Journal of Zoonotic Diseases, 2020, 4 (4): 36-50 **doi:10.22034/JZD.2020.11347**
https://jzd.tabrizu.ac.ir/article_11347.html

Original Article

Th1/Th2/Th17 pattern in pregnant mice inoculated with live *Echinococcus granulosus* Protoscolex

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(Received 2 August 2020, Accepted 30 Sept 2020)

Summary

The immune response to *Echinococcus granulosus* s.l. hydatidosis in an intermediate host is complicated. A T-helper-2 response can support parasite establishment, which is exacerbated by the pregnancy, whereas a T-helper-1 response would be destructive for the parasite. Thus, the present study was aimed to investigate the effects of pregnancy on the serum levels of some cytokines during parasite inoculation. Twenty Balb/c mice were divided randomly into four groups including: A) control group, B) healthy pregnant, C) pregnant inoculated with *granulosus* s.l. protoscolices, and D) non-pregnant inoculated with protoscolices. Subsequently, the Serum concentrations of tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin -4 (IL-4) and interleukin -17 (IL-17) were determined during four weeks by ELISA method. At 7th-day post-infection (d.p.i), the levels of cytokines increased in all groups, but there were no significant differences between increased levels. At 14 d.p.i, the levels of cytokines were nearly similar to the first week, except for IFN-γ and TNF-α, which their levels were significantly higher in group D than the other groups ($p < 0.05$). At 21 d.p.i, the levels of IL-17, TNF-α, and IFN-γ cytokines were significantly higher in the D group than the others ($p < 0/05$), but IL-4level was significantly higher in the C group than the other group ($p < 0/05$). At 28 d.p.i. level of IL-4 was significantly higher in the C group than the others ($p < 0/05$). Considering that pregnancy can increase the level of cytokine related to Th2, it can effectively survive of the *E. granulosus* parasite.

***Keywords*:** Cytokines, Hydatid cyst, Pregnancy, T helper, Th response.

Introduction

It has been shown that T and B cells are the major players of the immune system, forming innate and adaptive immune systems (Paciullo et al., 2017). Helper-T-lymphocytes (Th cells) are subtypes of CD_4^+, which are divided into four categories according to their cytokine profile: Th1, Th2, T-regulatory and Th17 cells. Tumor necrosis factor-α (TNF-α), interleukin-

2 (IL-2), and IL-12 as well as interferon-γ (IFN-γ) are produced by Th1 cells, consequently resulting in cell-mediated immunity, whereas Th2 cells contribute to the production of IL-4, IL-5, IL-6, IL-10, and IL-13 to stimulate humoral immunity. Th17 cells, as potential pro-inflammatory cells, contribute to the secretion of IL-21, IL-22, and IL-10. Notably, Th17 cells are able to convert into Th1 cells (Muranski et al., 2013). For a host to establish immunity against parasites and other invading insults, Th cells trigger the production of some responses such as IFN-γ, leading to the removal of infection (von der Weid and Langhorne, 1993). However, the story is a bit different about the role of Th2 cells. It has been demonstrated that Th2-related cytokines such as IL-4, IL-5, and IL-6 are in favor in terms of the establishment of *Fasciola gigantica* infection and evasion. Notably, cytokines present a distinct profile during pregnancy (Sheng et al., 2019). In contrast to Th2 cytokines, Th1 cytokines lead to adverse impacts on the pregnancy and may alter the outcome of pregnancy (Zhang et al., 2015). Abortion has been observed due to the increased concentrations of Th1 cytokines (Zhang et al., 2015). Furthermore, different data demonstrate the potential role of increased levels of Th17 cells in the stimulation of pregnancy disorders such as preeclampsia, mental illness, recurrent spontaneous abortion, and so on (Tesmer et al., 2008).

