Immunopathological Study of The Effect of Bile Salts on Secondary Hydatidosis Development inWhite Mice

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Abstract

This study was carried out to investigate theimmunopathological effects of bile salts on the immune response against hydatid cyst infection in mice.The treated groups of mice dividedinto two groups,the first group immunized with 0.3ml of2mg/mlof bile salts and the second group immunized with 0.2ml of1mg/mlof bile salts in 0 time after two weeks the same doses as repeated against secondary hydatidatid cysts, then delayed type hypersensitivity test was done on these groups and compared with the third group(that had been inoculated with 0.2 ml of sterile phosphate buffer saline as a control group)at day 27 post immunization using soluble bile salts.At day 30 half number of animals from each group were scarified to perform humoral immunity tests (ELISA).Then challenge with 2000 protoscolices were done for the remaining half ofall 3groupsand left them for three months then kill them for histopathologicalresults post challenge showed marked growth of cysts in the livers of the control infected group, with some degenerative and necrotic lesions accompanied byamyloidosis in spleen. While the immunized groups with bile saltsrevealed presence of focal mononuclear cellsinliver,kidney and lung tissue with lymphoid hyperplasiain spleen.Finally,this study showed that the bile saltswere highly immunogenicand this may related to the fact that one ofthecomponentsh of bile salts hasDeoxycholic acid init´s structure has the lysis effect on the protoscolsis against hydatid cyst infection in mice.

Key words :*Echinococcusgronulosns*, bile salts mice, Immunopathological effects,immunity.

دراسة مرضية مناعية في تأثير املاح الصفراء على الاكياس العذرية الثانوية في الفئرانالبيض

الخلاصة

أجريت هذه الدراسة لبحث بعض التأثيرات المرضية والمناعية لأملاح الصفراء على الاستجابة المناعية ضد الاصابة بالأكياس العذرية في الفئران المختبرية.وشملت هذه الدراسة تحصين المجموعة الأولى بجرعة ٠.٣ ملوالثانية بجرعة ٠.٢ مل من املاح الصفراء فمويا في الفئران البيض ضد المشوكة الحبيبية في زمن الصفر (بداية التجربة) ثم تم اعادة التحصين بعد اسبوعين وبنفس الجرع بعدها اجري اختبار نوع فرط الحساسية وقد تم مقارنتها مع مجموعةالسيطره (المجموعة الثالثة) والتي تم معالجتها بجرعة٠.٢ مل من المحلولالفسلجي في يوم ٢٧ وتم التضحية بنصف عدد الحيوانات من كل مجموعة في اليوم الثلاثين لإجراء الفحوصات المناعية الخلطية والتي اشتملت على اختبار الاليزا.شمحقنتالحيوانات المتبقية من المجاميع الثلاثة بجرعة التحدي الحاوية على مايقارب ٢٠٠٠ رؤويس حيوي/ لكل حيوان داخل الخلب وتركت الحيوانات لمدة مقاربة لثلاثة أشهر،بعدها قتلت الفئرانلاجراء الفحوصات المرضية النسيجية وكانت نتائجها كالاتي:بعد جرعة التحدي اظهرت نمو الأكياس العدرية في أكباد حيوانات السيطرة مصحوبة بآفات تنكسيةونخرية, مع ترسب النشوانفي الطحال اما المجاميع التي منعت بأملاح الصفراء اظهرت ارتشاح الخلايا الوحيدة النواة في انسجة الكبد والكلى والرئة مع وجود فرط تنسج لمفاوي في الطحال.أخيرا أظهرت نتائجنا أن الأملاح الصفراء اعطت مناعة عالية ضد الاصابة بالاكياس العذرية الثانوية، وهذا قد يعزى لحقيقة أن أحد مكونات املاح الصفراء هو حامض(Deoxy) acidcholic في بنيتها وهذا الأخير لديه تأثير تحللي للروئيسات الاولية في الفئران المصابةبالاكياس العذرية.

