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Comparative Feasibility of Oxidative Stress and Immune-Inflammatory Response Induced in the Secondary Intermediate Host by Different Viability Status of the Hydatid Cysts

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ABSTRACT

Background: Echinococcosis is a common health problem in the Mediterranean and the Middle East. Many hydatid cysts remain asymptomatic, even in advanced age due to the slow growth of the parasite.

Objective: The present study aimed to investigate the oxidative and inflammatory responses in rats' echinococcosis induced by three different viability statuses of the *Echinococcus granulosus* (G6) as diagnostic markers.

Methods: Forty-eight male albino rats were injected intraperitoneally with three different viability statuses of the hydatid cyst fluid of the camel strain. The groups included: the negative control group (1), the low viable protoscoleces (2), the high viable protoscoleces fluid (3) not viable and not completely transformed to the calcareous status of protoscoleces fluid (4). Serum was harvested at the end of each week from the 9th to the 12th week post-infection for measuring the oxidative stress by total antioxidant capacity (TAC), and lipid peroxide (Malondialdehyde) (Malondialdehyde or MDA). . Splenic tissues from different groups werecollected for histopathological examination.

Results: The results showed a histopathological change, a significantly decrease in TAC levels, and an increase in malondialdehyde, the TNF-α, and IL-10 levels of the infected groups compared with the uninfected group ($P<0.05$).

Conclusion: Our study conclude that the Echinococcosis induced severe oxidative stress and inflammatory responses including tissue necrosis and tissue degeneration the factors that can be used in the early stages of infection, avoiding hazards of contamination.

Keywords: *Echinococcus granulosus*, IL-10, MDA, TAC, TNF-α

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INTRODUCTION

The *Echinococcus granulosus* larval stage causes cystic echinococcosis, and since it appears to be a global food-borne infectious disease, it finds a critical public health issue in the developing countries. The crosstalk between the host's immune system and the parasite is considered to be the cause of the pathogenesis. However, there were attempts to show local immunological and circulatory responses (1). Several prevention systems have been implemented in many countries since 1963, owing to its role in the life cycle of animal hosts and humans (2). The *E. granulosus* infection is the most difficult, which can be very symptomatic or asymptomatic, with latent diagnosis (3). Multiple cysts cause non-returnable and severe changes in the vital organs of the host such as the liver, and the most dangerous stages are the rupture or puncture of the cyst which can infect multiple organs with larvae and cause serious morbidity and death (4).

The use of immunological and biochemical tests is a very successful tool for the identification and confirmation of parasitic diseases (5). Infection with different parasite species has increased the levels of free radicals which resulted in oxidative damage of host cells and tissues (6). Oxidative stress and antioxidant status can be assessed by several markers, which are both time-consuming and costly to measure separately (6). It has become more common to measure the total oxidant (TOS), the total antioxidant statuses (TAS), and the malondialdehyde (MDA) as the main indicator of the mechanism of tissue damage in the most of parasitic diseases (7, 8).

The hydatid cyst secretes and exposes numerous immunomodulatory and protective molecules that modulate the host immune response and elicit a granulomatous tissue reaction (9-11). The oxidative stress can be produced by phagocytic cells as part of the immune response with a significant cellular inflammatory response involving macrophage and lymphocyte infiltration within PSCs injected mice (10, 11), which plays an important immunomodulatory role to control infections and inflammatory pathologies and prevents excessive tissue remodeling (12, 13).

The *Echinococcus granulosus* dwells in host organs for a long time, so it has the ability to skew the peritoneal immune response away from a pro-inflammatory response and toward an anti-inflammatory response to avoid clearance (14). Th1 cells produce the TNF-α which has contributed to protective response and parasite elimination, whereas the IL-10 is expressed as Th2 cells response and they are associated with allergic anti-inflammatory responses and susceptibility to the disease (15). Fertile cysts expressed high Tumor necrosis factor-alpha (TNF-α) concentration, while infertile cysts had higher IL-10 concentration (16-18). The TNF-α and the IL-10 production play an important role in maintaining the immune homeostasis.

