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Research Article

Immunopathological effect of *Cryptococcus adeliensis* in treated rats with chitosan nanoparticles and antibiotics

Batool S. HAMZA[*1], Mohammed J. ALWAN[2]
[1]*Department of Pathology and Poultry Diseases, Baghdad University, Baghdad, Iraq.*
[2]*Department of Ansthesia, Al-Hady University College of Pathology and Poultry Diseases, Baghdad University, Baghdad, Iraq.*
[*]*Email: hamzabatool5@gmail.com*

Abstract

This study aimed to investigate the effect of immune stimulation by sonicated fungal antigens of *Cryptococcus adeliensis* using chitosan nanoparticles in rats treated with high doses of antibiotics. For this purpose, sixty rats of both sexes were divided randomly into six groups (10 animals, both males and females, in each group), as following: (G1) immunized with 0.5ml whole sonicated fungal Ag (0.83mg/ml protein concentration) /SC, 2 dose / 2 weeks interval. 2nd group (G2) immunized with 0.5ml whole fungal Ag as G1 and treated daily with high doses of antibiotic for six weeks (gentamicin 0.5mg/mL). 3rd group (G3) immunized I\P, with whole fungal Ag mixed with chitosan nanoparticles (1:1) 0.5 ml, 2 dose / 2 weeks intervals and treated with antibiotic as 2nd group, and (G4) was served as control positive group and (G5) was treated with antibiotic as 2nd group, and sixth group (G6) was served as negative control inoculated with normal saline 0.3ml. At 27 and 30 days post-immunization, skin test was done in 1st, 2nd, 3rd, and 5th groups and blood samples were collected to measure the serum level of antibody titers, after those animals of G1, G2, G3, G4, and G5 were inoculated SC, with 0.3ml, containing $1x10^8$ of the fungal cell, and the sixth group was injected with 0.3ml normal saline I/P. At 4 weeks post-infection. All animals were sacrificed and pieces of tissues were removed from the liver, lung, kidney, and brain for histopathological examination. The result revealed a high mean of skin thickness and high serum antibody titers in immunized animals but in 2nd group, the antibiotic treatment led to depressing values of skin test (ns) and serum of antibody titers (ns). Chitosan nanoparticles can improve values of skin test (***) and serum of antibody titers (***) in the 3rd group. The histopathological result showed severe pathological lesions in examined organs of the positive control group and severe lesions in these organs in animals treated with antibiotics post-infection. In addition, the current result showed mild to moderate lesions in examined organs of immunized animals with moderate lesions in immunized animal's treatment with antibiotics post-infection. The immunized animals with mixed fungal antigens and chitosan nanoparticles and treatment with antibiotics express mild to no clear lesions in examined organs post-infection. As a conclusion, the prolonged high doses of antibiotics lead to depressing immune response in immunized animal,s while immunized animals with fungal antigens mixed with chitosan nanoparticles can improve the immune response.

Keywords: Rat, Immune system, Chitosan nanoparticle, Serum antibody.

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Introduction

Fungal diseases are active infections caused by mycotoxins. These pathogenic agents can cause disorders such as bovine mastitis, respiratory disorder, fungal diarrhea in calves, superficial subcutaneous, mycotoxicosis, and systemic infection (Hassan et al. 2020; Tiew et al. 2020). Cryptococcosis is an important fungal disease in humans and animals caused by *Cryptococcus* genus, particularly *C. neoformans* and *C. gatti*, which cause the global incidence in HIV infected patients with approximately 1 million cases and 620 000 mortality every year (Park et al. 2009). The main route of *Cryptococcus* infection is the inhalation of the spores from the environment and the beginning of the infection may remain in the latent stage but the fungi may become active and disseminated to other organs based on the status of host immune responses (Park et al. 2009; Brown et al. 2012). Recently, an infection increase was recorded by *C. neoformans/ C. gattii* species complex such as *C.laurentii* (Castro-Lainez et al. 2019), *C. albidus,* and *C. adeliensis* (Choe et al. 2020). The status of host immune responses is considered an essential factor that influences the ability of invasive fungi to infect hosts in addition to the type of pathogenic fungi that most are opportunistic pathogens.

To control fungal infection, it is required to augment immune responses by vaccination (Chowdhary et al. 2017; Lockhart et al. 2017; Navalkele et al. 2017). An experiment revealed that the prophylactic vaccines activated the immune response against systemic mycosis even in the immunocompromised mice (Silva et al. 2017). Varies vaccine programs were developed experimentally against certain systemic fungal diseases such as cryptococcosis, histoplasmosis, candidiasis, and aspergillosis (Brown 2012), but till now, there is no licensed vaccine progressed to market (Brown et al. 2012; Travassos & Taborda 2017). Recently, numerous researches have been focusing on developing new effective fungal vaccines that provide long-term memory responses, particularly in patients suffering from insufficiency immune responses (Sandini et al. 2011). These researches focused on the application of immunomodulater agents and appropriate adjuvants as nanomaterials (Van der Lubben et al. 2001; Cassone & Casadevall 2012; Medici & Del Poeta 2015; Silva et al. 2017) and, chitosan and chitosan nanoparticles were used as immunomodulater that stimulated both arms of immune responses (Skene & Sutton 2006). In Iraq, there are researches on the influence of antibiotic on immune response and augment immune responses against *C. neoformans* infection; therefore, the present study aimed to investigate the influence of whole sonicated fungal antigens mixed with Chitosan nanoparticles on immune response against *C. adeliensis* (MZ031278) infection in rats treated with high doses of antibiotic.

Material and methods

Source of *C. adeliensis* (MZ031278): This fungus was isolated from the lungs of sheep and then identified by PCR and gene sequence in the central laboratory of Veterinary Medicine College/ University of Basrah. The source of chitosan nanoparticles was Shaanxi Sangherb BIO-TECH INC company (200mg).

