Experimental Trial with Glutathione S Transferase as Vaccine Candidate against Hydatid Disease

Hadi Mahdi Al-Sakee*

ABSTRACT

Background and Objective: Hydatid diseaseais is one of the most important zoonosis in the world. Because of economical and hygienic importance of the disease and no currently available drugs totally effective against it, researches in preventive methods in endemic areas are considerable. This study was undertaken to investigate the effectiveness of native protoscoleces glutathione S transferase as vaccine candidate against hydatid disease in mice.

Methods: Glutathione S transferase was partially purified from protoscoleces on sephadex G 150 column. Four groups of Balb/c mice (each of 20 animals) were immunized subcutaneously with 25µg, 50µg, 75µg and 100µg of the enzyme and three groups of mice were utilized as positive, negative and adjuvant controls.

Results: the study showed considerable protection (75%) and reduction (98.2%) in mice immunized with 75 µg of the enzyme. No significant differences were observed in total leukocyte number and in functions of the liver and kidney in all immunized groups as compared to control negative group. However, lymphocyte transformation response was highly significantly (P< 0.001) increased.

Conclusions: it can be concluded from the results, immunization of mice with 75µg of native protoscoleces glutathione S transferase conferred considerable protection against hydatid infection in mice.

Key words: Hydatid disease, vaccination, glutathione S transferase .

INTRODUCTION:

Hydatid disease caused by the larval stage of the tapeworm *Echinococcus granulosus* affecting both human and domestic animals is one of the most important zoonosis in the world [1,2]. The disease is primarily prevalent in areas where sheep or other herbivores are raised and where such animals are in close contact with dogs or other wild canine [3,4]. In Iraq cystic echinococcosis is prevalent in most parts of the country especially in rural areas where offal from slaughterhouses is incorrectly disposed of or where slaughtering is practiced on farms [5,6,7,8,9]. Because of economical and hygienic importance of hydatid disease and no currently available drugs are totally affective against hydatid cysts [10,11], preventive methods in endemic areas is considerable [12, 13, 14]. The use of enzymes derived from parasitic helminthes as vaccine candidate has been proposed by several authors [15,16,17,18,19,20]. Glutathione S transferases are a group of multifunctional proteins encoded by a multigene family. They perform functions ranging from catalyzing the detoxification of electrophilic compounds to protect against peroxidative damage [21]. This family of enzymes are ubiquitous among eukaryotes and have been found in a wide rang of parasitic helminthes [22,23,24,25], in which they have been shown not only to play a housekeeping role but also to protect against membrane damage induced by the cytotoxic products of lipid peroxidation, such as

*College of Medicine, Hawler Medical University

carbonyl species which generated as a result of host immune effecter mechanisms directed against the parasite [26,27]. Moreover the role of glutathione S transferase as a protective antigen in vaccination against parasitic helminthes has been well documented [28,16,18,19]. However, the effectiveness of *Echinococcus granulosus* protoscoleces glutathione S transferase as vaccine candidate has not yet been tested against hydatid disease. The present study is an experimental trial to use native *Echinococcus granulosus* glutathione S transferase as vaccine candidate against hydatid infection in mice.

PATIENTS METHODS:

Hydatid fluid and protoscoleces Hydatid cysts were obtained from infected livers of sheep slaughtered in Erbil slaughter house and processed according to Baz *et al* [12]. Briefly, hydatid cysts were aseptically punctured and cyst fluid (containing protoscoleces and membrane fragments) was aspirated. Protoscoleces were allowed to stand for 20 minutes at room temperature and the fluid was carefully removed and dialyzed against three changes of distilled water. The dialysate then dispensed in sterile screw cupped test tubes and stored at – 70 ºC until used as source of hydatid antigens. The protoscoleces, obtained as described, were washed thrice with 0.15 M phosphate buffered saline pH 7.2 and assessed for the viability by methylene blue (0.05 %) exclusion as described by Al-Saegh [39].