Introduction

EChinococcosis(hydatidosis) is a worldwide zoonotic infection, that affects humans and livestock, the causative agent of(CHD)(cystic hydatid disease is *Echinococcusgranulosus*metacestodestage)[1,2].*Echinococcusgranulosus* enhances both humoral and cellular responses in it's intermediate host [4,5]. Humoral responses will result in the production of immunoglobulins, which are important for the diagnosis of patients [6,7,8,9,10]. However, cellular responses also will take place in the infected host which are important for the prevention of the disease [11,12]. In particular, Th1 cell activation seems to be more related to protective immunity, while Th2 cell activation is linked to the susceptibility to the disease [13]. Bile acids are conjugated with taurine or glycine in the liver, forming bile salts.Primary bile acids are those synthesized by the liver. Secondary bile acids result from bacterial actions in the colon. The main function of bile acids is to facilitate the formation of micelles, which promotes digestion and absorption of dietary fat[14].Synthesis of bile acids is a major route of cholesterol metabolism in most species other than humans. The body produces about 800 mg of cholesterol per day and about half of that is used for bile acid synthesis producing 400–600 mg daily. The bile acid pool size is between 4–6 g, which means that bile acids are recycled several times each day. About 95% of bile acids are reabsorbed by active transport in the ileum and recycled back to the liver for further secretion into the biliary system and gallbladder. This enterohepatic circulation of bile acids allows a low rate of synthesis but with large amounts being secreted into the intestine [15].

The aim of this study was to study the effect of the bile salts on the immune-pathlogical change in albino mice that infected with secondary *Echinococcusgranulosus*.

Meterials and Methods

Experimental design:

This study was perfirmed in pathology department of collage of veterinary medicineinBaghdad University.Sixty white mice inwere randomly divided into three groups as follows:

1. The first group included 20 mice were immunized orally with 0.3 ml of bile salts at concentration of 2mg /ml for two doses with 14 days intervals first dose at day 0 and second booster dose at day 14.
2. The second group included 20mice were immunized orally with 0.2ml of bile salts at concentration of 1mg /mlfor two doses with 14 days intervals first dose at day 0 and second booster dose at day 14.
3. The third group20 mice were inoculated with 0.2 ml of sterile phosphate buffer saline as a control group,then these 3 groups were infected with hydatid cysts after 2 weeks from adaptation by injecting 2000 protoscolicescesintraperitonealy(1ml/mouse).

Parameter of the study:

1. Cell mediated immune response of experimental mice determined by skin test : Delayed type hypersensitivity test was done to detect the cellular immune response in all animals at day 27 post immunization and post challenge[16].
2. Humoral immune response detected by ELISA(enzyme linked immunosorbent assay): This test was done according to manufacturer (immunological consultents laboratory, Inc.)at day 30.
3. histological examination: At the end of the each period of experiment, five animals from each groups were sacrified under deep anesthesia and specimens from liver and kidney fixed in buffered formalin, sectioned (5 mm thickness) and stained with Hematoxylin and Eosin [17].
4. Data were analyzed statistically using the Microsoft Program (SPSS). Statistical analysis of data was performed on the basis of Two-Way Analysis of Variance (ANOVA) using a significant level of ($P<0.05$). Specific group differences were

determined using least significant differences [3].

Results and Discussion

Cellular immune response detected by skin test(delayed type hypersensitivity-DTH)

The results of the current study revealed that the mean values of the skin thickness after 24 hours were significantly ($p<0.05$) higher in the groups that immunized with bile salts for in both concentrations(2mg/ml) and (1mg/m) which were (1±0.06) and (0.70±0.4) mm respectively as compared with the control group (PBS) (0.12± 0.02) mm , while were significantly ($p>0.05$) lower in the groups that immunized by AgB for both concentrations (3.1mg/ml) and (2.8mg/ml) which were (0.47±0.02)and(0.35±0.04) mm respectively as compared to those that immunized by bile salts but significantly higher as compared with control the group (PBS) (0.12±0.02) mm. Also after 48h, the results were showed a significant difference ($p<0.05$) in the group that immunized with bile salts for both concentrations 2mg/ml and 1mg/ml which were (0.84±0.0)and (0.65±0.02) mm respectively as compared with control group (PBS) (0.10±0.002) mm no significant difference ($p>0.05$).

Table1: Showed the mean values of skin thicknessinthe three groups

time / group	24 hours	48 hours
1st group animal immunized with bile salt at *concretions* (2 mg /ml)	1.00±0.06 Aa	0.84±0.02 Ab
2nd group animal immunized with bile salt at concretions(1mg /ml)	0.70±0.04 Ba	0.65±0.060 B b
3rd control group	0.12±0.02 Ca	0.10±0.02 Cb

DifferenSmall t letters in row denot significant differences between periods (P≤0.05).Different Capital letters denot column significant differences between groups (P≤0.05).Data were analyzed statistically using the Microsoft Program (SPSS).