Experimental design: In the present study, fifty rats (*Rattus norvegicus*), from Biotechnology Research Center, Al-Nahrian University, both sexes, with an average age of 10-12 weeks, were divided randomly into six groups equally (10 animals in each group), and treatment as following: 1st group (G1) immunized with 0.5ml whole sonicated fungal antigens (0.83mg\ml protein concentration) /SC, 2 dose / 2 weeks interval. The 2nd group (G2) was immunized with fungal antigens as in 1st group and treated daily with high doses of the antibiotic (gentamycin 0.5mg/ml, globalforvet) for six weeks, orally. The 3rd group (G3) was immunized with 0.5 ml of whole sonicated fungal Ag mixed with chitosan nanoparticles (1:1), two-dose / 2 weeks intervals, and

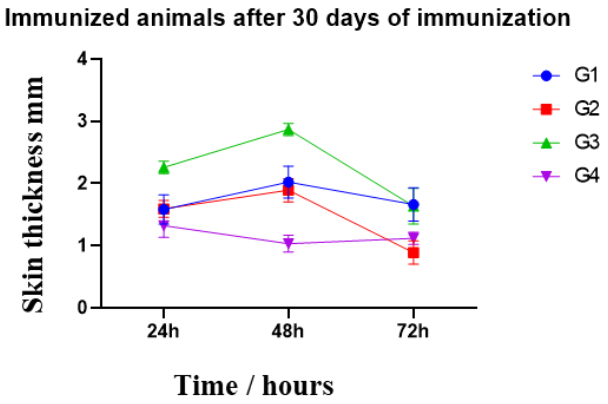

Fig.1. DTH test shows significant increasing after 24 hrs. to G3 (immunized + treated with chitosan nanoparticles and antibiotics), and G1 (immunized animals) after 30 days of immunization, then decreasing after 48, and 72 hrs. G2 (immunized animal + treated with antibiotics) no significant difference with G4 (positive control group). Significance (**P*<0.05, ***P*<0.01) was determined by using tow-way ANOVA and post-hoc Tukey's test for bar graphs and Mann–Whitney test for all groups data. Data are representative of at least three independent experiments.

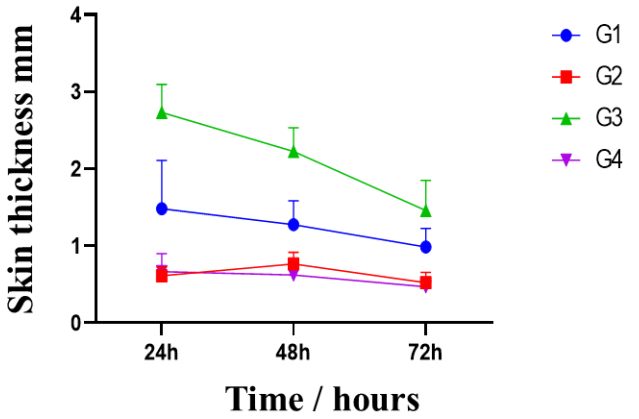

Fig.2. DTH test shows significant increasing after 48 hrs. to G3 after 30 days of immunization, then decreasing after 72 hrs. G1 and G2 show no difference. G4 (negative control) show significantly difference with G1(immunized) and G2 (immunized + treated with Antibiotics significance (*P<0.05, **P< 0.01) was determined by using tow-way ANOVA and post-hoc, Tukey's test for bar graphs and Mann-Whitney test for all groups data. Data are representative of at least three independent experiments.

treated with antibiotics as 2nd group. The 4th group (G4) was served as the positive control group, five groups were treated with the antibiotic, and six group was served as the negative control inoculated with normal saline 0.5 ml S/C only.

A skin test was done for G1, G2, G3, and G six at 27 days post-immunization. The blood samples were taken from immunized, and control negative groups to determine antibody titers at 30 days post-immunization. In G1, G2, G3, G4, and five groups were I\P inoculated with 0.3 ml of fungal suspension containing 1x10 ^{8} fungal cells \ml. All animals were sacrificed 4 weeks post-inoculation and small pieces were taken from the liver, kidney, lung, spleen, and brain for histopathological examination.

Results

Skin test: The results showed a significant change in DTH of skin test between G3 animals with G2 (immunized group + treated antibiotics), and negative control group (G4) after 30 days of immunization, while G1 with G4 (**P*<0.05) (Fig. 1). DTH reaction after 4 weeks of infection, the higher level between G3 and G4 (positive control) (*****P*<0.0001), then G2 with G3 (****P*<0.001), and finally between G1 with G3 and G4 (**P*<0.05) were observed (Fig. 2).

Antibody titers: The study revealed a high level of

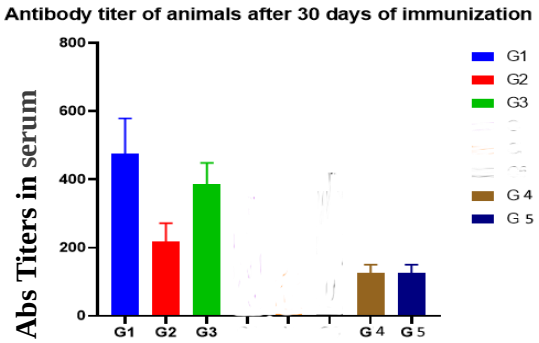

Fig.3. The serum antibody titers show high levels in G1, G3, and G2 respectively. While low levels in other control groups. Significance (*P<0.05, **P<0.01) differences were determined for ALL groups. Data are representative of at least three independent experiments.

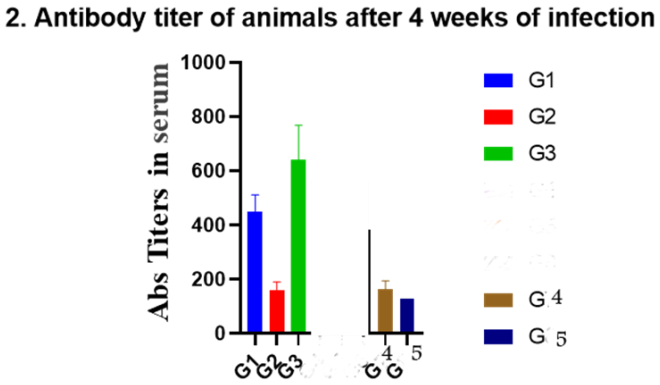

Fig.4. The serum antibody titers show high levels in G3 and G1 while low levels in other groups. Significance (*P<0.05, **P<0.01) differences were determined for ALL groups. Data are representative of at least three independent experiments.

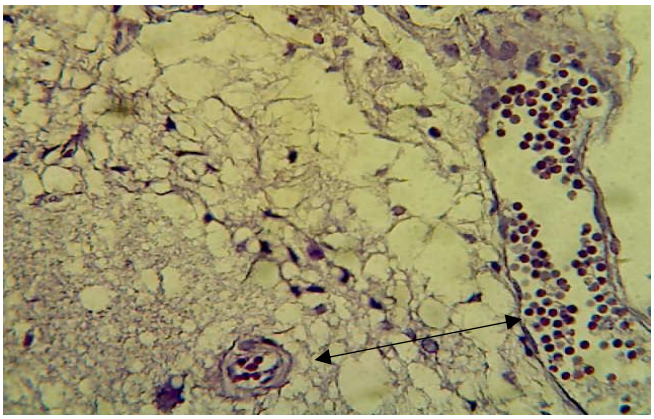

Fig.5. Section in the cerebrum of animal in G4 control positive group at 4weeks post-infection shows severe inflammatory cells particularly neutrophils in dilated congested blood vessels in pia mater as well as in parenchyma (arrow). (H & E stain 400X).

