immunopathological study of the fungus cryptococcus neoformans in mice

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Summary
The study aimed to investigate the effect of cryptococcal sonicated antigen and chitosan on the infection with this fungus which isolated from human skin lesions. Isolate of Cryptococcus neoformans was used to prepare the sonicated antigen, also 60 white mice from both sexes, at average age 8-10 weeks were getted and divided randomly into 4 groups equally and treated as following: First group: fifteen mice were immunized with 0.5ml of whole sonicated Ags(Wsc Ag) (protein concentration14mg/ml) subcutaneous two doses, one week interval. Second group: fifteen mice were fed diet supplemented with chitosan 1g/kg diet for 6 weeks. Third group: Mice were immunized as 1st group and at the same time fed diet supplemented with chitosan as 2nd group. Fourth group: Mice, which seted as control positive group.

At day 28-30 post-immunization, delayed hypersensitivity and indirect passive haemagglutination tests were done, then 1st, 2nd, 3rd and 4th groups were administrated intraperitoneally with 0.3 ml of fungal suspension containing 1X107 fungal cells, all animals were sacrificed at day 30 post-infection. The results showed that the immunized animals and fed diet containing chitosan had values of delayed hypersensitivity test(1.99±0.02 at 24hours and 2.11±0.03 at 48 hours)and value of antibodies(160±29.6) higher than that of immunized animals only(1.75±0.03 at 24 hours and 1.81±0.03 at 48 hours)and value of antibodies(136±28.2). The immunized animals with whole sonicated cryptococcal antigens (1st group) expressed high values of delayed type hypersensitivity and high levels of Abs titer, as well as the chitosan stimulate the immune system in the second group and the best results of immunization were getted in the third group while all animals of control positive(fourth group) were died at the first week post-infection.

The current study showed that all the non-immunized and infected animals of the fourth group were died during the first 2 weeks post-infection with severe fungal cells isolated from internal organs and severe suppurative inflammatory reaction in the examined organs during early stage of infection with granulomatous lesions appear at day 14 post-infection while no mortality rate was seen in animal fed diet supplement with chitosan with or without immunization(2nd and 3rd groups) moderate fungal isolation and granulomatous lesions were recorded in the examined organs of animals sacrificed at day 30 post-infection while the immunized animals and fed diet containing chitosan showed that there is no fungal isolation and nor fungal cells in the histological sections with few histological degeneration and small granulomatous lesions and sometimes absence of them in the examined organs. It concluded that cryptococcal sonicated antigen and chitosan have important role in protective immunity against C.neoformans infection.

Keywords: Immunopathological, Mice, Fungus Cryptococcus neoformans.

Introduction
Cryptococcosis, is an important zoonotic fungal disease worldwide distribution, caused by Cryptococcus neoformans or Cryptococcus gattii, these fungi can infect both immunocompetent and immunocomprised individual (1 and 2). During the past two decades, the prevalence of C.neoformans as emerging fungal pathogen was widely recognized throughout the world( 3 and 4) recorded that asymptomatic or mild infection with C.neoformans in the healthy individual while severe infection with significant mortality in the immunocompromised patients in which the disease progress to pneumonia and fatal meningoencephalitis ,with prevalence ranges from 8% in the United states to 30% in Africa (5 and 6). However,
these fungi can infect most of the internal organs in addition to skin, bone and joints (7 and 8) but the main target organs are brain and lungs (9).

The antifungal treatment of the cryptococcosis has major limitations, as well as the treated patients may exposed to relapse infection, therefore new strategies to control cryptococcosis were required (1). While(10 and 11) showed that immunomodulatory therapy and vaccine are effective programs in the control of cryptococcosis. Chitosan is a good immunomodulated that improve immune responses against certain pathogens such as E.coli infection, and Brucella melitensis.

In Iraq, there are little researches about the percentage of C.neoformans associated with skin wound and using chitosan as immunomodulater factors against fungal infection, therefore the aims of the present studies are to determine percentage of infected skin wounds with C.neoformans in the humans and study the influence of chitosan and C.neoformans antigens in the augment the immune response against infection with virulent viable Cryptococcus neoformans.

