BALB/c Mice Immunity to Hydatidosis Induced by *In-vitro* Reared Echinococcus granulosus Adult Worm Antigens

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ABSTRACT

Background: Echinococcosis is a zoonotic parasitic disease caused by the larval stage of *Echinococcus granulosus*. Several native and recombinant antigens, derived from different stages of *E. granulosus* life cycle, have been used for vaccine trials. *In vitro* reared adult worms are good candidates for vaccination as they do not produce fertile egg/s and do not have any risk of contamination for researchers. **Objective:** To evaluate different antigens derived from *in vitro* reared *E. granulosus* adult worms for the immunization of BALB/c mice against secondary hydatidosis. **Methods:** Viable protoscoleces (PCSs) of sheep hydatid cyst were cultivated in S.10E.H media. Excretory secretory (E/S) and crude antigens were prepared from reared adult worms. A total of fifty BALB/c mice, each 8-weeks-old, were divided into 5 groups of 10 mice. Three groups were subcutaneously immunized with crude, E/S and immunodominant antigens on days 1 and 28. The fourth group received only PBS and the fifth group had no injection. Three weeks following the second immunization, all groups were challenged, intraperitoneal, with viable PSCs. After the autopsy of the mice and opening their abdominal wall, cysts were counted and measured followed by histopathological observations. **Results:** The highest protective immunity (98.7%) against hydatidosis was induced by crude antigen, followed by E/S and immunodominant antigens. **Conclusion:** Antigens (crude antigens in particular) derived from *in vitro* reared *E. granulosus* adult worms, and their different protein components are suitable candidates for the vaccination of intermediate hosts against hydatidosis.

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Keywords: ***In vitro***, ***Echinococcus granulosus***, **Protective immunity, Hydatidosis, BALB/c**

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INTRODUCTION

Cystic echinococcosis (CE), one of the most important zoonotic parasitic diseases in the world, including the Middle East (1,2), is triggered by the metacestode stage of *Echinococcus granulosus* that lives in humans and a number of ruminants. Adult worms live in the small intestine of canids as definitive hosts with a high prevalence in the world (1,3). This parasite is particularly important in terms of clinical and economic issues (4). Diagnosis is a big problem in CE-endemic areas (5-7), as different human organs may be infected by CE, the treatment is very complicated and often surgery is required for the removal of the cyst from the infected organs (8). In order to prevent secondary cyst formation, the surgeons usually use protoscolicidal agents prior to operation to kill the protoscoleces, in case of the cyst/s are accidentally ruptured during surgery (9,10). Control programs have been implemented in several endemic areas to reduce or eliminate CE as a public health problem. The application of control instructions has reduced the incidence rate of CE in humans as well as other intermediate hosts. Furthermore, the prevalence of adult worms has also been decreased in canids with various control trails, including animal vaccination (11). Livestock or definitive host vaccination is an effective way of breaking the life cycle of the parasite, conducive to protection against infection. Vaccination of dogs with soluble native proteins isolated from PSCs of *E. granulosus* recognizably suppressed worm growth and egg production (12). The Antigens belonging to different stages of the parasite life cycle, especially oncosphere-related antigens, were evaluated in vaccination trials in intermediate hosts (11). To protect sheep, goats, and bovines against hydatid disease a vaccine was prepared as a recombinant fusion protein expressed in *Escherichia coli* (13).

Certain recombinant vaccines, originally prepared from oncosphere, are highly effective against infection with *Taenia ovis* in sheep, *Taenia saginata* in cattle, *Taenia solium* in pigs and *E. granulosus* in livestock (14). Eg95, as a recombinant vaccine have been successfully employed in vaccination trials in Australia, Argentina and Iran, against hydatidosis in sheep (11,15). Moreover, native antigens prepared from other stages of *E. granulosus* containing egg, homogenate protoscoleces (HPSCs), hydatid cyst fluid (HCF) and antigen B have been used in different vaccination trials (16). Certain studies have demonstrated that Th2 immune responses are dominant in definitive and intermediate hosts (17,18). These responses conduce to the establishment of parasite in their hosts. It has been documented that the vaccination of definitive and intermediate hosts induces Th1 immune responses that preclude the establishment of the worms (19). The crude antigens obtained from dog adult worms were successfully applied for the protection of BALB/c mice against secondary hydatidosis (20). These adult worms, on the other hand, can produce fertile eggs, thereby increasing human contamination. The Excretory/secretory (E/S) components of *E. granulosus* adult worms have not yet been utilized in vaccination trials in intermediate hosts. Since *in vitro* reared *E. granulosus* adult worms do not produce fertile egg/s in culture media (21,22). They carry no risk of contamination for researchers. Nonetheless, the immunization of BALB/c mice with crude and E/S antigens prepared from *in vitro* reared worms induce a dominant Th1 immune responses, producing high levels of IgG antibody (23). These antigens can be recommended as vaccine candidate antigens in intermediate hosts. Considering the aforementioned points, the current study aimed at utilizing different (crude, E/S, and