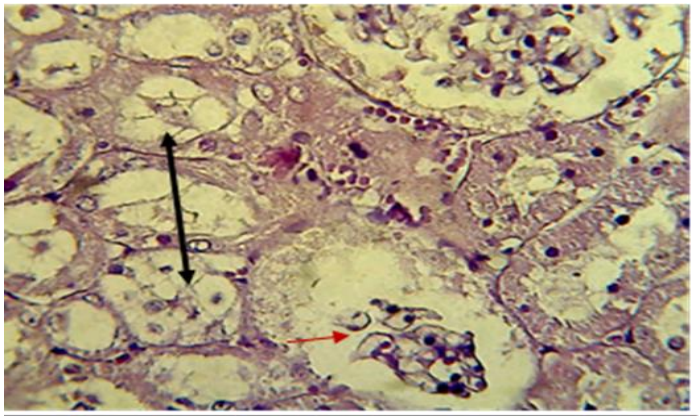

Fig.6. Section in the kidney of animal in G4 control positive group at 4weeks post-infection shows atrophy of glomerular tufts, protenious material in dilated Bowman space (red arrow), as well as acute cellular degeneration of renal tubules (black arrow) (H & E stain 400X).

antibody titers in the serum by ELIZA. The total titer of antibody after 30 days of immunization showed a significant difference G1 (immunized animals) with G2 (immunized and treated with antibiotics), and

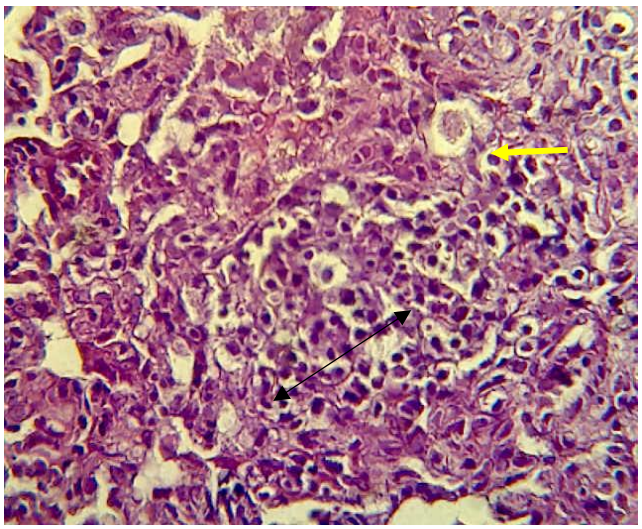

Fig.7. A section in the lung of animal in G4 control positive group at 4weeks post-infection shows pyogranulomatous lesion in the interstitial tissues consist from the aggregation of neutrophils and mononuclear cells (black arrow) in addition, yeast-like structures fungi (yellow arrow) and inflammatory cells in alveolar. (H & E stain 400X).

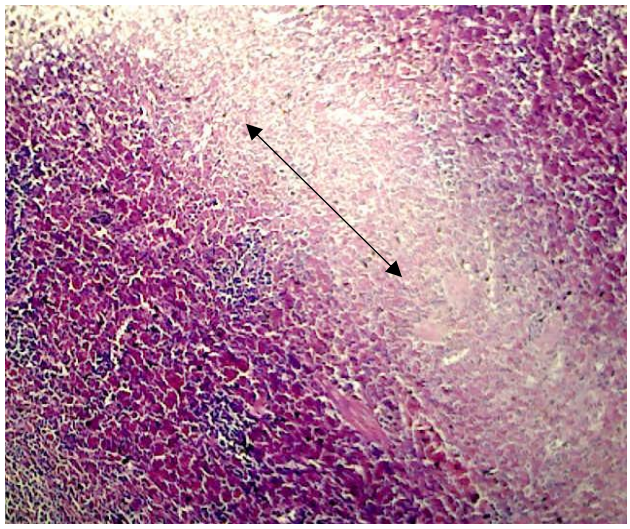

Fig.8. A section in the spleen of animal in G4 control positive group at 4weeks post-infection shows large necrotic area with congested red pulp (arrow). (H & E stain 400X).

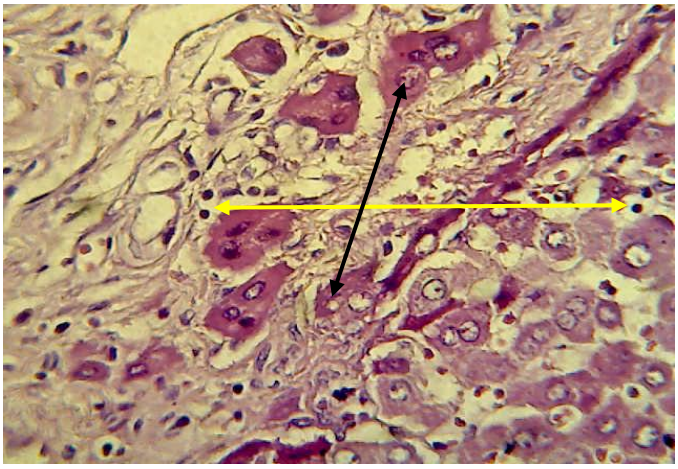

Fig.9. A section in the liver of animal in G4 control positive group at 4weeks post-infection shows fibrosis around regenerated hepatocytes (black arrow) with inflammatory cells in dilated sinusoids (yellow arrow) (H & E stain 400X).

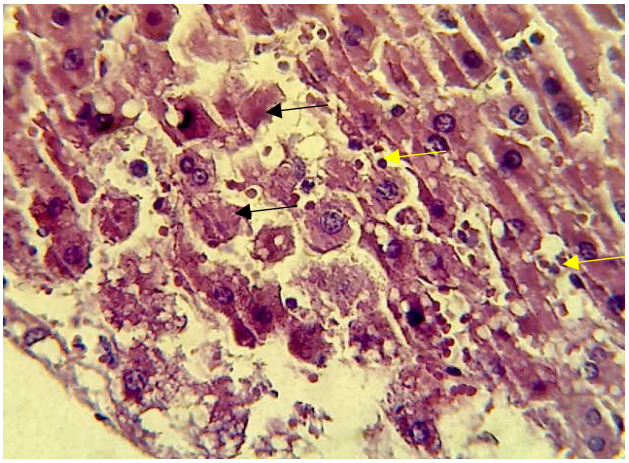

Fig.10. A section in the liver of animal in G4 control positive group at 4weeks post-infection shows fibrosis around regenerated hepatocytes (black arrow) with inflammatory cells in dilated sinusoids (yellow arrow) (H & E stain 400X).

control groups animals at levels (**P*<0.05, ***P*<0.01), respectively (Fig. 3). After 4 weeks of infections, the results showed high levels in G3 and other groups. There was a significant difference G1 at high-level antibody titers, while G2 and control one showed low levels. The G3 and G1 (****P*<0.001). nt The G3 and G1 show a significant difference (**P*<0.05, ***P*<0.01) (Fig. 4).