The study showed that the time required for the initial attenuation of protoscolesis directly proportional to the vitality and inversely with the number of protoscolesisand whenever there is increase in the concentration of bile salts there is decrease the time that required for attenuation of the protoscolesis[18].

2. Humoral immune response detected by ELISA(enzymelinkedimmunosorbent assay):

ELISA test was showed the means of antibodies)(IgG) titer. The results revealed that there were asignificant differences ($p<0.05$) in the means values of the groups that immunized by bile salts for both concentrations (2mg/ml) and (1mg/ml) which were (18.98±0.78)and (13.96±0.99)ng/ml respectively as compared with control group (PBS)(6.12±0.040).

Table2 : Mean and standard error of antibody (IgG) titers of the 1st and 2nd immunized groups and the 3rd control group at 30th day:

group	Mean&standard error ng/ml
1st group animal immunized with bile salt at *concretions* (2 mg /ml)	18.98±0.78 A
2nd group animal immunized with bile salt at concretions(1mg /ml)	13.96±0.99 B
3rd control group	6.12±0.40 C

Different capital letters in column denote significant ($p<0.05$) differences betweengroups.Data were analyzed statistically using the Microsoft Program (SPSS).

The results of the present study showed that the groups of mice that immunized with bile salts is a highly significant immune response against the *Echinococcusgranulosus* more than the control group of mice.This mean the increase in concentration ofbile salts lead to increase in titer of IgG.Thesesalts play an important roleinprotoscolesisdecomposion or may be duetothe effect ofbileacidsasalkaline phosphataseonthechemicalcomponentsofth eprotoscolesisormay bedue to disturbance in thecomponentof bile salt like sodium and potassium that induce changes in the osmotic and hydrostatic pressure [19] were reported that the bile consists of the alkaline phosphatase, fats,inorganic acids,urea and bile salts.

The bile of sheep may have the effect on the protoscolesis metabolism then lead to death of protoscolesis after short time[18].

Histopathological Examination

1-First group with 2mg/ml of ile salts:

No clear pathological changes were observed in liver parenchyma of this groupexceptthepresence of focalmononuclear cells consist of macrophage and lymphocyte aggregation seen mainly around central vein The main splenic histopathological changes revealed significant increase of megakaryoticandperiarteriolar lymphoid hyperplasia(figure-1).While the predominant pulmonary lesion was pulmonary edema in many alveoli spaces associated with MNCs perivasicular aggregation and evidence of neutrophil in the lumen of congested pulmonary blood vessels (figure-2).Also the result showed MNCs infiltration in intestinal submucosa associated with slight desqumation of epitheliallining intestinal villi.Microscopicpictures of renal tissue showed cellular swelling of tubular epithelial lining with perivasicular and periglomerular MNCs infiltration (figure-3) also the results showed no clear pathological changes in brain sections except slight cerebral edema with slight perivasicular cuffing (figure-4).

2-Second group with 1mg/ml of bile salts

The main hepatic tissue lesion showed multifocal MNCs infiltration mainly in portal area associated with vascular changesof liver parenchyma(figure-5). No clear pathological alterations were recorded in renal tissue, but other sections showed focal interstitial mononuclear cells aggregation (figure-6) in addition to slight perivasicular aggregation with moderate MNCs infiltration in adipose tissue in surrounding the kidney. Splenic lesions

revealed moderate hyperplasiaofmegakaryoctes(figure-7)associated with slight lymphoid depletion and evidence of apoptosis of some lymphocytealso thickness of splenic capsule was observed due to MNCs infiltration.The brain histopathological finding characterized by moderate to severeperivasicular lymphocytic cuffing with gliosis (figure-8).In addition to appearance of neutrophils in lumen of dilated congested blood vessles.