**Materials and Methods**

In this study isolate of the fungus Cryptococcus neoformans is used for preparing sonicated antigen and inducing infection. Soluble sonicated antigen was prepared after sonication of harvesting virulent C.neoformans, centrifuged and the sediment was resuspended by phosphate buffer solution (PBS), preserved in a screw cap bottle that put in a cooled sonicator oscillator and surrounded by enough amount of ice to prevent the temperature elevation during the sonication, the probe was put down inside the sample so it was reached to the bottom of the bottle without touching it. The actual work of the sonicator was 60 minutes and in range 1 minute work, 1minute rest with 50 voltage. During work, there was a replacement of ice that was surrounding the sample, and taking a loopful from sample and examined by staining with nigrosen stain to make sure that the sonication process was occurred, then taking a loopful from the sample and inoculated in sabouraud dextrose agar to make sure that no growth occur after 24-48hrs, then estimating its protein concentration which was(14mg/ml) by using Biuret kit (Randox Lab.) and this is called whole sonicated cryptococcal antigens (WSCAg). Certain amount of the sonicated suspension was centrifuged by cold centrifuge with 17000 rpm for 20 minutes and the supernatant was taken and the sediment was discarded. The supernatants passed through (0.22 µm) Millipore filter and the protein was estimated (14 mg/ml), and kept it under 4C until use in a skin test. Also 60 white mice were used in the immunopathological study of the fungus Cryptococcus neoformans.

**Results and Discussion**

The result showed that, at 24 hours post test, the mean values of the skin thickness against soluble sonicated antigens of C.neoformans in the 3rd group was higher(1.99±0.02) than those values in 1st group (1.75±0.03), and at 48hrs post-examination these values were arise in 3rd group (2.11±0.03) and 1st group 1.81±0.03 (Table,1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>After 24 hours</th>
<th>After 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.75±0.03</td>
<td>1.81±0.03</td>
</tr>
<tr>
<td>3</td>
<td>1.99±0.02</td>
<td>2.11±0.03</td>
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</tbody>
</table>

The results of skin test may indicate that SSCAgS using in this study stimulated the cell mediated immune response, since DTH reaction was considered one arm of CMI and this reaction control by activity of CD4+ and CD8+ T lymphocytes (12).

DTH reactions have been classically used to detect cell-mediated immune responses to cryptococcal antigens and these reactions can be elicited by cryptococcal sonicated antigen (13).

The present study showed that immunized animals with sonicated Ags expressed good values of DTH reaction but lower when compared with those values in immunized animal with this Ags and fed diet supplemented with chitosan. On bases of above evidence it was noticed that immunized animals with WSCAgS expressed high values of DTH reaction , moreover, the high values of cell mediated immune response
in immunized animals with WSCAgs may be due to these Ags have all types of fungal Ags that elicited both arms of immune response, these evidence was in agreement with (14) who reported that the MPs, a main constituents of C. neoformans Ags were capable of inducing DTH reactions, also (15) reported that WSCAgs elicited a good cell mediated immune response in immunized mice. these results was in consistent with previous researches which explained that chitosan potently activated macrophages and NK cells via activated macrophages to produce IL-12 that stimulated NK cells which produced IFN-y, these cytokines activated macrophage and immune cells as well as attracted these cells to the site of Ags (16 and 17).

Chitosan act to stimulate maturation of dendritic cells which play important role in protection the host against C. neoformans this idea is in agreement with finding of (18) who reported that during crytococcal infection, DCs are considered a major initiators of protective cell mediated immunity these cells are more efficient induction of T. response(19).

The mean values of Abs 3rd group were higher than in 1st group(Table,2). Table, 2: show the mean values of Abs titers in 3rd group were higher than those in 1st group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Means of Abs titers against C.neoformans ± SE</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>136±28.2</td>
</tr>
<tr>
<td>3</td>
<td>160±29.6</td>
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It was found that immunized animals with WSCAgs expressed high levels of Abs titers ,these results may be due to the WSCAgs have intact all fungal Ags that elicited both arms of immune response these results agree with (15) who found that WScAgs better elicited humoral and cellular immune responses.

The high levels of serum Abs titers in immunized animals fed diet supplementing with chitosan in the present result may be indicated that chitosan augment the production of Abs against C. neoformans Ags, these observations supported idea reported by (20) who found that chitosan induced increasing Abs titers over fivefold and splenic CD4+ T cell proliferation over 6 fold.

