et al*., 2014). This finding is not in conformity with a study conducted by Jamalzadeh *et al*.(2009) which reported an increase in total white blood cells (neutrophils, basophils and eosinophils) in fungal infection of Caspian salmon.

The mid-range cells comprised of monocytes, eosinophils, basophils and immature cells (Abacus 380 automated blood analyzer manual). Therefore, the increase in mean percentage of mid-range cells in positive control as compared to the immunized and negative control groups may be attributed to the presence of increased amount of *C. albicans* in the blood to meet up with the burden. Therefore, could be because there is a continuous proliferation of immature cells in circulation (left shift). This indicates that neutrophil

consumption in the tissues surpasses supply in the bone marrow (Honder *et al*., 2016).

Moreover, the significant increase in the percentage of lymphocytes in the immunized groups when compared to both controls could be because after immunization, the rats became sensitized and this result to the stimulation of memory cells to produce a substantial amount of lymphocytes (Delvis, 2014). The increase in lymphocytes is in conformity with a study conducted by Jamalzadeh *et al*.(2009) which showed a significant decrease in fungal infection.

Clinical observations reported by Mathews and Burnie, (2001) indicated that antibodies play an important role in host defense against disseminated candidiasis because individuals with defects in cell mediated immune response are particularly prone to superficial but not disseminated candidiasis. Similarly, the significant ($P<0.05$) increase in the level of antibodies of the immunized rats shows that inactivated *C. albicans* has the potential of stimulating humoral immunity as evident in this study. The UV group produced a mean antibody titers of 972.8µg/ml. This could be as a result of recognition of the immunogenic proteins and glycoproteins on the cell surface and subsequent stimulation of memory cells to produce significant quantity of antibodies on a second encounter of similar antigens (Delvis, 2014). Thus, this study correlates with findings of Cárdenas-Freytag*et al*. (1999) which reported a significant production of 3200 µg/ml immunoglobulins that were immunoprotective against candida infections in mice immunized with heat killed *C. albicans* in combination with adjuvant. Similarly, Thomas *et al*. (2006) also indicated a significant production of antibodies that are immune-protective during infection. In another study by Evron (1980), the circulating antibodies in immunized mice that were immunoprotective were greater than 256 µg/ml. Furthermore, in two separate studies conducted by Saville *et al*. (2008; 2009), showed vaccination with live attenuated *C. albicans* in mice challenged with a lethal dose of 5.2×10^6 virulent cells has a protective effect on candida infections. It is also interesting to note that UV inactivated *C. albicans* produced more antibodies, lymphocytes and granulocytes as compared to the positive control. This could not be unconnected with the fact that *β*-glucans of live cells are normally masked by mannan-mannoprotein layer thus precluding recognition. However, the *β*-glucans of UV inactivated *C. albicans* is exposed to recognition (Gow *et al*., 2007). This finding is in agreement with Gow *et al*. (2007) who reported that heat inactivated *C. albicans* induced significantly greater level of cytokines and chemokines which are known activators of immune cells.

There is a 75% survival of rats in UV inactivated group as compared to the un-immunized infected rats where 100% death was recorded before the end of the experiment. This agrees with the report of Thomas *et al*. (2006). The mortality was caused probably by the breakdown in osmotic balance when the tissues were destroyed by the penetration of the hyphae and the lethargy that resulted from excessive energy exerted to overcome infection stress. Therefore, this result is also in agreement with the research conducted by Shah (2002).

Conclusion

The findings of this study showed that heat inactivated *C. albicans* elicited significant immunological response in immunized albino rats. The study also clearly indicated an interesting immune-protection of the immunized rats against *C. albicans* infections. It was also shown that *C. albicans* infections or systemic candidiasis can cause immune-suppression, morbidity and even mortality as in the case of rats in the positive control group.

Recommendations

Further study should be conducted on different doses of UV on freshly isolated *C. albicans* that will elicit substantial amount of immunological response in rats.

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Author's Contributions

We participated in the conception, experinment and write up of this paper

Conflict of Interest

We declare that there is no conflict of interest.

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