3-Third group (control group)

The charactersticmicroscopical finding in the liver of infected non immunized animal,presence of multiple hydatid cyst with all 3 layers (germinated,laminated and adventia)(figure-9) with appearance of protoscoleses in the lumen of Some cyst(figure10),also the result showed sever vaculater necrotic changes with vaculation in liver parenchyma associated with aggregation of mononuclear cells, the result of pulmonary tissue showed sever deistruction in the epithelial lining bronchus and bronchules with vasicular changes as well as mononuclear cells.In addition to the development of granulates like lesion seen in pulmonary tissue.Focal lymphocytic aggregation seen between degenerated renal tubules with evidence of tubuler necrosis other section the result of infected kidney revealed severevaculation of renal epithelial lining with sever blood vessel congestion,the result of infected spleen reported predominant splenic lesion characterized by lymphoid deplesion with amyloid deposion with hyalinization of splenic arterioles seen in some section (figure-11),while the result showed neuronal swelling and degeneration wih focal necrosis associated with focal gleosis (figure-12) together with sever blood vessel congestion.

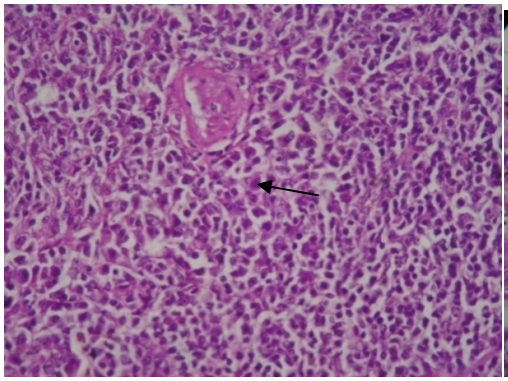

Figure1: Histopathological section in spleen of Immunized group with (2 mg/ml) of bile salts showed significant increase of megakaryotic(H & E stain 40 x).

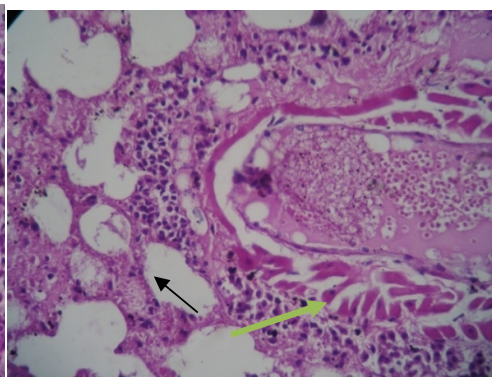

Figure2: Histopathological section in lung of Immunized group with (2 mg/ml) of bile salts showed pulmonary edema in many alveoli spaces associated with MNCs perivasicular aggregation (H & E stain 40 x).

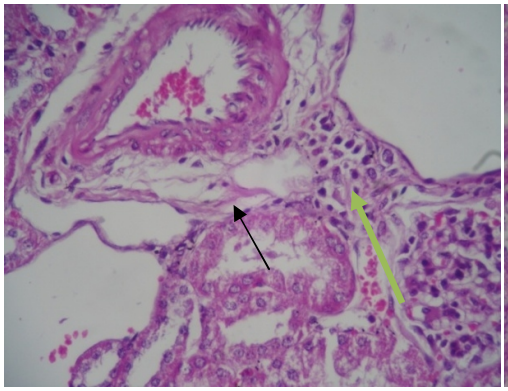

Figure 3: Histopathological section in kidney of immunized group with (2 mg/ml) of bile salts showed slight celluer swelling of tubular epithelial lining with perivasicular and periglomerular MNCs infiltration (H & E stain 40x)

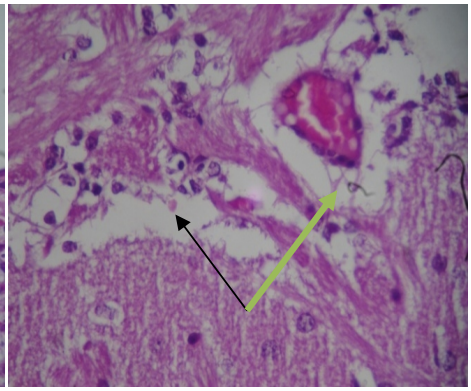

Figure 4: Histopathological section in brain of Immunized group with (2mg/ml) of bile salts showed slight cerebral edema with slight perivasicular cuffing (H&E stain 40 x).