Hydatid disease (HD), or cystic echinococcosis, is a globally distributed zoonotic disease caused by *Echinococcus granulosus* sensu lato complex (Craig et al., 2017; Thompson, 2017). During the recent decade, much attention has been made towards controlling this zoonotic disease, which is widely due to huge cost burden on nations, so that World Health Organization (WHO) has estimated a cost as much as $3 billion to control this infectious disease (Solomon et al., 2017; Harandi et al., 2012). Interestingly, the hydatid cyst of *E. granulosus* has sustainability as high as 50 years, resulting from two phenomena: passive escape and immunomodulation (Mihmanli et al., 2016). So, this parasite can be hidden from immune response, and also decreasing the effect of host responses. Canine definitive hosts and intermediate hosts such as sheep, cattle and camel involve in maintaining *E. granulosus* in our surrounding environment (Banks et al., 2006). The adult *E. granulosus* resides through the small intestine of the definitive host. Gravid proglottids release eggs in the feces, which are immediately infectious. After ingestion of the eggs by a suitable intermediate host, they hatch in the small intestine and then, release six-hooked oncospheres, which interpenetrate in the wall of the small intestinal

and migrate through the circulatory system into different organs, particularly the liver and lungs. In the affected organs, the oncosphere develops into a thick-walled hydatid cyst that expands progressively, producing protoscolices (PSC) and daughter cysts that fill the cyst interior. The cyst-containing organs of the infected intermediate host can infect the definitive host. After ingestion, the protoscolices release, attach to the intestinal mucosa, and develop into adult form pending 32 to 80 days (please insert the reference).

Humans are known as the aberrant intermediate hosts who become infected by the ingestion of the eggs. As expected, oncospheres are released in the small intestine, and hydatid cysts develop in the various organs. If cysts rupture, the released protoscolices may produce secondary cysts in other organs (secondary echinococcosis) (Moro et al., 2009).

In the present study, we provided a pregnant mouse model *E. granulosus* infection to investigate the alteration in levels of Th1/Th2/Th17-related cytokines.

Materials and methods

Animal source*:* 20 Balb/c mice were purchased from the laboratory animals rearing center of Pasteur Institute of Iran and divided into four groups, randomly, including A) control group; B) healthy pregnant group; C) pregnant group infected with PSCs; and D) non-pregnant group infected with PSCs. Each group of mice was kept in isolated cages and a clean room with a constant temperature of 25°C and a 12-hour cycle of light and darkness (L:D) and had access to adequate food and water. This study has been approved at the University of Tabriz ethics committee (Ref No. IR.TABRIZU.REC.1398.004).

PSCs preparation and viability assessment: In the fall of 2018, the fresh cysts of hydatid were provided from the naturally infected livers of sheep slaughtered in Tabriz industrial abattoir, East Azerbaijan province, Iran, and transferred to the laboratory of Parasitology at the Faculty of Veterinary Medicine, University of Tabriz. Initially, the hydatid cysts were surface disinfected twice with ethanol (70%). Then, cyst fluid was aspirated from each of the cysts by a 50 ml syringe, transferred into glass tubes and centrifuged (1500 rpm/3 min). The supernatant was elegantly discharged, and the deposited PSCs were washed with PBS solution (pH 7.2) several times. To determine the density of PSCs per ml, five microliters of the mixture containing PSCs were transferred on a microscopic slide, covered with a coverslip, and the prepared slide was then examined under a light microscope (40x). This step was performed in triplicates, and the mean

was recorded as PSCs density/mL. Finally, the PSCs density was adjusted up to 500 PSCs/mL in 0.9% saline normal over 90% viability for inoculation to mice. Also, to evaluate the viability of PSCs, by using Smyth and Barrett's method (Smyth and Barrett., 1980), 20 µl of PSCs mixture was mixed with an equal volume of eosin (Sigma-Aldrich, St. Louis, MO, USA) stain 0.1% (powder of eosin (1 g) in 1 L of distilled water) in a clean microtube. After 15 minutes of exposure to the eosin staining, the colorless PSCs (Fig. 1a) under the light microscope were considered as live while the colored red (Fig. 1b) ones were considered as dead. The viability percentage of PSCs was calculated by dividing the number of live PSCs to the total number of calculated ones by 100. This stage was also performed in triplicates (Mahmoudvand et al., 2017). To ensure, some mixture containing PSCs was heated (60°/30 min) in an oven (Fan Azma Gostar, Karaj, Iran) to kill the PSCs, and then stained with eosin 1% the same above mentioned.