immunodominant) antigens of *in vitro* reared *E. granulosus* adult worms so as to immunize BALB/c mice against secondary hydatidosis.

MATERIALS AND METHODS

Production of *in vitro* reared worms by the cultivation of protoscoleces. *E. granulosus* adult worms were prepared through the cultivation of sheep live PSCs (G1 strain) in biphasic S.10E.H medium culture as previously described (24). Briefly, *in vitro* reared adult worms were harvested from cultivation media 60 days following the cultivation of PSCs (Figure 1).

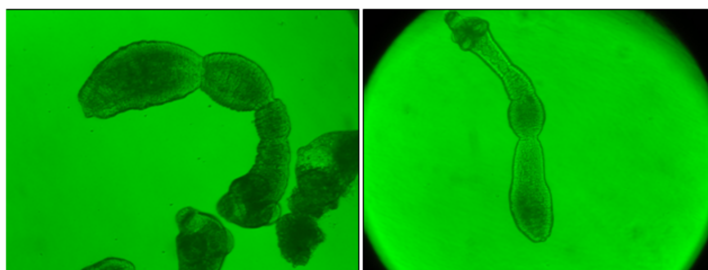

Figure 1. *In vitro* reared *Echinococcus granulosus* adult worm.

Preparation of different antigens. *In vitro* reared adult worms were harvested from cultivation media 60 days post cultivation (Figure 1). The excretory-secretory, crude and immunodominant (nearly 28 kDa) antigens were prepared as previously described (23). Briefly, to prepare immunodominant antigen, the crude antigens of *in vitro* reared worms were separated by SDS-PAGE and screened with a panel of human sera and healthy control. Contrary to the healthy subjects, the subunit which was presented in the immunoblotting of all CE patients was considered as the immunodominant antigen. The antigen was extracted from polyacrylamide gels using elution buffer. In order to prepare E/S antigen, the *in vitro* reared adult worms were transferred to the protein free media and incubated at 37°C in CO2 (5%). The supernatant was collected and dialyzed every 12 hours.

Immunization of BALB/c mice. In order to evaluate the immunogenicity of the prepared antigens, five groups (Positive control group: Pos.C, Adjuvant plus PBS group: Adj, Crude antigens of reared adult *Echinoccoccus granulosus* group: Crude Ag, Excretory/Secretory antigen group: E/S Ag. and Immuno dominant antigen group: ID Ag.) of eight-months-old inbred BALB/c mice (n=10) were selected. On days 1 and 28, three groups were subcutaneously injected with 100 µgr of crude, E/S or immunodominant antigens containing equal volumes of Freund's adjuvant (Sigma, USA). Freund's complete and incomplete adjuvants were respectively employed in the

first and subsequent injections. The fourth group of BALB/c mice received Freund's adjuvant plus PBS while group five underwent no injection and was considered as the positive control group.

Challenging immunized BALB/c mice with PSCs. Two weeks after the second immunization, all groups of BALB/c mice were intra-peritoneally challenged with 2000 of live PSCs in 300 µl of sterile PBS. The PSCs were extracted from sheep hydatid cyst while their viability was over 85%. The animal health was carefully checked and recorded for 8 months post challenge.

Determination of infection rate in BALB/c mice. All groups of mice were euthanized 8 months post infection through an overdose of ether. The animal peritoneal cavity was observed for the possible presence of hydatid cysts. The established cysts were removed from the peritoneal cavity in all groups of mice and their size (in millimeter) and number were recorded. Cyst load was determined by multiplying the size of cysts by the number of cysts in different groups of mice (19).