Preparation of protoscoleces extract solution Protoscoleces extract solution was prepared according to Seyyedi *et al* [26]. Briefly, protoscoleces, with viability of more than 95%, were suspended in three volumes of buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM phenyl methyl sulphonyl fluoride (PMSF) (Sigma, UK), 0.15 M NaCl, 0.5 % (v/v) Triton x-100 and sonicated for two periods of 15 seconds, separated by a 10 seconds cooling period, using a MSE Soniprep fitted with an exponential probe at 4 μm amplitude. The disrupted protoscoleces, were then centrifuged at 5000 g for 30 minutes at 4ºC with the resultant supernatant being stored at – 70ºC until used.

Partial purification of glutathione S transferase Glutathione S transferase was purified from protoscoleces by single step purification scheme involving gel filtration on sephadex G 150 (Pharmacia fine chemicals) at 4 ºC. GST was eluted from the column with 4-fold PBS pH 7.4. Fractions of 3 ml each were collected at a flow rate of 40 ml/hour. GST activity in the eluted fractions was measured according to the procedure described by Seyyedi *et al* [26], using 1-chloro, 2,4- dinitrobenzene as substrate. Fractions with highest enzyme activity were used as vaccine in the experiments. The protein concentration was determined according to the method of Lowry *et al* [29].

Immunization protocol The immunization protocol was conducted as described by Hashemitabar *et al* [14] as follows:

Eighty 6-8 old BALB/c mice were divided into 4 groups (each of 20 animals) and immunized subcutaneously with 25 μg, 50 μg, 75 μg and 100 μg of partially purified native *E. granulosus* protoscoleces glutathione S transferase diluted in 100 μl of 0.15 M PBS (pH 7.2) plus 100 μl of Freund's complete adjuvant (FCA) (Sigma, UK) for first immunization. Second immunization was performed four weeks later with the same preparations except that Freunds complete adjuvant was replaced by Freunds incomplete adjuvant (FIA). Four weeks after the last immunization, blood samples were collected from four mice of each group for lymphocyte transformation response, total leucocyte count and assessment of liver and renal functions. Remaining mice were then challenged intra-peritoneally with 2000 viable protoscoleces. All mice were necropsied 90 days after the challenge infection and the internal organs were carefully examined for the site, number and size of the developing

Control groups

1 – Adjuvant control: a group of 20 mice injected subcutaneously with 100 µl of normal saline mixed with 100 µl of FCA for first immunization. Second immunization was conducted after four weeks with same preparation except that FCA was replaced by FIA.

2 – Positive control: a group of 20 mice injected subcutaneously with 200 µl of normal saline for both immunization measures and infected intra-peritoneally with 2000 viable protoscoleces.

3- Negative control: a group of 8 mice injected subcutaneously with 200 µl of normal saline for both immunization measures.

Lymphocyte transformation response

Lymphocyte transformation response was performed as described by Shubbar and Allak [30].

Total leucocyte count

Total leukocyte count was estimated according to the method described by Powrs [31].

Assessment of liver and renal functions

Commercial kits from Biolabo reagents (France) were used for determination of liver related enzymes, alanin aminotransaminase (ALT) and aspartate aminotransaminase (AST) activities in the sera of experimental mice. Renal function was assessed by estimation of blood urea nitrogen, using commercial kit provided by Biomerieux (France).

$$\text{Protection rate (\%)} = \frac{\text{No. of injected mice} - \text{No. of infected mice}}{\text{No. of injected mice}} \times 100$$

$$\text{Reduction rate (\%)} = \frac{\text{Mean of no. of cysts in group P*} - \text{Mean of no. of cysts in each group}}{\text{Mean of no. of cysts in group P*}} \times 100$$

P *: Control positive group

RESULTS:

Chromatography pattern of GST in the protoscoleces extract solution was as shown in Figure.1. In which three peaks (A, B and C) of GST activity were detected and highest enzyme activity was recorded at fractions 37, 38 and 39 which collected and utilized as source of antigens in the immunization experiments.