Histopathological changes: Positive control animals' group of G4 at four weeks post-infection in the cerebrum showed severe congested blood vessels with hemorrhage adjacent to congested vessels. In another section, it had inflammatory cells, particularly neutrophils, in dilated congested blood vessels in pia mater and parenchyma (Fig. 5). The kidney revealed severe acute cellular degeneration of epithelial cells of renal tubules characterized by severe vacuolar degeneration, necrosis, and

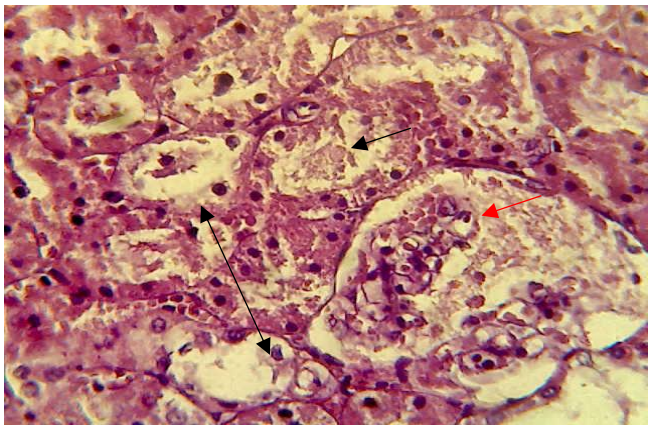

Fig.11. A section in the kidney of animal in group5 at 4 weeks post-infection shows inflammatory cells atrophy of glomerular tufts (red arrow) with protenious materials in Bowman space (black arrow) with inflammatory cells in congested blood vessels and acute cellular degeneration of renal tubules (black arrow) (H & E stain 400X).

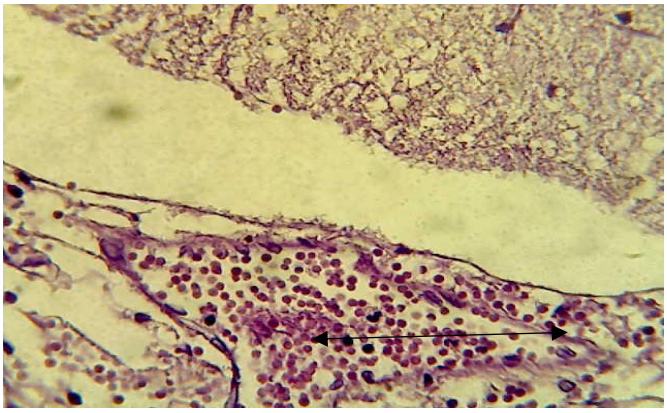

Fig.12. A section in the cerebrum of animal in group5 at 4 weeks post-infection shows severe inflammatory cells in congested blood vessels in pia mater arrow (H & E stain 400X).

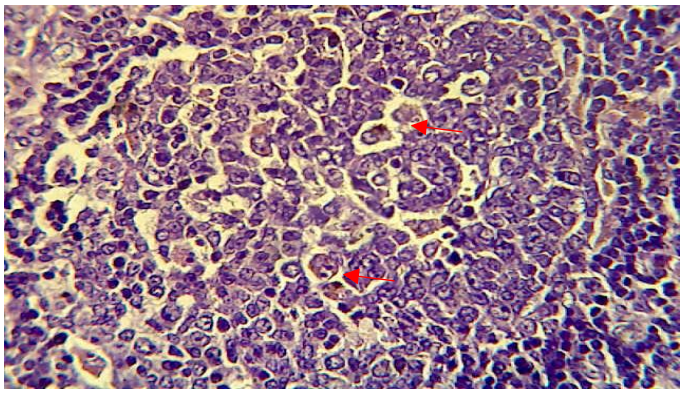

Fig.13. The section in the spleen of the animal of group5 at 4 weeks post-infection shows apoptotic of lymphocytes in white pulp left multiple spaces contain cellular debris (arrow) (H & E stain 400X).

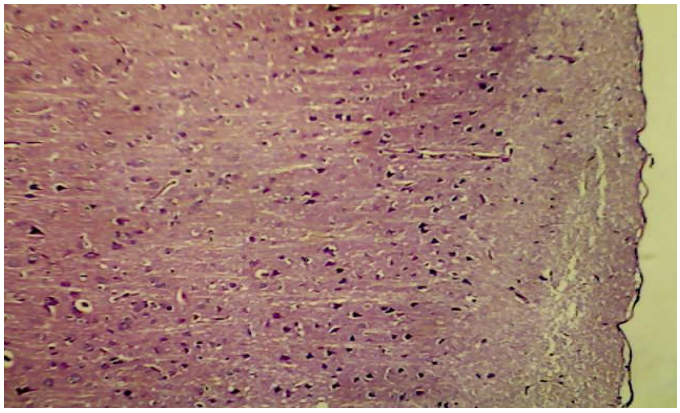

Fig.14. The section in the cerebrum of animal in group1 at 4weeks post infection shows no clear lesions (H & E stain 400X).

sloughing of epithelial cells in addition to thrombus in blood vessels. The main lesions in the kidney were congested blood vessels between renal tubules, atrophy of glomerular tufts, and proteinous material in dilated Bowman space (Fig. 6). The main lesions in the lung were severe inflammatory cells, particularly neutrophils in and around congested blood vessels and proliferation of alveolar macrophages in alveolar space. In other animals, the pyogranulomatous lesion in the interstitial tissues was recorded, consisting of an aggregation of neutrophils and mononuclear cells. In addition, yeast-like fungi and inflammatory cells in alveolar spaces and congested blood vessels were observed (Fig. 7).

The section in the liver showed fibrosis around regenerated hepatocytes with inflammatory cells in dilated congested sinusoids and blood vessels, in addition to single necrosis of hepatocytes. In other samples, pseudo lobule was recorded surrounded by fibrous connective tissue infiltrated with mononuclear cells (Fig. 8). Necrotic area replacement by RBCs characterized other lesions in the liver. The spleen revealed depletion of white pulp and a large necrotic area in the red pulp (Fig. 9).