However, the Abs titers elicited by immunized Ags with chitosan supplementing diet were higher than those values in immunized animals alone, these results may be indicated that chitosan activated B. cells to proliferate and differentiate into plasma cell producing Abs. This evidence is in agreement with (20) who reported that chitosan increase Abs titer over 5 fold and stimulated specific splenic CD4+ T cell proliferation more than 6 fold.

The present study shows that immunized animals fed diet supplementing chitosan expressing high values of CMI and high levels of Abs titers, these results may support idea that chitosan stimulated both arms of immune responses(20), as well as it may be indicated an interaction between CMI and humoral immunity, this observation is in agreement with results of (21) who recorded a relationship between CMI and humoral immune response.

Histopathalogical changes of immunized animals with Sonicated Ags at 30 day post-infection:There are no clear lesions in the kidney and brain and the lesions in the spleen characterized by Proliferation of lymphocytes in the periarteriolar sheath were the main lesions in spleen, in addition to proliferation of mononuclear cells around sinuses form cord like appearance . The main lesions in the lung characterized by marked mononuclear cells aggregation in the wall of the bronchiols and blood vessels and in the wall of bronchi which showed muscular hyperatrophy, in addition to mononuclear cells in the interalveolar septa and in the alveolar space , the liver showed mature granulomatous lesion consisting from aggregation of active macrophages in the liver parenchyma, in other animals, The liver showed multiple granulomatous lesions in portal area consisting from aggregation of epithelioid cells, macrophages, lymphocyte around few degenerative yeast cells and surrounded by fibrous connective tissue, the fibrosis infiltrated with large number of mononuclear cells extended to capsular region.

Histopathalogical changes of Immunized animals with whole sonicated Ags and fed diet supplemented with chitosan:
The microscopic examination showed no clear lesions in the heart and brain at day 30 post-infection and the main lesions in the spleen were marked proliferation of lymphocytic cells in the periarteriolar sheath and proliferation of mononuclear cells around sinuses form cord like appearance (Fig. 1), in addition to multiple granulomatous lesion scatter in liver parenchyma at day 30 post-infection (Fig. 2). Histopathological section in the lung revealed aggregation of mononuclear cells around blood vessels and airways, as well as macrophages in the alveolar space, with moderate mononuclear cells infiltration in the interstitial tissue at day 30 post infection.

The main lesions in the liver characterized by marked mononuclear cells aggregation in portal area around portal vein and bile ducts, in addition to hyperatropy of kupffer cells. In other section, there were granulomatous lesions consisting from aggregation of macrophages and lymphocytes around degenerative yeast cells and encapsulated by fibrous connective tissue capsule, in other animal, the capsular region of the liver showed granulomatous lesion as well as severe mononuclear cells infiltration and marked fibrous connective tissue proliferation around degenerative yeast cells, the fibrosis was extended to portal area of the liver.

Lung:

The lung showed aggregation of mononuclear cells around blood vessels and airways, as well as granulomatous lesions in the interstitial tissue and proliferation of alveolar macrophages in the alveolar space and increased thickness of interalveolar septa.

Gliaosis were seen in the brain parenchyma (Fig. 3) as well as inflammatory cells particularly neutrophils were seen around congested blood vessels in pia metter.

![Histopathological section in the spleen of immunized animal with sonicated Ags shows proliferation of lymphocytes in the periarteriolar sheath and proliferation of mononuclear cells around sinuses (H&E stain 40X).](image1)

![Histopathological section in the liver of immunized animals with sonicated Ags and treated with chitosan at 30 days post-infection shows multiple granulomatous lesions in the liver (H&E stain 40X).](image2)

![Histopathological section in the brain of animal treated with chitosan at 30 days shows gliosis in the brain (H&E 40X).](image3)

Histopathological changes of infected animals with Cryptococcus neoformans died during 2 week: All animals were died during the first week post-infection. The main lesions in kidney characterized by congestion of blood vessels with inflammatory cells in their lumen particularly neutrophils as well as acute cellular degeneration of renal tubules, yeast cells with few inflammatory cells were recorded between renal tubules, in other section, the kidney showed yeast cells with few neutrophils, eosinophils and mononuclear cells in capsular region as well as...
vascular degeneration of epithelial lining cells of renal tubules (Fig.4), but in other animal, yeast cells were noticed in adipose tissue without inflammatory reaction. In other section, mononuclear cells infiltration between renal tubules with cellular debris in these tubules.