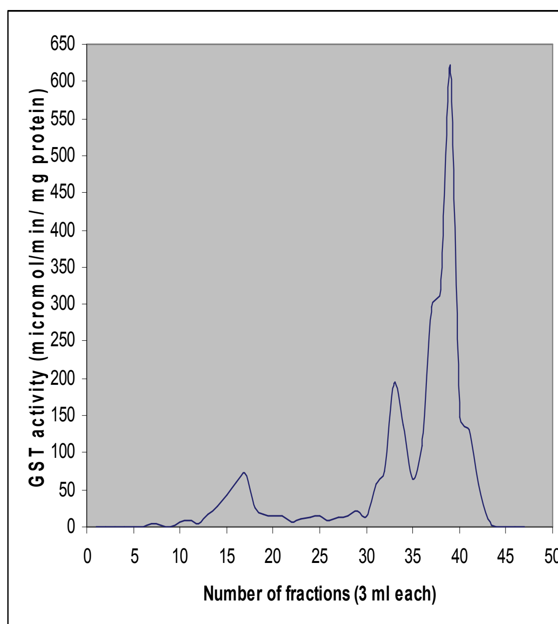

Figure 1: Chromatography pattern of glutathione S transferase in protoscoleces extract solution, passed through Sephadex G 150 column.

Immunization study As can bee seen in Table.1, immunization of mice with 25 µg and 100 µg of GST resulted in relatively minimum protection against challenge infection when compared with those mice immunized with 50 µg and 75 µg of the enzyme. It has also been shown that, highest protection rate (75 %) was in mice immunized with 75 µg of the antigen and the reduction rate was shown to be in the range between 92.07 % - 98.2 %. The results also showed that the size of developing hydatid cysts was significantly ($P < 0.01$) reduced in all immunized groups in comparison with control positive group (Table.1). The localization of developing hydatid cysts on the internal organs was variable as shown in (Figures 1,2,3,4), although, liver and peritoneal cavity have shown to be the major target of infection. Total leukocyte number was shown to be in range between 4367 ± 1419- 5100 ± 556.8 cells/ cmm, however, no significant ($P > 0.05$) differences were observed in

total leucocyte number between immunized mice and non-immunized control negative group (Table.2), whereas the lymphocyte transformation response was highly significantly ($P<0.001$) increased in all groups of immunized mice in comparison to control negative group and even to those mice immunized with the adjuvant alone (Table.2). As seen in (Table.3), the function of the liver and kidney was non-significantly affected ($P> 0.05$) by the vaccination procedure.

Table 1: Reduction rate of secondary hydatid cysts, protection rate, number and size of cysts in mice immunized with partially purified glutathione S transferase.

Experimental groups	No. of mice	No. of infected mice	no. of cysts (Mean ± St)	Mean of cyst size(mm) ±St.	Protection (%)	Reduction of cysts (%)
Immunized with 25 µg	15	9	83 (5.53 ±8.19) (p<0.01)	0.2 + 0.14 (p< 0.01)	40	92.07
Immunized with 50 µg	13	4	45 (4.15 ±8.96) (p<0.01)	0.23 ± 0.212 (P< 0.01)	69.23	94.1
Immunized with 75 µg	12	3	15 (1.25 ± 2.26) (p<0.01)	0.28 ± 0.2 (p< 0.01)	75	98.2
Immunized with 100 µg	12	5	66 (5.5 ± 8.58) (p<0.01)	0.31 ± 0.43 (p<0.01)	41.67	92.11
Immunized with adjuvant	14	14	344 (24.57 ± 16.73)	0.48 ± 0.36	0	64.77
Positive control	16	16	116 (69.75 ± 38.83)	0.44 ± 0.6	0	