Histopathological evaluation of cyst layers. All cyst samples were fixed in buffered formalin (10%), embedded into paraffin blocks, cut into different sections (5µm) and stained with hematoxylin and eosin (H&E). All histopathological slides were ultimately evaluated microscopically, using 1000 x magnification.

Statistical analysis. The mean size and number of cysts in different groups of mice were statistically analyzed using SPSS (version 16) for windows (SPSS Inc., Chicago, IL, USA). Kruskal-Wallis test (non-parametric independent group comparisons) was used to compare the mean values of the size and number of the cysts among the groups. Mann-Whitney test was carried out so as to compare the data in paired groups.

RESULTS

The size and number of the established cysts in different groups.

All groups were euthanized 8 months post challenge. Different cysts were observed in all PSCs-injected mice including positive control, those immunized with immunodominant antigen and mice which received Freund's adjuvant plus PBS (Figure 2).

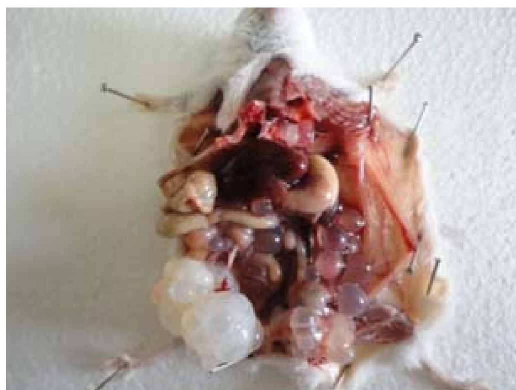

Figure 2. Hydatid cysts in peritoneal cavity of mouse in positive control group.

No cyst was observed in 80% (8 mice) of the mice immunized with *in vitro* reared worms' crude antigens (Figure 3), whereas only 90% (one mouse) of the mice immunized with E/S antigen were stricken with cysts.

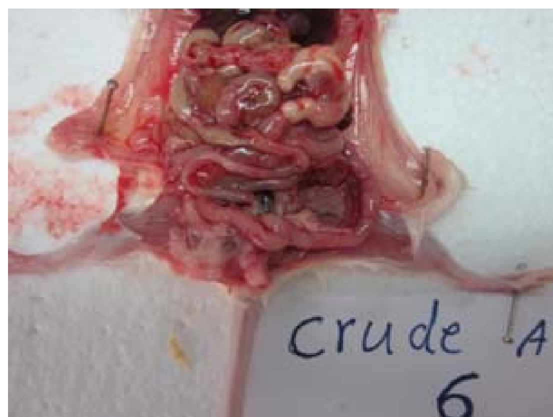

Figure 3. Immunized mouse with crude antigens eight months post challenge.

The highest (128.3 ± 34.92) and the lowest (1.58 ± 3.7) mean number of cysts was respectively observed in the control positive mice group and the mice immunized with crude antigens of reared *E. granulosus* adult worms. The average numbers of cysts were 12.5 ± 11.53 and 30.43 ± 9.46 in mice immunized with E/S and immunodominant antigens obtained from reared adult worms, respectively.
The mean number of cysts was 87.89 ± 22.88 in mice receiving Freund's adjuvant plus PBS. Kruskal-Wallis test indicated statistically significant differences among the five groups (except between Freund's adjuvant plus PBS group and the positive control (P=0.09)) in so far as the mean number of cysts is concerned (P<0.001). Moreover, Mann-Whitney test showed no statistically significant difference in the mean number of cysts between mice which received Freund's adjuvant plus PBS and positive control (P=0.09). The mean numbers of cysts in the mice groups are shown in Figure 4.

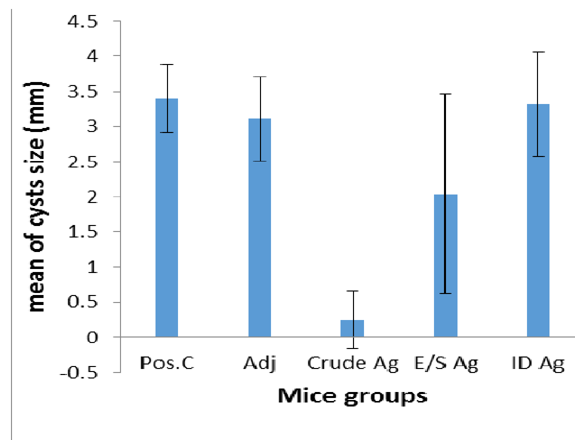

Figure 4. Mean size of cysts in different groups of mice eight months post challenge.