Preparation of animal model: To induce *E. granulosus* infection, 500 PSCs (Yumin et al., 2019, Valizadeh et al., 2017) was injected into each mouse (mice in the groups of C and D) intraperitoneally (i.p.). The control group received only normal saline i.p. One week before PSCs induction, the male and female mice were kept together to adapt and provide pregnancy. The pregnancy was recognized through the pregnancy plaques formation in the vagina 10 days after keeping together.

Evaluation of cytokine levels: At 7, 14, 21, and 28 d.p.i., the blood samples were taken from the tail vein, and the TNF-α, IL-4, IFN-γ, and IL-17 levels were determined using commercial kits according to the manufacturer's instructions Bender Med Co. Austria; R&D Systems, the United Kingdom). Briefly, each of the cytokines was individually assayed in a sandwich ELISA method, which described simultaneously due to the similarity of the work process. Two wells were considered for each sample, one pair of the cells as blank controls and seven pairs of cells as standard samples. Initially, the plate wells were washed twice with a buffer, and each time with a volume of approximately 300 µl before returning and discharging the content of the plates, it was given for 10 to 15 s, and then extra water of the plate was taken by a tissue. Standard dilutions with the assay of each cytokine were provided in wells. For this purpose, 225 µl of sample diluents were added to all seven pairs of wells. 225 µl from the cytokine standard stock solution was added to each of the first wells, and mixed completely with the contents of the wells several times. Then 225 µl of this mixture was added to the

second well and the work proceeded in the same way; eventually 225 µl of this mixture discarded from the seventh wells. Test samples (conditioned media of spleen cells culture) were added to the wells at 50 µl. Following this, 50 µl of the dilution buffer was added to each of these wells and mixed with the test sample. 100 µl of the dilution buffer was added to the blank wells. In the next step, 50 µl of secondary antibody conjugated with biotin (x1) was added. Then the plates were covered and incubated at room temperature for 2 hours. Then, the plates were washed with washing buffer for three times, as in the first step. 100 µl of Streptavidin to the HRP (1x) solution were added to all wells and, after covering the plate surface, were incubated at room temperature for an hour. Similar to the first step, the plates were washed three times, and 100 µl of the HRP enzyme-substrate (TMB) was immediately added. Once again, the surface of the plates was covered and incubated at room temperature for about 10 minutes. The intensity of the color created at the 620 nm wavelength was evaluated by ELISA and, as soon as standard No. 1 showed an optical absorption of 0.9 to 0.95, the reaction stopped by adding 100 µl of 1M phosphoric acid, and plates were immediately read by an ELISA reader with 450 nm wavelength. The average OD was calculated, and the standard curve was plotted on logarithmic paper. By using the standard curve, the amount of each of the cytokines in the sample was determined as pg/ml, and the standard curve was depicted between 15.7-2000 pg/ml. The sensitivity of the test was 4.0 pg/ml for IL-17 and IL-4 and 1.4 pg/ml for TNF-α and 6.3 pg/ml for IFN-γ, according to the manufacturer. The criterion for ensuring work accuracy is a lack of difference of more than 20% of each sample with the average calculated. It should be noted that since the test sample is diluted in a 1 to 2 ratio in the third step, the final concentration of cytokines is determined by doubling the numbers obtained from the standard curve. The results from five subjects were expressed as a mean ± SEM (the standard error of the mean).

Data analysis: Statistical analysis was performed using SPSS software version 19 and ANOVA test and *t*-test with $P< 0.05$ as a significant level. All data were expressed as mean ± SEM.

Results

The levels of cytokines at 14 d.p.i: After *E. granulosus* PSCs induction, the levels of TNF-α, IFN-γ, IL-4, and IL-17 increased in the C and D groups compared to the A and B groups, but the differences were not significant ($P < 0.05$) (Fig. 2).

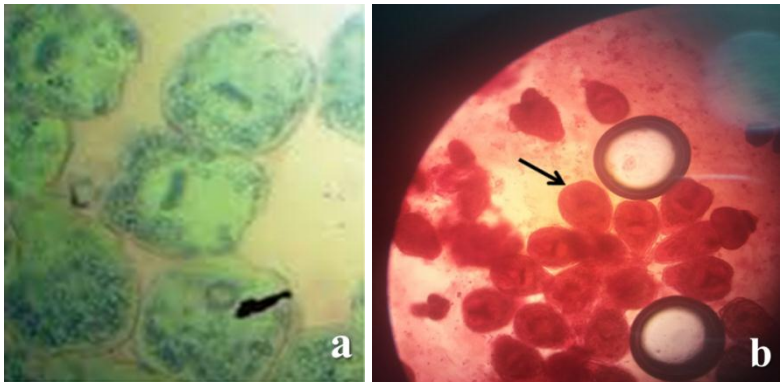

Fig. 1. The live colorless protoscolices (**a**) and the colored red killed protoscolices (**b**) after exposure to eosin 1%.