According to the World Health Organization (WHO, 2001), the most important consequences for screening, treatment, and commonly used classification for hepatic hydatid cyst have been categorized into 6 stages according to its structure (CL, CE1, CE2, CE3, CE4, and CE5) (19). Khammari (2019) (20) found that the stage of the active cysts (CE2) was more fertile than the other stages, so this study concerned two separate active phases and statuses the low and high viable protoscoleces; reflect CE4 stage, mixed hypo- , and hyperechoic material with absent daughter cysts indicating the degenerative existence of the cyst) to investigate the oxidative and inflammatory response in infected rats, usage some mediators as diagnostic markers and confirm the infection at these different stages.

MATERIALS AND METHODS

Ethical Approval

Animal experiments were carried out according to Institutional Animal Care and

National Research Centre Animal Care Unit compatible with the guidelines of the International Animal Ethics committee (*8th Edition 2011, Record number: 13799, legacy ID: 8247)* and according to the local laws and regulations.

Collection of Protoscoleces

Protoscoleces of the *E. granulosus* were collected aseptically from the lung hydatid cysts of naturally infected camels, slaughtered in El Basateen abattoir located in Cairo Province (Egypt). The protoscoleces were washed several times with phosphate buffer saline (PBS pH=7.2) and preserved in labeled sterile tubes containing the same volume of warm Hanks' Balarlced Salt Solution (HBSS) for the experiment (21). The viability of protoscoleces (G6 train) was determined by 0.1% Gentian violet dye according to (22).

Animals and Experimental Design

Forty-eight male albino rats of *Sprague Dawley* strain, with an average weight of 160- 200g were obtained from the National Research Center, Dokki, Giza, Egypt. Animals were examined to be free from parasitic infection to prevent the development of antibodies before the experiments and held in the animal house, the Desert Research Center, Cairo, Egypt under standard 21°C, laboratory treatment, with food and water ad libitum at 16% moisture content. Rats were acclimatized 7 days before the initiation of the experiment. Animals were randomly divided into four groups (12 rats/ each) and injected intraperitoneally with three different viability statuses of the hydatid cyst fluid of camel strain (G6) as follows: the first negative control group administrated with only Hanks' Balarlced Salt Solution (HBSS), the second group intraperitoneally (IP) injected with 1 ml of low viable protoscoleces (Low Prot.) suspension in HBSS Solution (HBSS), the third group intraperitoneally injected with 1 ml of high viable protoscoleces (High Prot.) in HBSS, the fourth group injected with 1ml suspension of semi-calcareous fluid before completely calcified of protoscoleces (Semi-Calc.) in HBSS. After the injection, the rats were daily examined to monitor the external and morphological changes for 2 months before scarifying. Three animals per group were sacrificed at the end of the 10th and the 12th week for post-mortem examinations.

Blood Collection and Preparation

The rats were anesthetized using cotton wool soaked in chloroform and the abdominal cavity was opened to the sternum using medical scissors (3rats/group) at the end of each week from the 9th to the 12th week for post-mortem examinations. The blood samples were directly drawn from the heart using a 5ml sterile syringe into clean and dry centrifuge tubes that were allowed to stand for 10 minutes at room temperature, then centrifuged at 3000rpm for 15 minutes using a laboratory centrifuge (SM 800B, Surgifriend Medicals, England). Sera were carefully removed, stored and frozen at -80°C for further analysis.

Oxidative Stress Assays

Total antioxidant capacity (TAC) and malondialdehyde (MDA) analyses were estimated by commercial kits (Biodiagnostic Co., Dokki, Giza, Egypt) as described in the manufacturer's instructions by spectrophotometer.

Cytokine Assays

Serum tumor necrosis factor-α (Rat, TNF-α ELISA Kit, Abcam, ab46070) and interleukin-10 (Rat IL-10 ELISA Kit, Abcam, ab214566) were estimated using a sandwich enzyme-linked immunosorbent assay (ELISA). The resulting optical density was read on a microtiter plate reader (ELX-808, BioTek Instruments, Winooski, VT, USA) with 450 nm wavelength correction.