The highest mean size of cysts (3.4 ± 0.48) in millimeter (mm) was seen in positive control group of mice. The lowest mean size of cysts (0.25 ± 0.6mm) was seen in mice immunized with crude antigens of reared *E. granulosus* adult worms. The mean size of cysts was 2.04 ± 1.51 mm and 3.23 ± 0.74 mm in mice immunized with E/S and immunodominant antigens, respectively. The mean size of cysts was 3.11 ± 0.59 in mice which received Freund's adjuvant plus PBS. Figure 5 shows the mean size of cysts in different mice groups. Kruskal-Wallis test shows statistically significant difference in mean size of cysts in five different groups of mice (P<0.001). Moreover, Mann-Whitney test showed no statistically significant differences in mean size of cysts in positive control and mice which received Freund's adjuvant plus PBS (P=0.221) as well as mice immunized with immunodominant antigens (P=0.770). Also, no statistically significant difference was observed in the number of cysts between the mice immunized with immunodominant antigen and mice which received Freund' s adjuvant plus PBS (P=0.711).

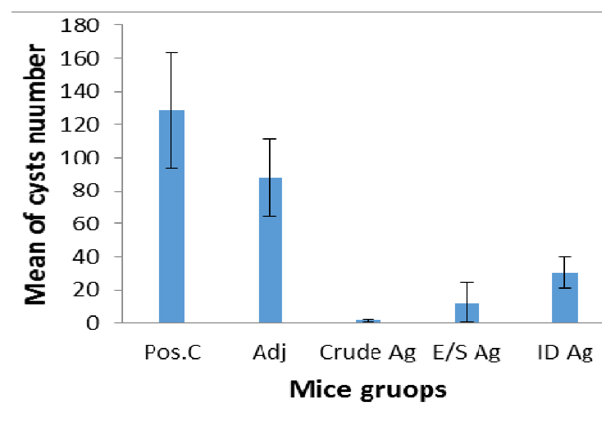

Figure 5. Mean number of cysts in different groups of mice eight months post challenge.

Infection rate of BALB/c mice (Cyst load).
Cyst load index was calculated for each group by multiplying the mean number of cysts by the mean size of the cysts in millimeters. In this regard, the highest (436.22) and the lowest (0.395) cyst loads were respectively seen in positive control and crude antigens groups. Figure 6 shows the cyst load in different mice groups.

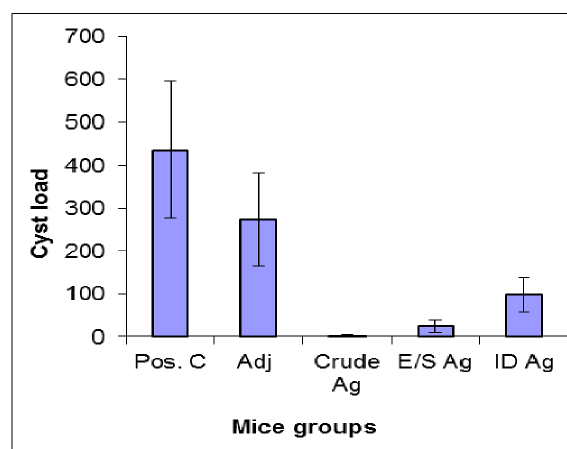

Figure 6. Cyst load in different groups of mice.

Protective immunity index.
Protective immunity index was calculated based on the following equation.

$$\%\text{Protective immunity} = 1 - \frac{\text{Average of cysts in test group}}{\text{Average of cysts in control group}} \times 100$$

Considering the number of cysts, the highest protective immunity (98.7%) belonged to the mice immunized with *in vitro* reared *E. granulosus* adult worms' crude antigens; the protective immunity was 90%, 76.2% and 30% in mice immunized with E/S and immunodominant antigens and Freund's adjuvant plus PBS.

Confirmation of cysts as hydatid cyst.
The laminated and germinal layers of the hydatid cysts were observed in the histopathological sections of the mice (Figure 7). No PSCs were observed in cyst sections.