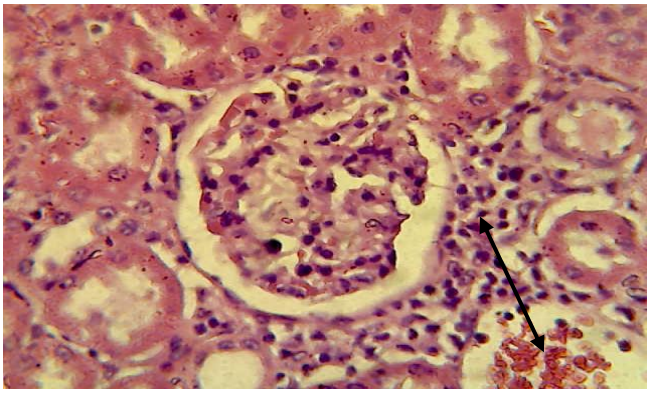

Fig.15. A section in the kidney of animal in group1 at 4 weeks post-infection shows mononuclear cells infiltration around glumeruli with congested blood vessels (arrow). (H & E stain 400X).

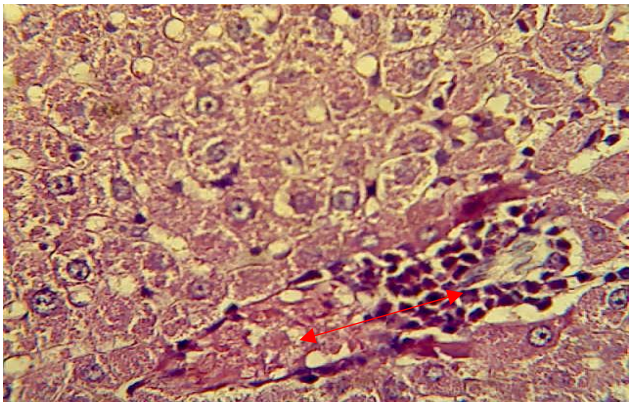

Fig.16. A section in the liver of animal in group1 at 4 weeks post infection shows mononuclear cells aggregation around vessels and bile duct in portal area (arrow). (H & E stain 400X).

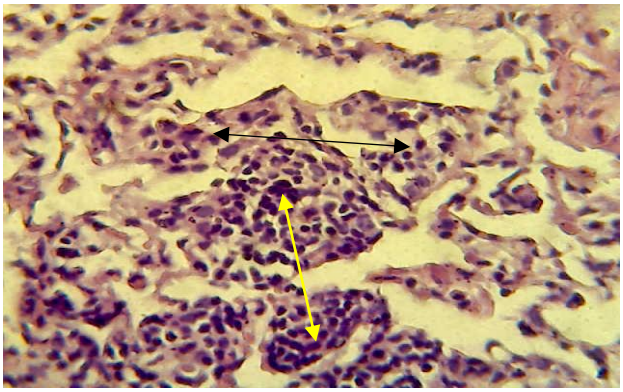

Fig.17. A section in the lung of animal in group1 at 4 weeks post-infection shows proliferation of alveolar macrophages and few neutrophils infiltration in the alveolar space (black arrow) with hyperplasia of lymphoid tissue in the wall of airways (yellow arrow) (H & E stain 400X).

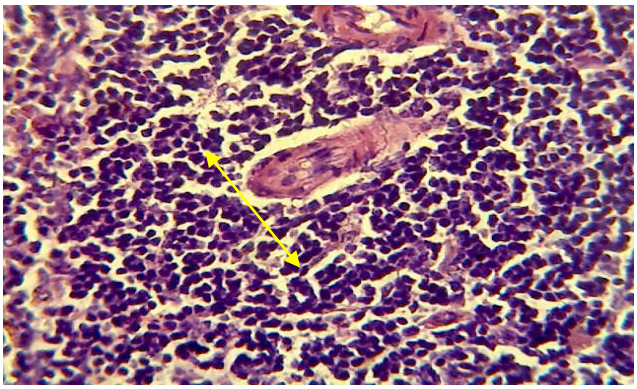

Fig.18. A section in the spleen of animal in group1 at 4 weeks post infection shows moderate hyperplasia of white pulp (yellow arrow). (H & E stain 400X).

Animals treated with antibiotics drug G5: The main lesions in the liver were inflammatory cells aggregation around congested blood vessels and bile duct in the portal area, in the dilated congested blood vessels and sinusoids, and necrotic of hepatocytes (Fig. 10). In other animals, it found inflammatory cells in the necrotic area under fibrosis of the capsular region and inflammatory cells in dilated sinusoids. The kidney section showed severe acute cellular degeneration of epithelial cells of renal tubules that led to destruction and fused these tubules. In another section, atrophy of glomerular tufts with proteinous materials in dilated Bowman space, congested blood vessels, and edema between renal tubules were recorded (Fig. 11). The cerebellum section marked central chromatolysis of Purkinje cells characterized by round pinkish cytoplasm with eccentric or loss of nuclei (Fig. 12). The cerebrum expressed severe inflammatory cells in congested blood vessels in the parenchyma and ependymal region, to the hemorrhagic area was found. In another section, severe inflammatory cells in congested blood vessels in the pia mater were observed. The main lesions in the cerebrum are characterized by perivascular edema, marked proliferation of astrocytes, oligodendrocytes, and microglial cells in addition to necrosis of neurons attached. The spleen showed apoptotic lymphocytes in white pulp characterized by multiple spaces filled with cellular debris (Fig. 13). The lung showed severe inflammatory cells

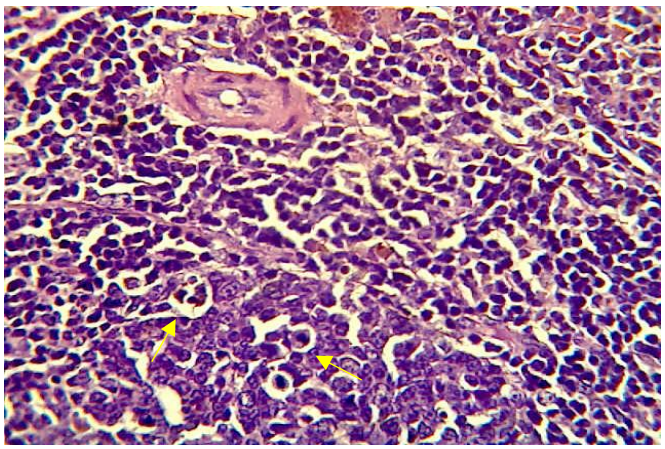

Fig.19. Section in the spleen of animal in G2 at 4 weeks post infection shows apoptosis of lymphocytes in white pulp left multiple spaces containing cellular debris (arrow). (H & E stain 400X).