Histopathological Preparation

Specimens of rat spleen in different groups were fixed in 10% formalin for 24 hours at room temperature. They were then subjected to washing, de-hydrating in ascending grades

of alcohol, clearing in xylene, embedding in paraffin wax, sectioning (5 micrometers), and staining with hematoxylin and eosin according to Bancroft and Stevens (23) for histopathological examination by Olympus BX51 light microscope.

Statistical Analysis

The results were expressed as the Mean±standard deviation (SD) of the mean. The significant difference between the means was evaluated by one-way analysis of variance (ANOVA) followed by a post hoc test for the comparison of significance using the Statistical Package program (SPSS version 23). Values of $P<0.05$ were considered statistically significant.

RESULTS

Parasitology Results

The current study revealed several external changes that represented in weight loss, body hair loss, and back curvature in the 4th group (Semi-Calc.) at the end of the experiment, however, the visual inspection, and after the autopsy, showed different changes as internal bleeding and splenomegaly (Figures 1 and 2). The severity of changes appeared in low viable protoscoleces (Low Prot.) and high viable protoscoleces (High Prot.) on the 6th week, while semi-calcareous fluid (Semi-Calc.) group appeared on the 3rd-week post-treatment as the following in Table 1.

Figure 1. External changes in echinococcosis infected rats; Loss of body hair in semi-calcerous stages.

Oxidative Stress Result

MDA Concentration in the Blood

In the present study, a significant increase ($P<0.001$) in the sera MDA levels of the *E. granulosus* infected rats was revealed compared to the control rats (Table 2, Figure 3A). Meanwhile, this elevation in the MDA levels was a time-dependent manner in all the treated groups and the highest levels were recorded in the semi-calc. group started with moderate elevation of MDA and still increased further gradually until reaching the highest value (68.7±0.58) on week 12.

TAC Concentration in the Blood

Interestingly, after 9 weeks of the *E. granulosus* infection, it showed a significant increase in both the Low Prot. And the High Prot. groups. However, a time-dependent significant decrease ($P<0.001$) in the TAC levels in both groups was represented in the other weeks of investigation (Table 2). Distinctively, the infected group with the semi-calcareous *E. granulosus* revealed a significant reduction ($P<0.001$) in the TAC levels in a time-dependent manner with the lowest level after 12 weeks of infection compared to the control (Table 2, Figure 3B).

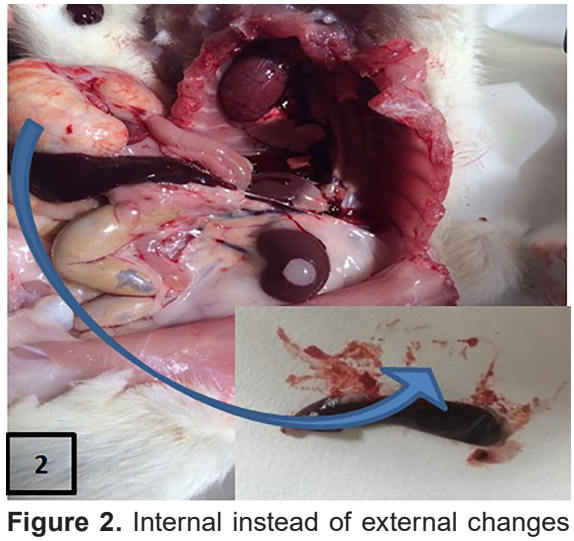

Figure 2. Internal instead of external changes in echinococcosis infected rats; splenomegaly in semi-calcerous stages.