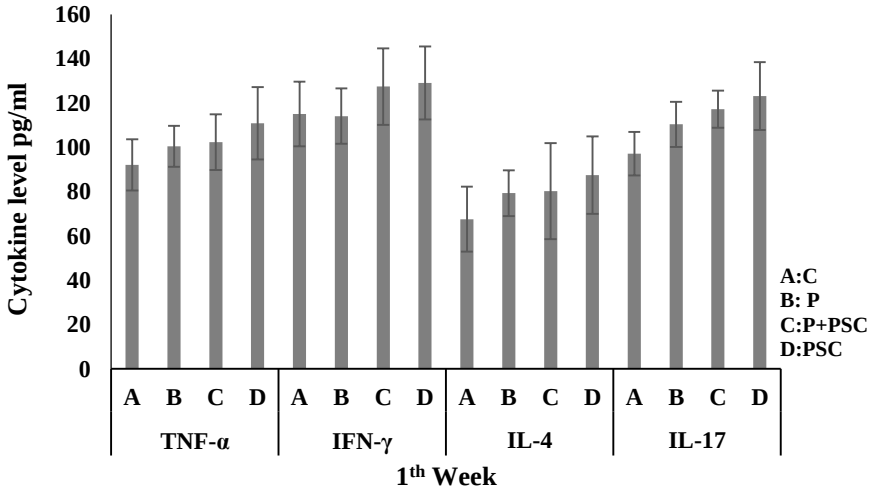

Fig. 2. The levels of cytokines in different groups in the first-week post-experiment (PE). The values are shown as mean ± SEM ($P < 0.05$).

The cytokines levels during the second week: TNF-α, IL-17, and IFN-γ serum levels in group D, remarkably increased compared to the others, but the difference concerning IL-17 was not significant ($P < 0.05$). Notably, in the second week, the level of IL-4 in group C was more than group D but the difference was not significant (Fig 3). Also, IFN-γ and TNF-α serum levels in group C were higher than group B, but the differences were not significant.

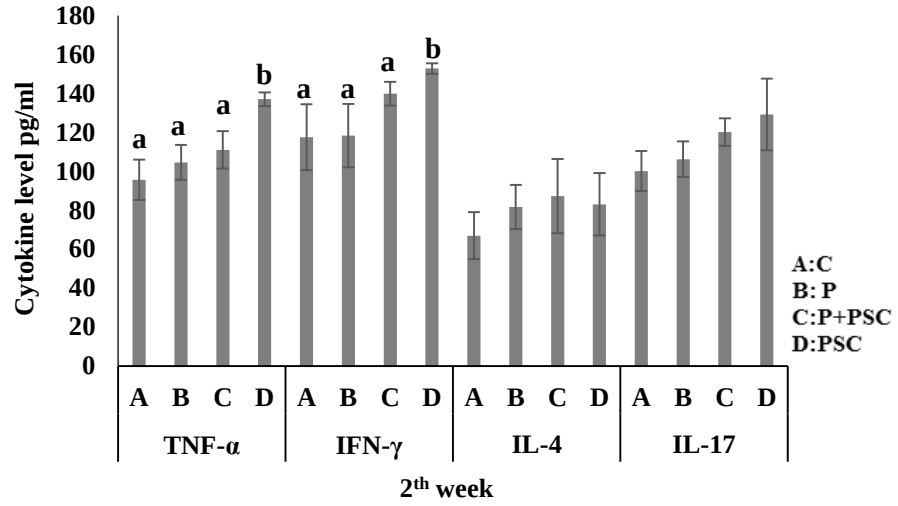

Fig. 3. The levels of cytokines in different groups in the second week PE. The values are shown as mean ± SEM. Different letters show significant differences between groups ($P < 0.05$).