Figure, 4: Histopathological section in the kidney of animal at - 3days post-infection shows yeast cells in capsular region with few neutrophils, eosinophils and mononuclear cells infiltration well as acute cellular degeneration of renal tubules (H&E 40X).

Histopathological sections in the liver revealed neutrophils, eosinophils, macrophages and magokarocytes in dilated sinusoids and diffuse distribution of yeast cells in the liver parenchyma without inflammatory cells, in addition to vacuolation of enlargement hepatocytes and hemorrhage in necrotic area of hepatocytes, in other animal, multiple granulomatous lesion consisting from aggregation of neutrophils and macrophages with or without yeast cells in their center.

There were depletion of white pulp with PMNs, mononuclear cells and fibrin networks in the red pulp as well as inflammatory cells in the congested blood vessels. In other section, hemorrhage and congestion of red pulp were noticed, in other animals, multiple cystic structure containing yeast cells were seen between renal tubules, increase thickness of capsular region due to PMNs and mononuclear infiltration, fibrin network deposition, proliferation of fibrous connective tissue and multiple cystic structure containing large number of yeast cells and the lesion was extended into pancreas interstitial tissue.

The microscopical section of the lung revealed congested blood vessels with PMNs and mononuclear cells in their lumen and in the interalveolar septa, in addition to alveolar space were filled with PMNs, alveolar macrophages, RBCs and fibrin networks as well as granulomatous lesions were seen. In other animals, large space filled with large number of yeast cells surrounded by few inflammatory was recorded in the interstitial tissue.

The heart showed PMNs and mononuclear cells aggregation between fragmentation cardiac muscle fiber and cystic structure filled with yeast cells in cardiac muscle fiber were seen.

Histopathological examination of the brain expressed inflammatory cells particularly neutrophils in the lumen of congested blood vessels in meninges, in addition to aggregation of inflammatory cells around congested blood vessels in the brain parenchyma, also hemorrhage in brain tissue was seen, as well as large multiple cystic structure filled with yeast cell without inflammatory cells were seen in brain parenchyma.

The current study revealed very high degree of tissue damage in all examined organs of non-immunized –non-chitosan supplemented diet (control positive group) induced by the local isolated strain of Cryptococcus neoformans, the tissue damage associated with this fungi is a complex process occur either by direct effects of the fungi or immune complex resulting from combination Abs against C.neoformans Ags with these Ags, this idea was supported by suggestion of (22) who explained that the damage tissue reflects a complex interplay of pathogen – induced damage, host-induced damage or both. Also a very high degree of damage tissue in the present study may be indicated that the strain using in the study is highly virulent and these damage caused by C.neoformans virulence factors. The GXM in capsular polysaccharide of C.neoformans have wide array of deleterious effects(23). Also tissue damage associated with these fungi are related to immune complex and or Th1-type inflammatory response. C.neoformans may classified as class 4 pathogen and the damage response of pathogen is determine the outcome.
of host-pathogen interaction in susceptible host (24).

It was recorded that the animals fed diet supplemented with chitosan showed low degree of tissue damage as compared with control positive group, these may be due to chitosan stimulated the phagocytic cells and immune cells to produce cytokines that facilitate killing of these fungi via activation the macrophage, a host cells of C.neoformans these idea was in agreement with (20) who explained that the chitosan, a positive group ions have high affinity for the body cells and stimulated the immune system. Also (25) found that the chitosan activated the macrophages through upregulated IL-1, TNF-a and INF-y production.

The main lesions in liver, and lung of animals fed diet chitosan supplementing were granuloma at day 30 post infection with mononuclear cells infiltration around blood vessels in other organs, this result was supported that chitosan elicit cell mediated immune response that it is responsible for development granulomatous reaction, these idea was in consistent with (26) who reported that chitosan increase immune activity of DCs via up-regulate the major histocompatibility complex class II antigens and produce IL-12, also lymphoid tissue hyperplasia may be due to the chitosan activated lymphocytes proliferation, (26) revealed payers patches hyperplasia in animal oral administration with chitosan. However, lymphoid tissue hyperplasia may indicated activation of CMI respose (27).