Table 1. External changes in rats infected with different viability statuses of cystic echinococcosis

Group	Exposure period (Time of scarifying/Weeks)	External and internal changes
low viable protoscoleces (Low Prot.)	9th and 10th weeks	*Weight loss
	11th and 12th week	*Weight loss *Loss of body hair *Splenomegaly
High viable protoscoleces (High Prot.)	9th - 12th weeks	*Weight loss *Splenomegaly *Internal bleeding
Semi-calcareous fluid (Semi-Calc.)	9th - 12th weeks	*Weight loss *Loss of body hair *Splenomegaly *Internal bleeding *Back curvature

Table 2. TAC and MDA in rats infected with different viability statuses of cystic echinococcosis and the healthy controls (N=3).

Time/Weeks	Groups	Control	Low Prot.	High Prot.	Semi-Calc.
MDA (mmol/ ml)	9th	24.7±0.33	29.3±0.66***	39.3±0.920***	55.1±0.56***
	10th	22±0.91	33.5±0.24***	42.1±0.23***	59.9±0.29***
	11th	24.1±0.38	35.0±0.08***	48.8±0.13***	66.2±0.26***
	12th	23.3±0.033	39.4±0.20***	50.5±0.01***	68.7±0.58***
TAC (mmol/ ml)	9th	0.41±0.006	0.45±0.003***	0.46±0.003***	0.2±0.006***
	10th	0.43±0.003	0.42±0.006***	0.33±0.003***	0.18±0.001***
	11th	0.44±0.007	0.39±0.007***	0.30±0.001***	0.16±0.005***
	12th	0.41±0.001	0.35±0.006***	0.30±0.005***	0.12±0.001***

Values are expressed as the mean±standard deviation (SD), n= 3 rats.
Low prot.: low viable protoscoleces, high prot.: high viable protoscoleces, semi- calc.: semi-calcareous, MDA: malondialdehyde and TAC: total antioxidant capacity.
Significance versus the control * at P<0.05, ** at P<0.01 and *** at P<0.001.

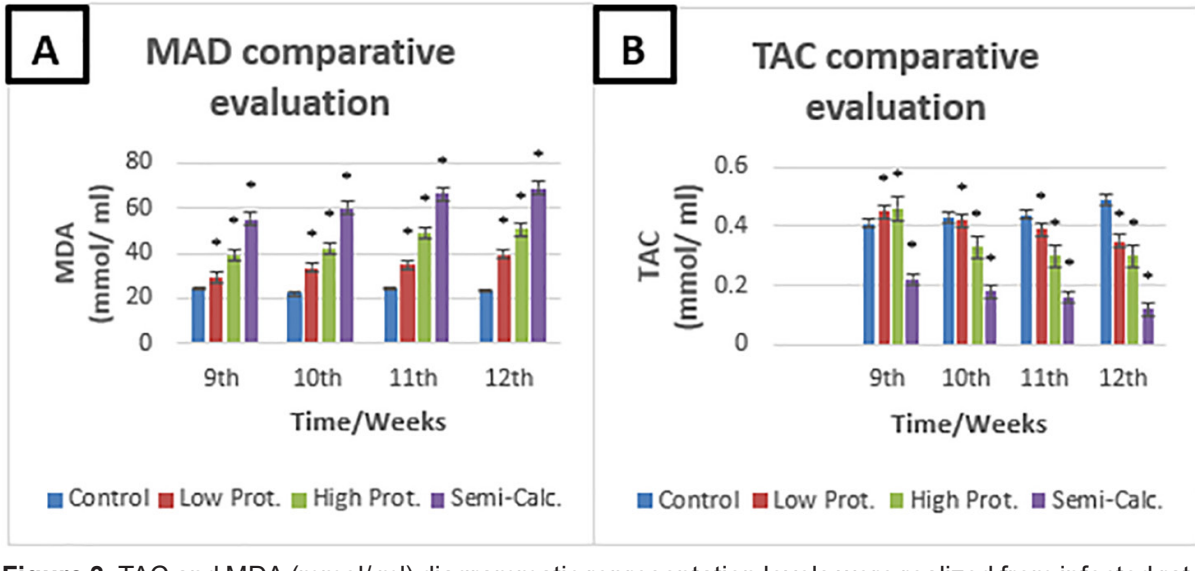

Figure 3. TAC and MDA (mmol/ ml) diagrammatic representation levels were realized from infected rats with different doses of the *E. granulosus* as compared to the control group. (*P<0.05 vs. the control group).