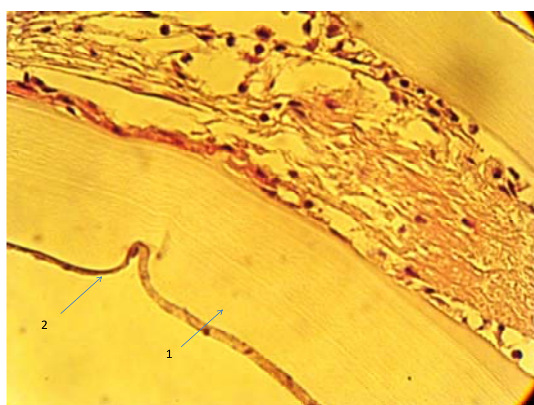

Figure 7. Laminated layer (1) and germinal layer (2) in histopathologic section.

DISCUSSION

The highest vaccine efficacies concerning cystic echinococcosis have been achieved by utilizing different recombinant oncosphere antigenic components, among which the Eg95 is considered as the most effective vaccine for protection against hydatidosis in sheep (11,14,26-28). A host of studies have also evaluated other antigens from different stages of *E. granulosus* life cycle containing HCF, homogenate PSC (HPSC), antigen B and egg (16,19).

In the previous study, we demonstrated that antigens of *in vitro* reared *E. granulosus* are appropriate for application in vaccine trials (23). These antigens, especially the crude ones have induced high levels of Th1 immune responses in BALB/c mice, which is necessary for protective immunity against secondary hydatidosis (23).

In the present study, for the first time, *in vitro* reared *E. granulosus* antigens were used in vaccination against CE in BALB/c mice. Given the previous studies, inbred BALB/c mice were considered as suitable experimental intermediate hosts for such investigation (32,33). The findings pointed to the fact that crude, E/S and immunodominant antigens of *in vitro* reared *E. granulosus* adult worms induce protective immunity against the secondary hydatidosis in 98.7%, 90% and 76.2% of the immunized mice, respectively. In line with this study, subcutaneous immunization of BALB/c mice with crude antigens prepared from intestinal *E. granulosus* adult worms created a high (100%) level of protective immunity (20). In another study, the subcutaneous immunization of lambs with the whole body homogenate protein of *E. granulosus* entailed a high level (90.9%) of protective immunity (31).

In the present research, the protective immunity of crude antigens of reared worms proved higher. Vaccination of sheep with Eg95 recombinant protein induced a very high level of protective immunity (96-100%) against hydatidosis (11,15,32).

Immunization of BALB/c mice with antigen B, crude sheep hydatid fluid (CSHF) and protoscoleces homogenate (PSH) antigens generated 98.3%, 79% and 71% protective immunity, respectively (19). Such results corroborate the fact that the establishment of protective immunity by antigen B is almost equal to the immunity induced by crude antigens (98.7%) in the present study. Our results showed that E/S and

immunodominant antigens were more effective compared with CSHF and PSH in as far as inducing a protective immunity is concerned (19).
Intramuscular immunization of buffaloes with antigens derived from *E. granulosus* eggs and onchosphere resulted in 76.7% and 83.5% protective immunity, respectively (16). The acquired protective immunity in the foregoing study was less than that induced by crude and E/S antigens in the current study.
Different excretory/secretory protein components have been recognized in adult worm and PSC of *E. granulosus*. These components are important for the protection against and diagnosis of hydatidosis (33-36). An immunodominant protein component of *in vitro* reared *E. granulosus* adult worm (nearly 28 kDa) was detected by SDS-PAGE and western blotting in our previous study (23).
Subcutaneous immunization of BALB/c mice using 100 µg of this antigen induced 76.2 % immunity. It can, therefore, be postulated that utilizing higher doses of this antigen may induce higher levels of protective immunity. Eg95, an appropriate vaccine candidate, also has a 23 and 25 kDa component (26).
In the current study, the reduction of cyst numbers in the mice group receiving adjuvant plus PBS compared with the positive control group might be attributed to the augment in the number of monocytes and macrophages in the early stage of the challenge.
Taken together, utilizing *E. granulosus* adult worms originated from dog intestine which contains fertile eggs may ensue the risk of contamination for researchers. *In vitro* reared *E. granulosus* adult worms, however, do not produce fertile egg/s and are suitable materials for research. Our findings indicated that the components of *in vitro* reared worms can induce an acceptable protection in BALB/c mice against secondary hydatidosis.

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