The cytokines levels during the third week: At the third week, TNF-α, IL-17 and IFN-γ serum levels were remarkably increased in group D compared with the other groups ($P < 0.05$). Besides, similar to the second week, the level of IL-4 was higher in group C compared to with others ($P < 0.05$) (Fig 4). Moreover, TNF-α, IL-17, and IFN-γ serum levels in group C were increased compared with group B, but the difference was not significant.

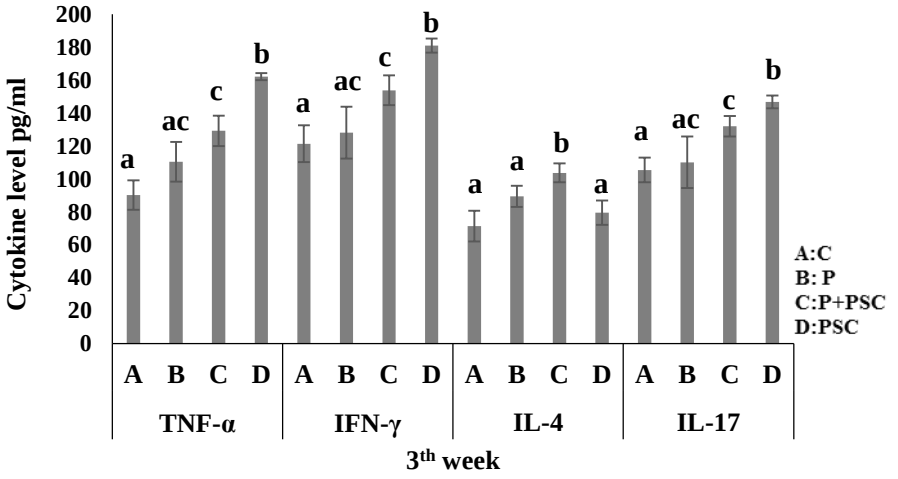

Fig. 4. The levels of cytokines in different groups in the third week PE. The values are shown as mean ± SEM. Different letters indicate significant differences between groups ($P < 0.05$).

The levels of cytokines during the fourth week: In the fourth week, there was a trend like other weeks, and the levels of cytokines significantly increased. Furthermore, similar to the second and third weeks, the level of IL-4 in the fourth week was higher in group C compared with group D (Fig 5).

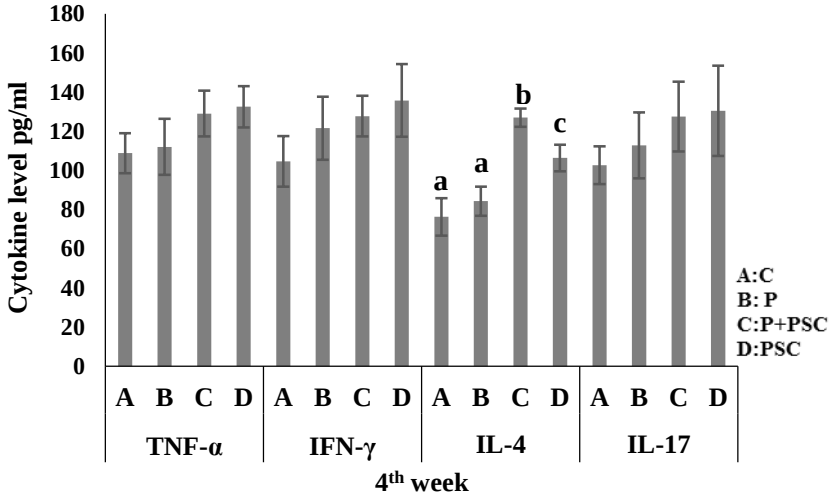

Fig. 5. The levels of cytokines in different groups in the fourth week PE. The values are shown as mean ± SEM. Different letters indicate significant differences between groups ($P < 0.05$).

Discussion

We observed in this study that at 7th-day d.p.i, the levels of cytokines increased in all groups, but there were no significant differences between increased levels. At 14 d.p.i, the levels of cytokines were nearly similar to the first week, except for interferon -γ and tumor necrosis factor -α, which their levels were significantly higher in group D than the other groups ($p < 0.04$). At 21 d.p.i, the levels of interleukin -17, tumor necrosis factor –α, and interferon -γ cytokines were significantly higher in the D group than the others ($p < 0.05$), but interleukin -4 level was significantly higher in the C group than the other group ($p < 0.05$). At 28 d.p.i. level of interleukin -4 was significantly higher in the C group than the others ($p < 0.05$).