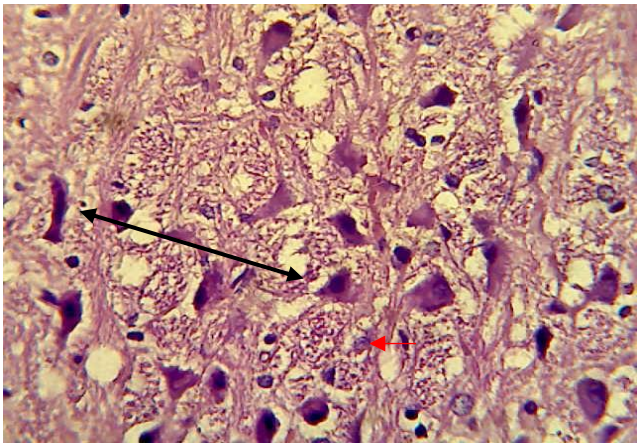

Fig.20. A section in the brain of animal in G2 at 4 weeks post infection shows proliferation of astrocytes (black arrow), oligodentrocytes (red arrow) and microglial cells (brown arrow) in addition, to oligodentroctes contact with necrosis neuron (black arrow) (H & E stain 400X).

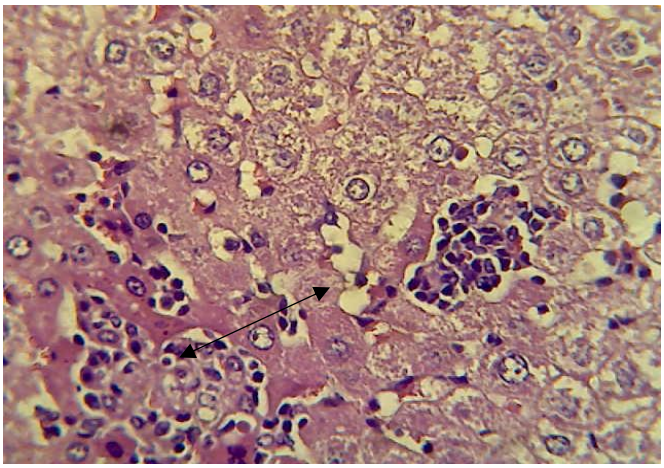

Fig.21. The Section in the lung of animal in group2 at 4weeks post infection shows neutrophils and RBCs in alveolar spaces and neutrophils in congested blood vessels in addition to fibrin networks (arrow) (H & E stain 400X).

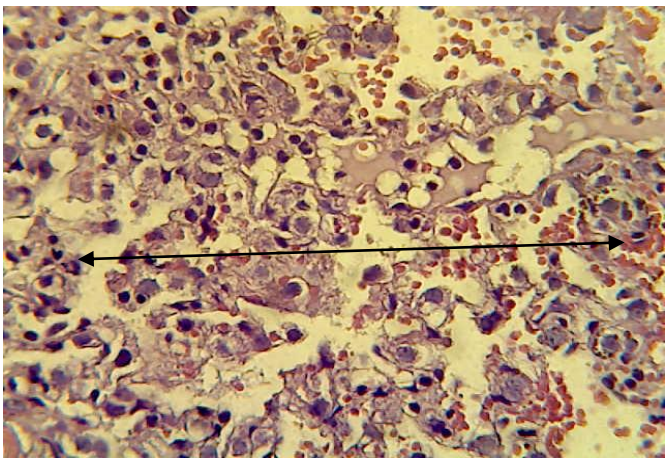

Fig.22. The section in the liver of animal in group 2 at 4weeks post infection shows necrosis of hepatocytes with multiple granuloma in liver parenchyma with mononuclear cells in sinusoids (arrow). (H & E stain 400X).

particularly neutrophils and mononuclear cells in the interstitial tissue and alveolar spaces.

Immunized animals with whole sonicated fungal antigens G1: The cereberum showed no clear lesions (Fig. 14). The kidney revealed mild degenerative changes in the epithelium cells of renal tubules with congested blood vessels. The lesions in the kidney were characterized by mononuclear cells infiltration around glomeruli with congested blood vessels (Fig. 15). The liver showed inflammatory cells in congested blood vessels, and sinusoids with single-cell necrosis, with mononuclear cells aggregation around blood vessels and bile duct in the portal area (Fig. 16). The main lesions in the lung are characterized by proliferation of alveolar macrophages and few neutrophils infiltration in the alveolar space with hyperplasia of lymphoid tissue in the wall of airways, and multiple granulomatous lesions in the interstitial tissues. The spleen showed moderate hyperplasia of white pulp (Fig. 17).

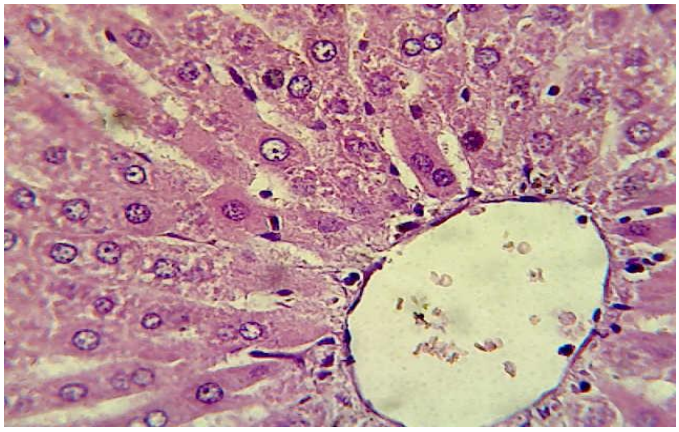

Fig.23. The section in the liver of animal in group3 at 4weeks post infection shows proliferation of kupfer cells with few mononuclear cells infiltrated a round central vein (arrow). (H & E stain 400X).