Table 3. TNF-α and IL-10 (pg/ml) comparative evaluation levels (the Mean±SD) were realized from infected rats with different doses of Echinococc*us granulosus* as compared to the control group (N=3).

Time/Weeks	Groups	Control	Low Prot.	High Prot.	Semi-Calc.
TNF-α (pg/ml)	9th	300±0.001	350±0.006***	380±0.005***	400±0.003***
	10th	301±0.006	380±0.003***	411±0.001***	420±0.004***
	11th	300±0.005	340±0.004***	420±0.008***	425±0.006***
	12th	300±0.001	335±0.003***	400±0.003***	410±0.001***
IL-10 (pg/ml)	9th	105.74±0.03	106.33±0.01***	106.93±0.03***	107.6±0.03***
	10th	105.74±0.04	106.05±0.05***	107.57±0.05***	108.99±0.04***
	11th	105.75±0.02	106.12±0.06***	108.08±0.08***	110.45±0.04***
	12th	105.74±0.03	106.45±0.04***	109.11±0.06***	112.51±0.03***

Values are expressed as mean±standard deviation (SD), n= 3 rats.
Low prot.: low viable protoscoleces, high prot.: high viable protoscoleces, semi- calc.: semi-calcareous, TNF- α: tumor necrosis factor and IL-10: interleukin-10.
Significance versus the control * at P<0.05, ** at P<0.01 and *** at P<0.001.

Cytokines Expression

This is a case-control study which revealed the mean of the TNF-α and the IL-10 concentrations in comparison with the control (without the induction of infection) at every stage, the TNF-α and the IL-10 concentrations significantly increased (P<0.001) in three different viability statuses of the *E. granulosus* groups than those in the control group after 9, 10, 11 and 12 weeks, the statistical analysis of the data is summarized in Table 3, Figure 4. The TNF-α concentrations significantly increased (P<0.001) in the Low Prot., the High Prot. and Semi-Calc. groups after 9th, 10th, 11th, and 12th week p.i. (*P<0.05) compared to the control healthy group. The TNF-α and, the TNF-α levels showed a significant reduction after 12 weeks of infection (P<0.001) in all the infected groups as concerns its levels in the other investigated times (Table 3, Figure 4A).

Furthermore, the concentrations of the IL-10 showed significantly elevated (P<0.001) in different infected groups at all studied intervals as compared to the control group, which expressed the lowest values at the 9th week and the highest values at 12th week post infection with reverse correlation to the TNF values (Table 3, Figure 4B).