During pregnancy, the mother's immunity activities are suppressed to prevent abortion, which is immunologically allograft. Since allograft rejection is mainly occurred due to cellular immunity, physiological immunity suppression during pregnancy may be due to Th1 suppression with the increase of Th2 (Shimaoka et al., 2000).

The findings of the present research on group B mice, which were pregnant mice, showed that a series of changes in the level of cytokines and the response to the immune system during pregnancy occurred in the mother's body compared to the control group. The results of this study in group B showed an increase in the level of all four cytokines i.e., TNFα, IFN-γ, IL-17, IL-4, every four weeks after the experiment compared with the control group, but these changes were not significant.

Different experiments have been conducted on changes in the level of cytokines associated with Th1 and Th2, which show different and sometimes various results. Despite the fact that all these different results in the ratio of Th1/Th2, the importance of mother immunity tolerance during pregnancy is undeniable. The mechanism of these changes is not fully understood. But, several pregnancy-related proteins are known to orient the immune response to Th2, including the leukemia inhibitor factor (Trowsdale et al., 2006), progesterone, progesterone-induced blocking factor (Szekeres-Bartho et al., 2001), and estradiol (Sykes et al., 2012). It should be noted that the regulation of Th1: Th2 ratio during pregnancy occurs through both suppression of Th1 and the increase of Th2 (Sykes et al., 2012). However, the immunity modulation from hormone changes must also be considered (which severe hormonal changes occur in the body during pregnancy) (Mpairwe et al., 2014). These immunological changes in pregnancy are based on the precise balance of immunity tolerance and immunity suppression. It has been suggested that this balance is caused by a suppression of the immune system by shifts from Th1 to Th2 response during pregnancy (Raghupathy et al., 2000). In a study supporting the theory of suppression of Th1 cytokines response during pregnancy (Keski-Nisula et al., 2003), it was shown that reduction of the levels of IFN-γ and TNF-α cytokines secreted by T helper cells occur during pregnancy (Sykes et al., 2012), that is in line with our results. Another study showed that the dominant cytokine profile was th2 in pregnant mice. (Wegmann et al., 1993).

The reason for these contradictory results may be due to the simplification of different observations during pregnancy. In several studies, pregnancy has been evaluated as a single event, while in reality, it has three distinct immunological stages characterized by distinct biological processes (Mor et al., 2008). As a result, pregnancy is an anti-inflammatory and pre-inflammatory condition that depends on the stage of pregnancy (Gil et al., 2010).

Therefore, the present study was performed on rate every four weeks after starting pregnancy, which showed an increase in the level of

TNFα, IFN-γ, IL-17, IL-4 cytokines in group B, which were pregnant mice without parasitic contamination compared to those in the control group. Regarding the increase of IL-4, it is concluded that pregnancy increases the Th2 response.

In the present study, in addition to group B, group C, which were pregnant mice induced with protoscoleces (PSCs) of E.*granulosus*, and group D, which were non-pregnant mice induced with PSCs of *E.granulosus*, were investigated. There were no significant results in the first week, but in the second week, IFN-γ and TNFα in the D group were significantly higher than the other groups. In the third week, the increase in levels of IFN-γ, TNFα, and IL-17 cytokines in group D was significantly higher than other groups and, in contrast, the increase in IL-4 cytokine levels in group C was significantly higher than group D. Finally, the results on the fourth week showed no significant difference between the four groups, except for the level of IL-4, which in the group C was significantly higher than the other groups.

It should be taken into account that the function of cytokines is quite complicated in host immune responses to *E. granulosus* infections. In addition, there are contradictory reports about the profile of cytokines secreted against hydatid cyst. These different responses depend on the difference in dosage of PSCs, the infection period, and the strain of the used experimental animals (Bayraktar et al., 2005; Dematteis et al., 2003).