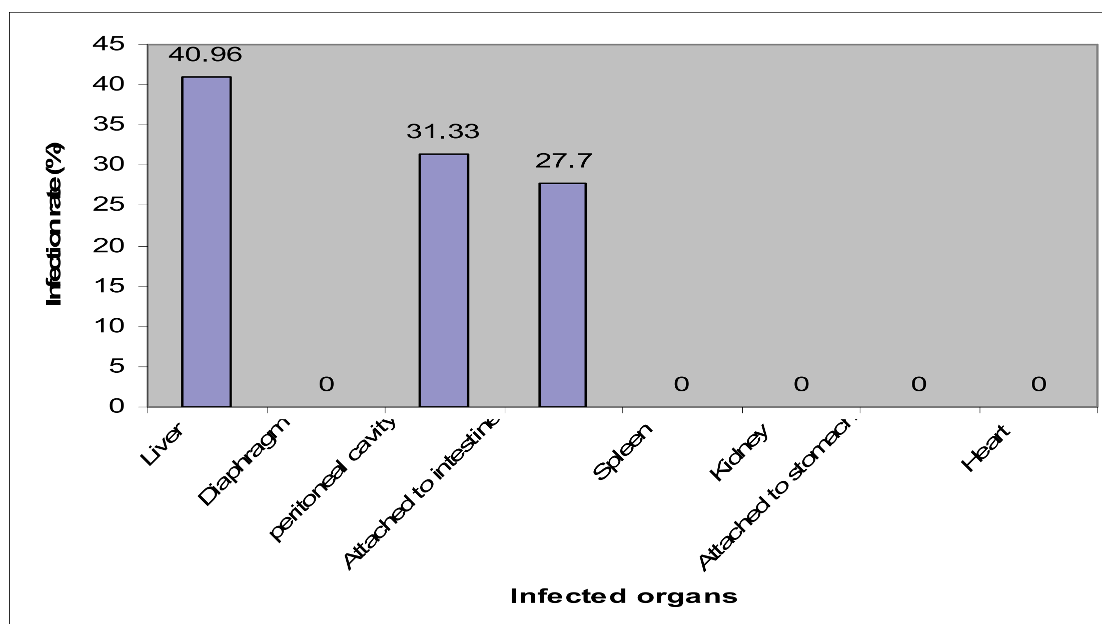

Figure 2: Localization of secondary hydatid cysts in mice immunized with 25 µg of partially purified glutathione S transferase.

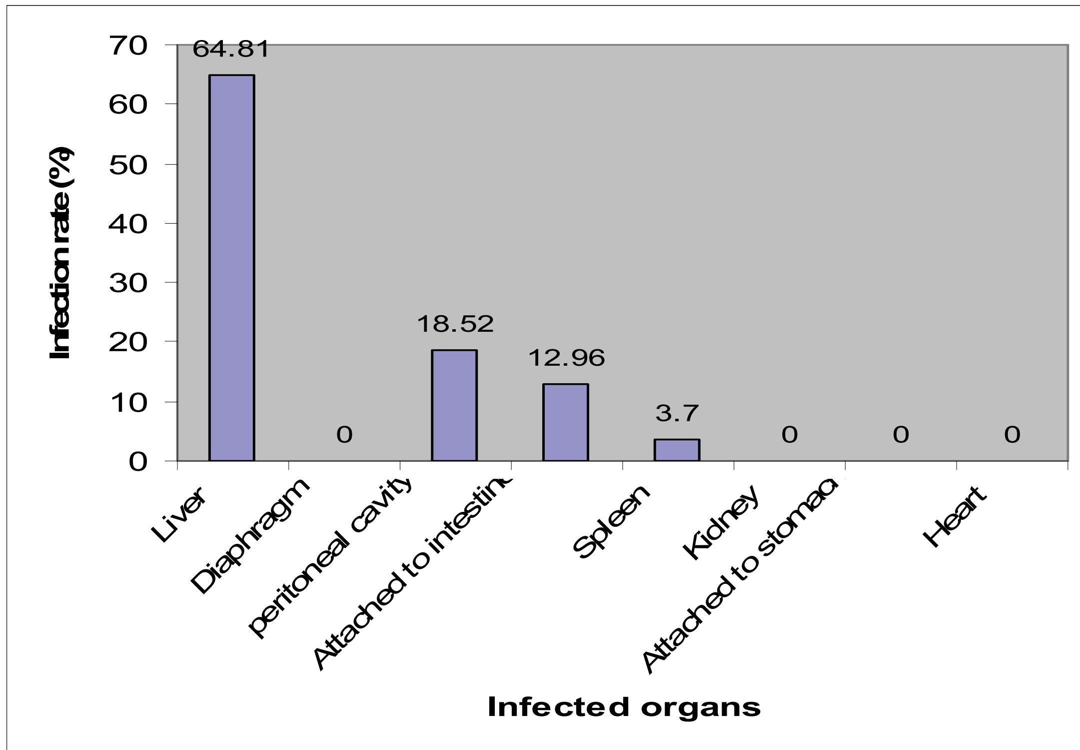

Figure 3: Localization of secondary hydatid cysts in mice immunized with 50 µg of partially purified glutathione S transferase.