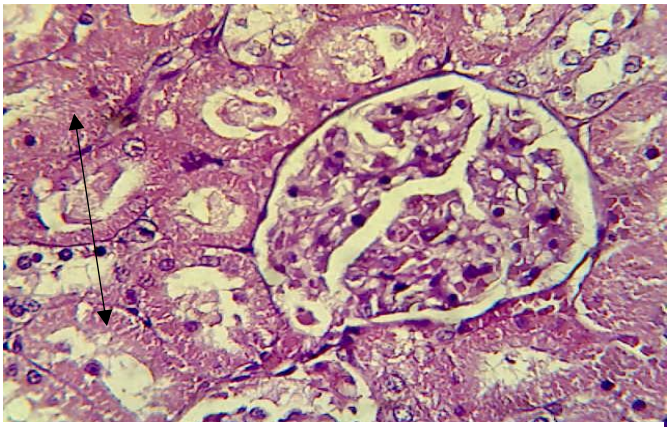

Fig.24. The section in the kidney of animal in group3 at 4weeks post infection shows moderate acute cellular degeneration of renal tubules (arrow). (H & E stain 400X).

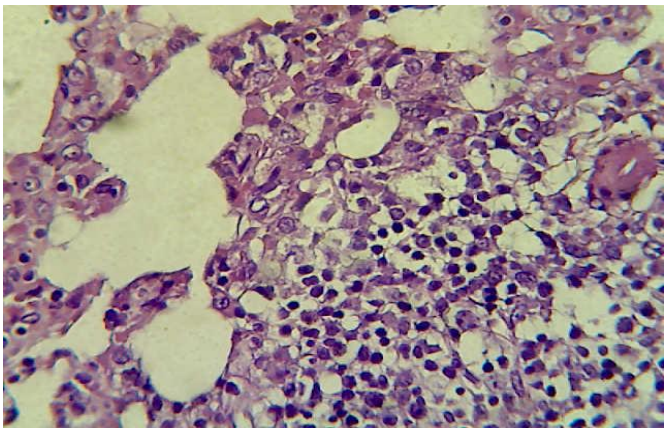

Fig.25. The section in the lung of animal in group3 at 4weeks post-infection shows mononuclear cells infiltration in the interstitial tissues (red arrow). (H & E stain 400X).

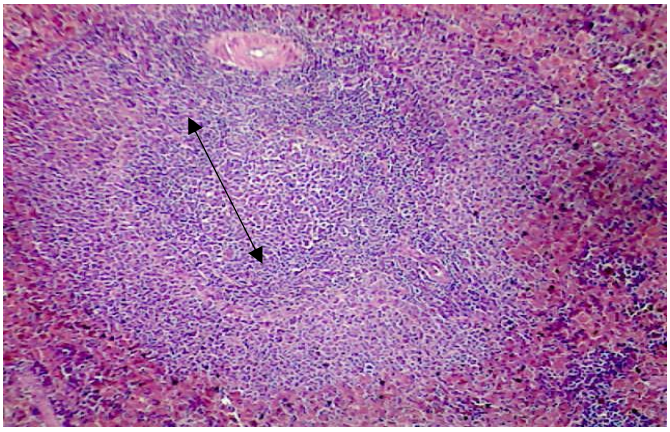

Fig.26. A section in the spleen of animal in group3 at 4weeks post infection shows proliferation of lymphocytes in periarteriolar sheath (arrow). (H & E stain 400X).

Immunized animals treatment with antibiotic G2: At 4 weeks post-infection, the spleen showed apoptosis of lymphocytes in white pulp left multiple spaces containing cellular debris (Fig. 18). Histopathological examinations also revealed the proliferation of astrocytes, oligodentrocytes, and microglial cells, oligodentroctes contact with a degenerative neuron (Fig. 19). The lung showed mononuclear cells aggregation in the interstitial tissue and alveolar spaces, neutrophils and RBCs in alveolar spaces, and neutrophils in congested blood vessels and fibrin networks (Fig. 20). Liver alternations were granuloma in parenchyma with mononuclear cells in sinusoids, aggregation of inflammatory cells particularly neutrophils and mononuclear cells in portal area and sinusoids, and necrosis of hepatocytes (Fig. 21). Findings also showed acute cellular degeneration of epithelial cells of renal tubules and mononuclear cells infiltration between mucosal glands.

Group 3 of immunized animals with whole sonicated antigen, and treatment with chitosan nanoparticles and antibiotic: The liver showed proliferation of Kupfer cells with few mononuclear cells infiltrated around the central vein (Fig. 23). The kidney had moderate acute cellular degeneration of

renal tubules (Fig. 24). The lung showed mononuclear cells infiltration in the interstitial tissues (Fig. 25). No clear lesions were seen in the cerebrum and cerebellum. The spleen revealed a proliferation of lymphocytes in the perarteriolar sheath (Fig. 26).

Discussion

Immune responses: The results showed an increase in skin tests of immunized animals with WSC Ags, indicating that these antigens can activate cell-mediated immunity. The skin test is a main type of immunity based on Th1 cytokine, including FNγ, and our finding agrees with findings of Mahmood and Alwan (2019), who recorded that WSC Ag can stimulate CMI that DTH reaction can be controlled by CD4 and CD8 T-cells (Rosen et al. 1995) and activating these cells can produce IFNγ. In addition, the present study showed that immunized animals' treatment with antibiotics expressed a low DTH reaction. These results may indicate that prolonged the application of antibiotics can depress immune response and influence on vaccine program. The present finding showed that applying chitosan nanoparticles in immunized animals can improve cell-mediated immunity and humoral immunity, similar to that of Maeda & Kimura (2004), who found that chitosan can activate macrophages to produce IL 12 that activates NKs to produce IFNγ. The present results may indicate that chitosan can stimulate the maturation of DCa, which is a major initiator of protective cell-mediated immunity (Osterholzer et al. 2009).

Control positive group: Severe pathological alternation in all examined organs of control positive animals at 4 weeks post-infection, may indicate that the fungal strain in the present study is a highly virulent pathogen, that overcome normal host defense mechanisms and spread to multi organs, as reported by Kwon-Chung et al. (2014) and May et al. (2016). They reported that the classic cryptococcal virulence factors such as melanin formation, phospholipase, and urease are associated with fungal survival, disease initiation and infection progression. The lesions in the brain of the control positive group may indicate that *C. adeliensis* can cause neurological cryptococcosis that agrees with Sabiiti et al. (2014), who demonstrated that *C. neoformans* can induce encephalitis, meningoencephalitis, ventriculitis.