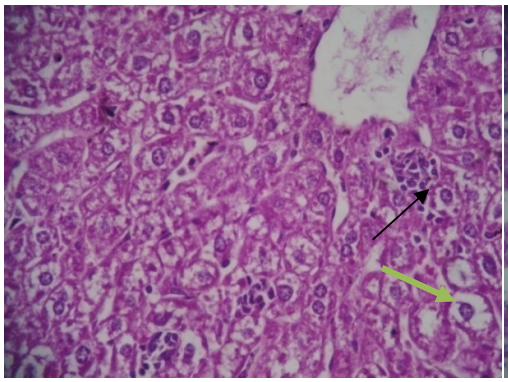

Figure5: Histopathological section in liver of Immunized group with (1mg/ml)of bile salts showed multifocal MNCs infiltration mainly inportal area associated with vacular changes of liver parenchyma (H&E stain40x).

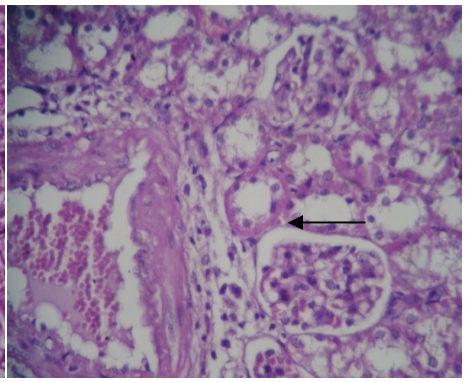

Figure6: Histopathological section in kidney of Immunized group with (1 mg/ml) of bile salts showed slight perivasicular aggregation with moderate MNCs infiltration (H&E stain40x).

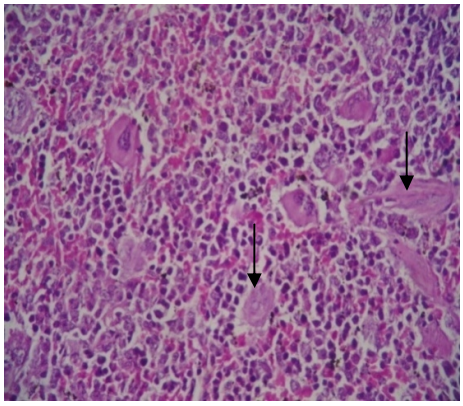

Figure 7: Histopathological section in spleen of Immunized group with (1 mg/ml) of bile salts showed moderate hyperplasia of mega-karyoctes(H & E stain 40x).

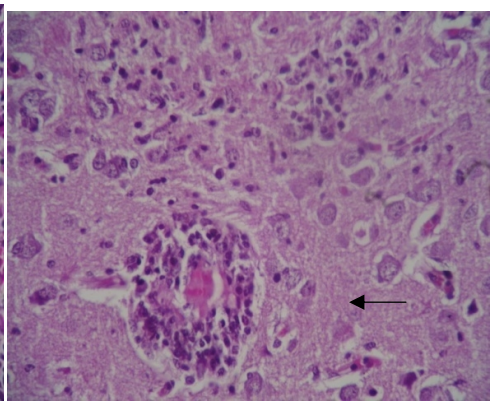

Figure 8: Histopathological section in brain of Immunized group with (1mg/ml) of bile salts showedmodrate to severeperivasicular lymphocytic cuffing with gliosis (H & E stain 40x).

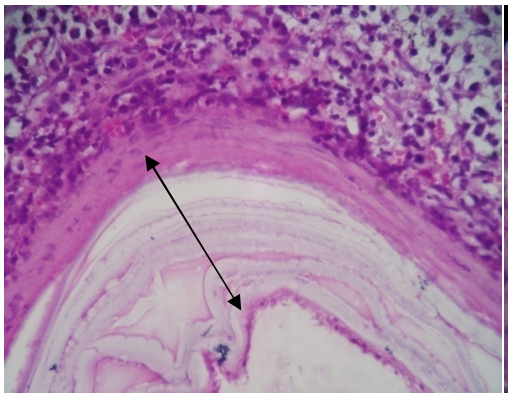

Figure9: Histopathological section in liver of controlgroup showed precense multiple hydatid cyst with all 3 layers (H&E stain 400x).

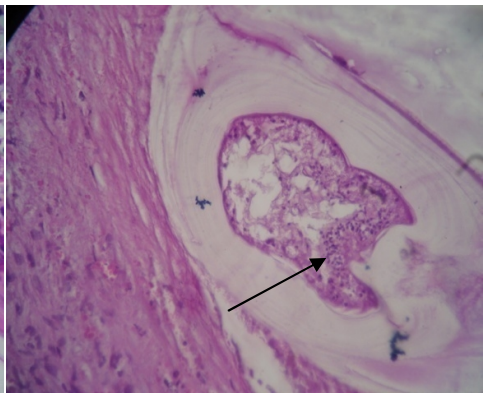

Figure10: Histopathological section in liver of infected non immunized animal with appearance of protoscoleses in the lumen of some cyst (H&E stain400x).