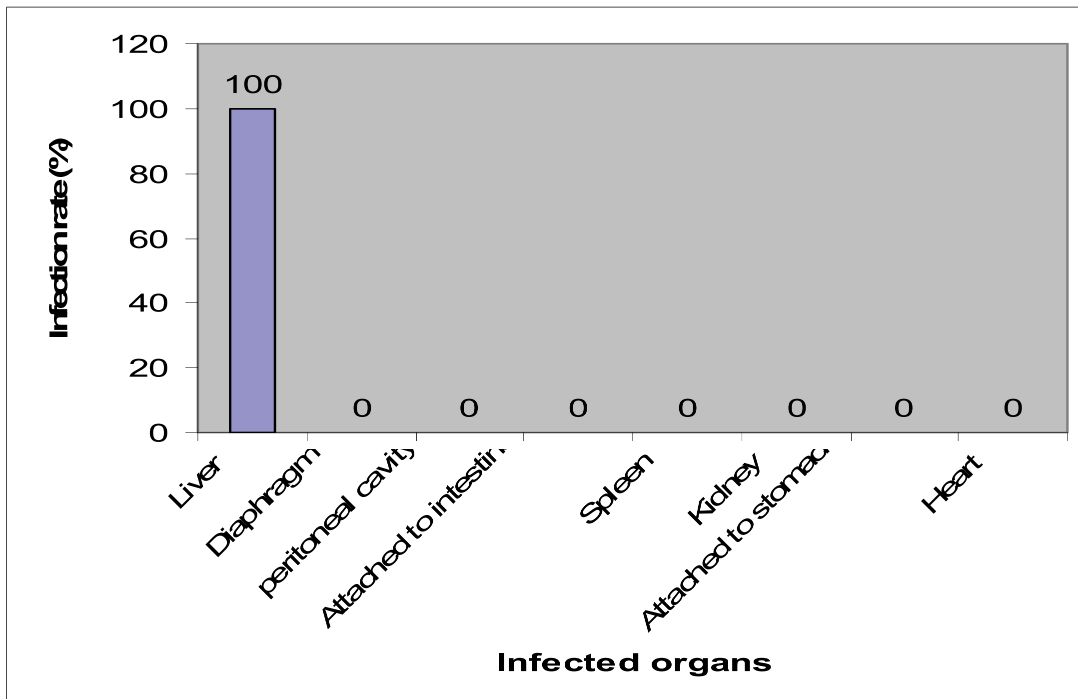

Figure 4: Localization of secondary hydatid cysts in mice immunized with 75 µg of partially purified glutathione S transferase.

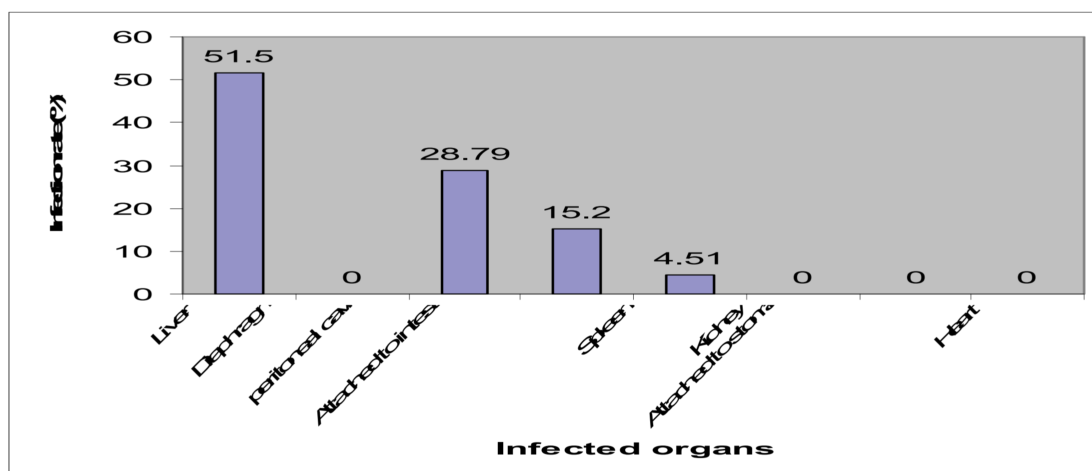

Figure 5: Localization of secondary hydatid cysts in mice immunized with 100 µg of partially purified glutathione S transferase.

Table 2: Total leukocyte count and lymphocyte transformation response in mice immunized with partially purified glutathione S transferse.