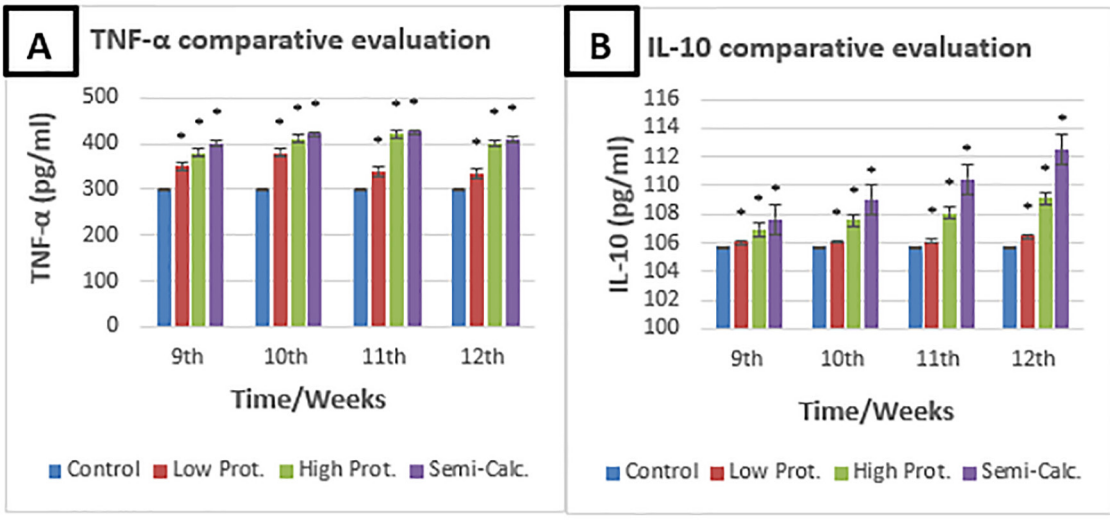

Figure 4. The TNF-α and the IL-10 (pg/ml) diagrammatic representation levels were realized from infected rats with different doses of the *E. granulosus* as compared to the control group. (*P<0.05 vs. the control group).

Histological Studies

Histopathological Results

The inflammatory reaction of splenic tissue to the *E. granulosus* infection (visualized by hematoxylin and eosin-stained histology) at weeks 10 and 12 post-infection (Figure 5 ×200), showed marked granulomatous inflammation, delamination of the distorted capsule, increased number of connected and distorted white pulps with increased proliferation of lymphocytes all over the experimental groups, but red pulps showed somewhat normal architecture in the splenic tissue of the Low Prot. group after 10 weeks of treatment (B), in addition to the highly destructed and thickened walls of the central