A good protection afforded by immunized animal with (WSCAgs) with or without supplemented diet with chitosan were reported in the present study, based on few tissue damage, clearance of yeast cells from all organs during 15-30days post-infection prolonged survival till the end of the experiment and prevent dissemination of the fungal cells to the brain and other target organs, this result was in agreement with (15) who demonstrated that the WSCAgs induced a better immune responses against C.neoformans, also she demonstrated high level of serum IFN-y and TNF-a in immunized animals by these Ags and she suggested that the control of cryptococcal infection depends on CMI responses.

WSCAgs containing all types of fungal Ags particularly MPs which is a protein in nature and it considered a good CMI stimulator, that was in agreement with (28) who found that DCs are faster maturation and activation by MPs. CD5 produce IL-12 which is play critical role for effective response against C.neoformans (29). IL-12 play role in differentiated Th0 into Th1 cells that produce INF-y which efficiency activated of the macrophages to produce oxidatative and nitrosative burst(30).

The present granulomatous lesions in organs of immunized animals may be indicated that some fungal cells reached to these organs and the immune response localized and kill these organs since granulomatous response was considered a stronger body defense against virulent pathogens and persistent till destroyed the invading agents(31), moreover, the present finding demonstrated very low degree of tissue damage in internal organs of immunized WSCAgs-chitosan group.

References


دراسة مناعية مرضية للفطر المكورات الخبيئة في الفئران

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المصطلحات

هدفت الدراسة الحالية لدراسة تأثير الأنتيجين المكسر للفطر المكورات الخبيئة في إجراء رابع جرعة من عسل فطر مجهز بالكيتوسان وتأثير مادة الكيتوسان على الأصابات بعد ذلك.

في يوم 28-30 بعد التعقيم، اجري فحص سمك الجلد وفحص التلازيم النموي غير المباشر بعد ذلك، والمجموعة الأولى، والثانية، والثالثة، والمجموعة الرابعة تم حفظها داخل البريمون بـ0.3 ملم من مغلق طفري بـ170 خلايا فطرية في يوم 30 من الأصابات بـب吏رة المكورات الخبيئة. يندرج النتائج أن الحيوانات الممنعة والمبكرة على عينية تجري الكيتوسان أظهرت معدلات من فص في الحيوانات الممت洲 (1.99±0.17) بـ0.25 ملم وكمية مجهزة بالكيتوسان مثل المجموعة الثانية.

المجموعة الرابعة كانت بمعدلة موجهة لمجمعة في يوم 10-28 بعد التعقيم، اجري فحص سمك الجلد وفحص التلازيم النموي غير المباشر بعد ذلك، والمجموعة الأولى، والثانية، والثالثة، والمجموعة الرابعة تم حفظها داخل البريمون بـ0.3 ملم من مغلق طفري بـ170 خلايا فطرية في يوم 30 من الأصابات بـب吏رة المكورات الخبيئة. يندرج النتائج أن الحيوانات الممنعة والمبكرة عالياً على عينية تجري الكيتوسان أظهرت معدلات من فص في الحيوانات الممتوز (1.99±0.17) بـ0.25 ملم وكمية مجهزة بالكيتوسان مثل المجموعة الثانية.

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لا يوجد هائل في الدراسة الحالية أن كل الحيوانات غير ممنعة والتي تم إصابتها ما بين الأسبوعين الأولي بعد الأصابات في عسل فطري كليغ في جميع الأعضاء الداخلية مع تفاعل النسبي في المجموعة الأولى، والثانية، والثالثة، والمجموعة الرابعة تم حفظها داخل البريمون بـ0.3 ملم من مغلق طفري بـ170 خلايا فطرية في يوم 30 من الأصابات. يندرج النتائج أن الحيوانات الممنعة والمبكرة على عينية تجري الكيتوسان أظهرت معدلات من فص في الحيوانات الممتوز (1.99±0.17) بـ0.25 ملم وكمية مجهزة بالكيتوsan.

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