Experimental groups	Leukocyte count (cells/ cmm)±S.D.	LTT (%) ± S.D
Immunized with 25 µg	4367 ± 1419 (P>0.05)	16.65 ± 0.82 (P< 0.001)
Immunized with 50 µg	4933.3 ± 1701 (P>0.05)	16.1 ± 0.19 (P< 0.001)
Immunized with 75 µg	4600 ± 953.9 (P>0.05)	15.57 ± 0.77 (P< 0.001)
Immunized with 100 µg	5100 ± 556.8 (P>0.05)	12.6 ± 0.56 (P <0.001)
Immunized with adjuvant	7800 ± 700	4.5 ± 1.53 (P > 0.05)
Control negative	6400 ± 692.8	3.6 ± 0.7

Table 3: Biochemical parameters in the sera of mice immunized with partially purified glutathione S transferase.

Experimental groups	ALT activity (IU/ml)	AST activity (IU/ml)	Blood urea (mg/dl)
Immunized with 25 µg	61.285 ± 8.65 (P>0.05)	93.89 ±22.65 (P>0.05)	37.25 ±5.73 (P>0.05)
Immunized with 50 µg	51.7 ± 3.96 (P>0.05)	114.7 ± 8.2 (P>0.05)	31.1 ± 5.52 (P>0.05)
Immunized with 75 µg	59.89 ± 4.69 (P>0.05)	110.0 ± 14.84 (P>0.05)	36.36 ± 4.25 (P>0.05)
Immunized with 100 µg	66.53 ±9.62 (P>0.05)	121.0 ± 10.54 (P>0.05)	33.25 ± 3.9 (P>0.05)
Immunized with adjuvant	39.02 ± 7.77	83.5 ± 1.41	33.55 ± 7.24
Negative control	46.79 ± 0.495	83.46 ± 8.15	33.31 ± 0.93

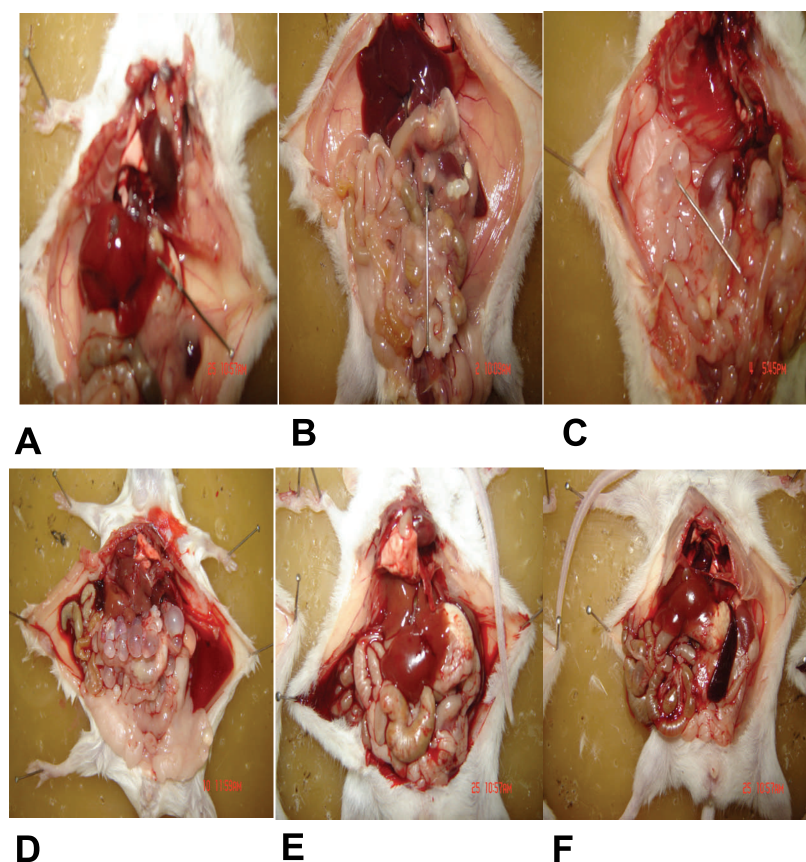

Figure 6.A-E: Immunized mice 90 days post infection. **F:** Mouse of control positive group 120 days post infection.