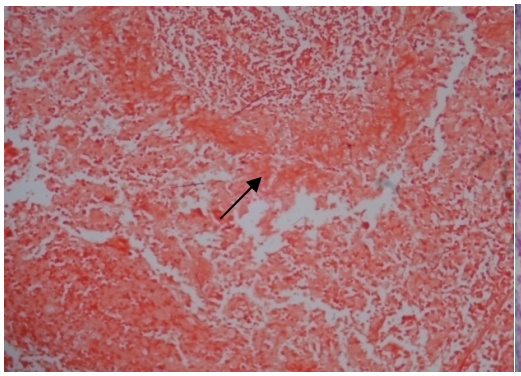

Figure 11: Histopatological section in spleen of control group showed lymphoid deplesion with amyloid deposion(H & E stain40x).

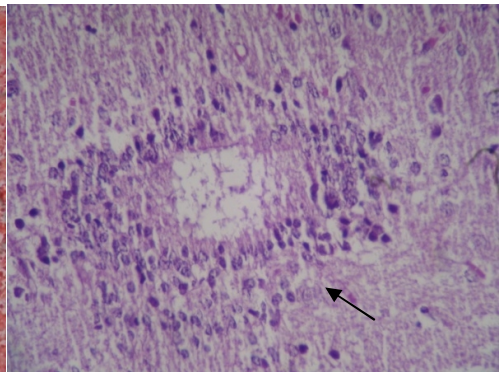

Figure 12: Histopathological section in brain of control group showed focal necrosis associated with focal gleosis(H & E stain 40x).

The results revealed (figure-5)to the absence of cyst in the liver of immunized groups with bile salts indicate that the ox bile was found to lyse protoscoleses of *Echinococcusgranulosus*.This result accept with result of [18] who mentioned that the sodium deoxycholic acid(one of the bile components) can killtheprotoscoleses.Also thecystsdisappearedfrom the hepatic tissue in current study so this result identical with [19] which noticed that the deoxycholic acids can fragmented the mitochondria which located in the cover of protoscolices that lead to protoscolices decomposition.

While results of the current study showed to infiltration of MNCs in the liver and kidney tissue(figure3,5).These result similar to results of [15,25].These results due to both primary and secondary bile salts can activate T-cell TGR5 and farnesoid X receptor(FXR), and several bile salts have been reported to be natural ligands of TGR5 is expressed in several tissues, with the highest levels detected in the gall bladder, followed by the ileum and colon. TGR5 expression is not detectable in primary hepatocytes [15]. In contrast, FXR is highly expressed in the liver, intestine, kidney and adrenal glands.FXRexpression in immune cells, such as $CD14^{+}$monocytes.In same causes therecent studiesthey found expression of TGR5 on $CD14^{+}$ peripheral blood monocytes. Furthermore, the presence of the TGR5-specific agonist promoted the differer tiation of IL-12 hypo-producingdenteric cell in a similar manner to that seen in the presence of bile salts. Taken together, these results suggest that BAs can regulate the denteric cells differentiation process through TGR5 expressed on primary peripheral blood monocytes[26].

The results in (figure9,10)revealed tothe appearance of hydatid cysts in the liver of control infected group associated with vacuolar and necrotic changes The result of current study showed that all samples (germinal layer,protoscolece) indicate to infect with*E. granulosus*and agreement with theresults of[22],as well as atrophy of splenic white pulp indicate that PSC reached the liver through the periton developed and parasite overcome the host defense with its secretion of immune complexes that inhibit immune mechanism of infected host according to[20].Liver is the common site of cystic development.Other study was mentioned that the oncosphereistrapped in the central veins of the hepatic lobules and the resulting cyst may be deep or superficial [21].

Perhaps theMNCs infiltration in different organs tissuesbelonged finding

of parasite inside cells of tissues.This results agreement with[23],whileamyloidosis in spleen (figure-11) due to immunoglobulin deposition in this organ so this result identical to[24].

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