The result revealed a granulomatous lesion in the liver the of control positive group. These lesions may indicate that being infected by *C. adeliensis* can elicit a granulomatous reaction. Since *C. neoformans* can induce tumor-like masses in the lung and brain that give rise to the name *neoformans*. The granulomatous lesions in the liver of the control positive group may indicate that the host body attempts to control fungal infection, which agrees with Baker & Haugen (1995), who recorded that the granulomatous inflammation is one features of picture of host limiting Cryptococcal growth. the present result may give indication that granuloma response is a main feature of cryptococcal infection similar to those recorded by Abd Elgadir et al. (2016), who mentioned that granuloma originated from *C. neoformans* called Crptococcomas. Also, the present finding may indicate highly virulent fungal agent used in this study, similar to those described by Pagán & Ramakrishnan (2018), who recorded that highly virulence fungal strain associated with progress of active macrophages into epithelioid granuloma containing accessory features of the granuloma such as neutrophils, B and T cells. The lesion in the lung was agreement with Baker & Haugen (1995), who found that pulmonary cryptococcocal infection associated with macrophages proliferation, epithelioid cells, and giant cells. The marked present foamy alveolar macrophages in alveolar spaces of control positive group post fungal infection may indicate that alveolar macrophages can recognize and destroy fungal spores (Waldorf et al. 1984).

Animal's treated with antibisotic: The current

finding revealed that the histopathological changes were severe in the examined organs showing that prolonged using of high dose of antibiotic can cause suppression of normal host defense mechanisms that fascilated severe fungal infections. Other researches pointed out that the antibiotic can cause damage of intestinal microbiota which mediated development immune cells (B, and T cell). Our results was consistent with Kabat et al. (2014), who showed that the intestinal microbiota can regulated the development of different immune cells, including Th17, DCs and NKs. Deformity of renal tubules with severe destruction of their epithelial cells may indicated that the antibiotic can cause depress normal defense mechanisms of the host that lead to increase their susceptibility to fungal infection as immunocompromised host. Zonios et al. (2007) and Saijo et al. (2014) found that immunocompromising conditions are a major risk factors for cryptococcosis and immunological perturbation are predisposed the host to cryptococcosis. Our results showed that the brain lesions were more intense in the animal treatment with antibiotic compared to control positive group. These maybe due to impair immune system by antibiotic. Garcia-Hermoso et al. (1999); Goldman et al. (2001); Saha et al. (2007); and Dromer et al. (2010) showed that active pulmonary cryptococcosis (PC) can disseminate throughout the body to the CNS causing cryptococcal meningitis (CM) during an immunosuppressive event.

Immunized animals with whole sonicated fungal antigens: The present result showed that the immunized animals had mild to moderated pathological lesions indicating that the cell-mediated immunity play important role in the protection of the host against *Cryptococcus* infection, since, values of skin test were high in these group. Cell-mediated immunity (CMI) plays a critical role in anticryptococcal defense (Park et al. 2009). Both innate and adaptive arms of the immune system is critical for the defense against the fungal infection (Olszewski et al. 2010; Kronstad et al. 2011; Coelho et al. 2014). Also, the immunized group in the present study expressed high result of skin test and high levels of serum antibodies titers. These results may indicate that WSFAgs can stimulated both cellular and humoral immune responses, and both arms of adaptive immunity can protective the host against fungal infection. Wormley et al. (2007) and Wozniak et al. (2011) found that protective response against *C. neoformans* can mediated primarily by Th1 cell immune response without required of B-cell mediated processes. The current result also showed concomitant between levels of skin test, CD4, CD8 T cells phagocytic index and pathological changes. Skin test reaction associated with production of Th1 cytokines including IFNγ that play crucial role in protective immunity against cryptococcal infections, these idea was in consistent with Wormley et al. (2007), who, found that vaccination with *C neoformans* can stimulated significant increasing in the levels of Th1-type proinflammatory cytokines and chemokines in the lung, associated with decreases in the levels of cytokines that are mediators of Th2-type anti-inflammatory activities and protective immunity against a lethal pulmonary infection with wild-type *C. neoformans*

Immunized animals treated with antibiotics: The current finding showed that immunized animals with antibiotic showed intense lesions compared to immunized animals at 4 weeks post infection. These results indicated that application high dose of antibiotic can influence on the vaccine activity associated with defect in immune responses through its effect on the intestinal microbiota, which play role in development immunity of the hosts (O'Hara & Shanahan 2007; De Vadder et al. 2014; Tilg et al. 2020). Also, Ivanov & Honda (2012) showed that the Intestinal microbiota play a pivotal role in the protective of a healthy immune system of host. Depletion of white pulp of the spleen indicated that prolonged application of antibiotic can cause necrosis of lymphocytes. Also, the RBCs, neutrophils and fibrin networks in alveolar spaces of the lung at 4 weeks post infection of this group indicated severe fungal infection due to impairment of immune

responses by antibiotic. Ekmekciu et al. (2017) showed that mice treatment with antibiotics decrease in the immune cells such as cytotoxic T cells, T-cells memory and effector T cells, B lymphocytes, Treg and activated DCs. Acute cellular degeneration in the kidney can supported idea that treatment with high dose of antibiotic can diminished stimulated immune response by fungal antigens. Ekmekciu et al. (2017) rshowed that mice treatment with broad spectrum antibiotic for 8 weeks cause depletion of the intestinal microbiota composition d with reduced number of CD8 T cells, in the small intestine, colon, mesenteric lymph nodes (MLN), and spleen. Also, they recorded decreased production of IFN-γ, IL-17, IL-22, and IL-10 of $CD4^+$ cells.

Immunized animals with mixed antigens and chitosan nanoparticle treatment with antibiotics: Mild to no clear lesions in the examined organs of animals immunized with WSF Ags mixed with chitosan nanoparticles and treatment with antibiotic indicated that chitosan can stimulated immune against fungal infection and prevent influence of prolon application of antibiotic on immune system. Bueter et al. (2014) demonstrated that purified chitosan can stimulated immune responses via activation dendritic cell proinflammatory cytokines production. Also, the present finding showed less intensity of pathological changes in the immunized animal treated with chitosan and antibiotic. These results may indicate that this way of immunization can activated both innate and adaptive immune response to prevent or decrease fungal loads in examined tissue. The main lesions in the liver, kidney and lung were mononuclear cells infiltration. These results may indicate that the fungal antigens with chitosan can activated immune responses. Also, the present result indicated that immunized animals with WSFAs mixed with chitosan can provide immune response that completely protective the animals against *C. adeliensis* infections. The current finding showed high levels of CD4T and CD8 T-cells in immunized animals' treatment with chitosan and antibiotics, associated with decrease of pathological lesions, showing these cells play crucial role in activated cell-mediated immune response against Cryptococcal infection through production of IFN-γ. Gozalbo et al. (2014) and Ikeda-Dantsuji et al. (2015) showed that the increase in the number of $CD4^+$ T producing IFNγ play essential role in control of opportunistic mycosis. It is concluded that prolong application of antibiotic lead to depress immune responses and immunized with whole sonicated fungal antigen mixed with chitosan nanoparticles can improved protective immunity against fungal infection.

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