DISCUSSION:

The life cycle of *E. granulosus* includes two hosts an intermediate hosts and definitive hosts. Effective cystic echinococcosis control programs show that prevention of transmission to either host can reduce or even eliminate the infection in human and livestock populations [13]. In the present study partially purified GST was used at different doses as the primary immunogen, since it has been suggested that a systemic immune response may be induced by administration of large doses of antigen [17]. However, this antigen was highly immunogenic with doses of 50 µg and 75 µg. Demonstration of considerable increase in the protection rate in mice groups immunized with 50 µg and 75 µg of the antigen could be explained by the fact that antigen concentration (dose) and its factors that determine the efficiency of the vaccine as that indicated by studies with a variety of experimental models [14,20, 33,34]. Previous studies have shown that GST participated in detoxifying the exogenous toxins. The *E. granulosus* protoscoleces GST, like other cytosolic GST of helminthes, may involve in catalyzing conjugation of glutathione to electrophilic compounds [22,26]. Studying of vaccine potential of native or recombinant parasite glutathione S transferase had been adopted by several authours. Rathaur *et al*[19] have shown that vaccination of jirds with glutathione S transferase purified from *Setaria* cervi (nematode-filaria) against lymphatic filariasis resulted in significant (82.75%) reduction in adult parasite burden 90 days post Brugia malayi challenge infection. On the other hand vaccination of sheep with native glutathione S transferase

significant reduction in adult *Fasciola hepatica* burden (78 %) with significant antibody response [28]. Narin *et al* [20] have shown that vaccination of mice with recombinant *Fasciola gigantica* GST conferred protection rate ranged between 77-84 %. Apart from reducing worm burden, *Schistosoma mansoni* 28 KDa GST vaccine formulation was also significantly reduced female fecundity and fecal egg out put in a primate models [35]. Experimental trial with hydatid elements to prevent hydatid infection have been attempted since thirties of the last century [36]. Recently the results of three vaccine trials in Australia and Argentina have shown that vaccination of sheep with Eg95 (95 KDa protein isolated from *E. granulosus* onchosphere) conferred considerable protection (86 %) against challenge infection [37]. On the other hand immunization of mice with recombinant P-29 protein conferred significant protection (96.6%) against experimental hydatid infection [34]. Al-Sakee [38] revealed that immunization of mice with 60 µg and 90 µg of heat shock protein 70 which purified from protoscoleces of *E. granulosus*, conferred absolute protection approximately 90 days post challenge infection. In the present study the results showed that, the liver is the predilection site of occurrence of secondary hydatid cysts with the peritoneal cavity being the next. These findings were in agreement with that obtained by previous studies involved experimental models [32, 38,39,40,41]. However, disagreed with studies involved naturally infected herbivores [4,42,43,44,45] and human [5,6,46,47], who revealed that lung is the second target of infection after the liver. Obviously the exact mechanisms governing the predilection sites of infection are as yet not clear. However, the higher rate of hepatic involvement could be attributed to the fact that, liver acts as primary filter in the body [5,48,49]. In the present study the total leukocyte count was non-significantly altered in all immunized groups when compared with mice of control negative invaded pathogens and from foreign substances that might be harmful to the body. Non- significant differences in the total leukocyte count may be attributed to the fact that the time elapsed since blood collection was performed four weeks after last vaccination, which might be allowed the leukocyte number to return to its normal state [38]. However, the lymphocyte transformation response was significantly increased in all immunized groups when compared with those mice of control negative groups. Recognition of T-dependent antigens by specific clones of T-lymphocytes, which previously sensitized with that antigen, induces earlier and stronger activation of sensitized lymphocytes (a feature of secondary immune response) [50]. Assessment of liver and renal functions revealed that native *E. granulosus* glutathione S transferase as vaccine candidate has no-pathological side effects on the liver and kidney of the mice. The present study suggested that, subcutaneous injection of 75 µg of partially purified protoscoleces glutathione S transferase confer considerable protection against hydatid infection in BALB/c mice. Further studies to asses the effectiveness of native and/or recombinant protoscoleces glutathione S transferase as vaccine candidate in other intermediate hosts such as sheep and against challenge infection with viable *E. granulosus* eggs or onchospheres are recommended.

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