Another point is that one of the characteristics of worm parasites infections is that they carry cytokines profile in the host body towards the response of Th2 (IL4, IL5, and IL10 production) (Baz et al., 2006). A study showed that after induction of PSCs, and at the time onset of infection, Th1 (INF-γ, TNFα, IL-2) cytokines increase, In contrast, Th2 (IL-4) cytokines decrease, but several weeks after infection, Th1 cytokines are reduced, and Th2 cytokines are increased, which can be the result of the activation of immunosuppressive cells (Rostami-Rad et al., 2018). In another study, intraperitoneal insemination of mice with PSCs of the parasite initially showed a dominant concentration of IFN-γ, IL-2 and IL-15 (Th1 cytokine), but then the concentration of IL-4, IL-5, IL-6, IL-10, and IL-13 (Th2 cytokine) increase (Mourglia-Ettlin et al., 2011), which was similar to the results of the present study in a parasite-induced non-pregnant group. Thus, the Th2 response to *E. granulosus* helps to escape Th1 protective immune responses (Baz et al., 2006). Of course, it should be noted that cell populations other than T helper cells can also secrete Th1 or Th2 cytokines, suggesting that the Th2

reaction in parasitic infections should not only be related to the induction of Th cell (Baz et al., 2006). In general, the Th1 toward the Th2 cytokine profile shift may be proposed as a cause of resistance to *E. granulosus* parasite infection (Rostami-Rad et al., 2018). In addition to the above, and as previously mentioned, in this study, in addition to the groups B and D mice, another group of mice that were PSCs-induced pregnant (C) have been investigated. The results of the first and second weeks showed an increase in TNFα, IFN-γ, IL-17, IL-4 cytokines compared to groups A and B. In the third week, IL-4 was more in group C than in other groups. In the fourth week, except that IL-4 was more in group C than other groups, there was no significant difference between them.

The worm infections regulate the host immune response through various mechanisms (van Riet et al., 2007), which leads to prolonged survival of the parasite in the host body (Mpairwe et al., 2014). In mammals other than humans, the increase of sensitivity to worm parasites during pregnancy is well-known, which the immune modulation caused by hormone changes and nutritional deficiencies are among the causes of this increase in sensitivity (Beasley et al., 2010). The studies in mice also show that pregnancy increases the damage caused by Schistosoma, which this is due to the reducing IFN -γ secreted against the parasite and the increase in IL-4 production, which is consistent with present results in the pregnant group (group B) (Farah et al., 2007).

Since pregnancy increases, the response of Th2 and Th2 is less effective in defense against the parasites, so it is effective in surviving the parasite in the body. In other studies on other parasitic infections such as *Malaria*, *Toxoplasma, Trypanosoma,* and *Schistosoma*, it has been concluded that such parasitic infections are exacerbated in the pregnant host (Diaz-Lujan et al., 2012). In this regard, in a study, the burden of parasitic contamination in the skeletal muscle of contaminated male rats was significantly lower than that of contaminated pregnant rate (Cardoni et al., 2004). In other studies that examined the effect of infection on pregnancy, it is concluded that the probability of a successful pregnancy decreases due to an increase the response to infections caused by intracellular pathogens, which indicates the importance of shifting the immune response to Th2 during pregnancy (Wegmann et al., 1993). It is noteworthy that the activity of Natural killer cells, Th1, CD8 Thyroid + cytotoxic cells, and the production of IL-2 and TNF-α in pregnancy decreases and all contribute to the protective immune response against these pathogens (Hunter et al., 1994). And in another study, it is observed

that the induction of IL-2 or IFN-γ in pregnant mice could significantly increase their resistance to *T. gondii* infection (Shirahata et al., 1993). Also, during human pregnancy, the level of infection with Malaria is higher and the more severe disease (Dematteis et al., 2003). Similarly; in rat model, pregnancy infection has adverse effects on pregnancy (Bayraktar et al., 2005).

Conclusion

In summary, it can be concluded given that pregnancy can raise the level of cytokine related to Th2, it can be effective in survival of the *E. granulosus* parasite.

Ethical approval

This study has been approved at the University of Tabriz ethics committee (Ref No. IR.TABRIZU.REC.1398.004).

Conflict of interest statement

There is no conflict of interest.

Acknowledgments

We would like to express our thanks to the University of Tabriz and the Faculty of Veterinary Medicine for financial support.

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