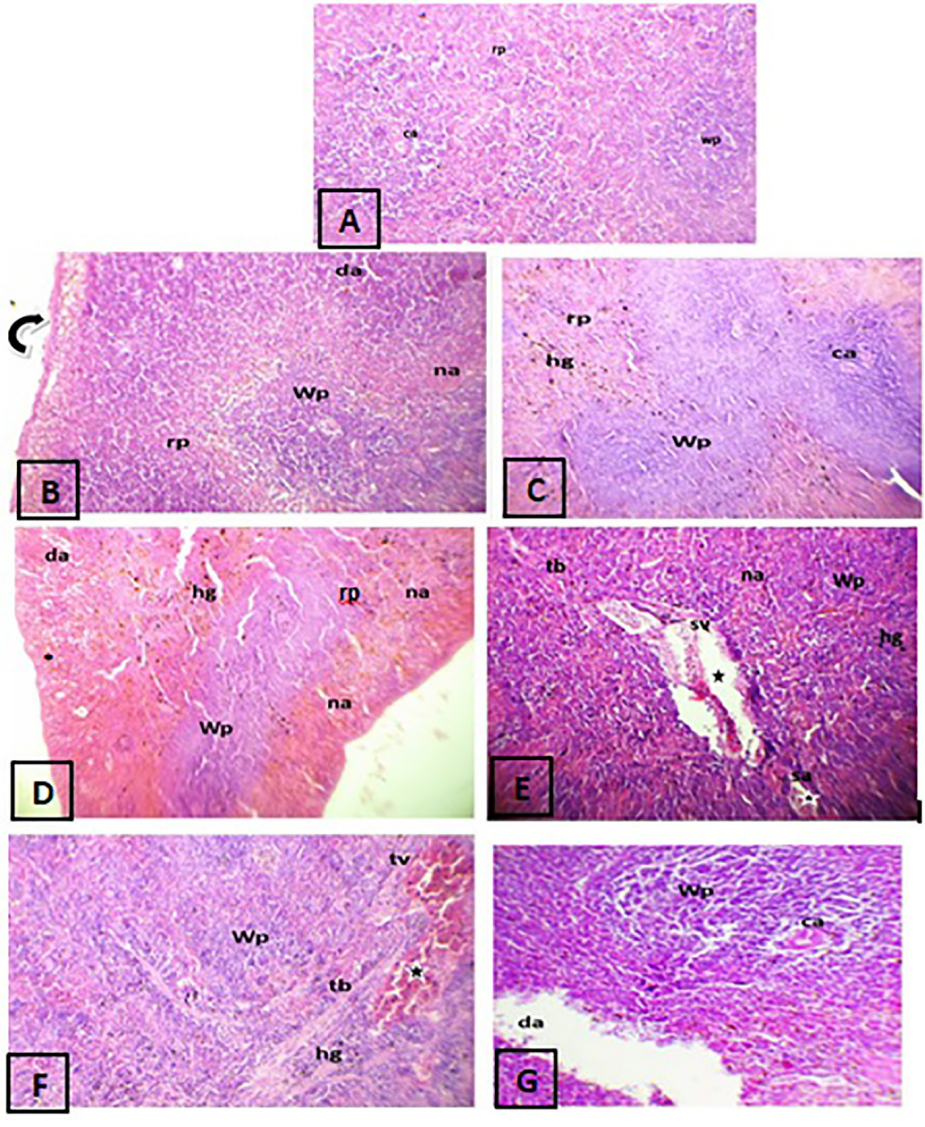

Figure 5. One representative photomicrograph of splenic tissue for the control and infected groups after 10 and 12 weeks of infection with different stages of the E. granulosus respectively. **(H & E X200).** (A) Normal structure of the control group. **(B)** Inflammatory reaction visualized within splenic tissue of the Low Prot. group after 10 weeks of infection, marked a granulomatous inflammation, delamination of the distorted capsule ( ), connected and distorted white pulps (Wp), somewhat normal architecture of red pulps (rp), degenerated (da) and necrotic areas (na). **(C)** The Low Prot. group after 12 weeks p.i. with highly destructed and thickened walls of the central arteries (ca), and numerous hemosiderin granules (hg). **(D)** The High Prot. group after 10 weeks of treatment showed expanded red pulps hemolyzed blood cells ( ). **(E)** The High Prot. group widened highly and elongated splenic vein after 12 weeks of treatment (sv) and artery (sa) were noticed and contained hemolyzed blood cells. **(F & G)** Bizarre arrangement of splenic tissue of Semi-Calc. group after 10 & 12 weeks respectively with highly dilated and elongated trabecular vein (tv), which contained hemolyzed blood cells.

arteries after 12 weeks of treatment (C). High Prot. group showed a bizarre arrangement of WBCs hyperproliferation in the white pulps after 10 weeks of treatment, and expanded red pulps (D), while, after 12 weeks of treatment highly widened and elongated splenic vein and artery were noticed and contained hemolyzed blood cells (E). Splenic tissue of Semi-Calc. group lost its normal architecture after 10 and 12 weeks with bizarre arrangement all over the tissue with highly dilated and elongated trabecular vein, which contained hemolyzed blood cells (F & G); compared to splenic tissue of the control group, which showed normal structure (A). Notice: numerous hemosiderin granules, degenerated and necrotic areas with the debris of degenerated cells all over the splenic tissues of infected groups.

DISCUSSION

In the present study, the infection with the *Echinococcus granulosus* revealed several external changes like weight loss, body hair loss, and back curvature of the infected animals that come following the findings of Edo and Kebede (24). Our postmortem findings represented in the internal bleeding and splenomegaly are similar to the findings of Al-Kuraishi et al. (25) that summarized severe pathologic changes in the livers and the spleens of the infected animals , especially on the second and fourth-month post-infection.

The parasitic infections can be diagnosed by detecting changes in some biochemical parameters (26). The free radicals are greatly involved in the pathogenesis of many parasitic infections (27) and are produced as a result of host reaction to the parasite (28) causing oxidative stress (29). Malondialdehyde (MDA) results from lipid peroxidation of polyunsaturated fatty acids is a good biomarker of oxidative stress (30). The production of several antioxidants is one of the mechanisms that protect the host cells against excess free radicals (31). Determining serum total antioxidant capacity (TAC) may help to identify the conditions affecting the *in vivo* oxidative status rather than the simple sum of measurable antioxidants (32). Consequently, the present study examined the effect of different viability statuses of hydatid cyst fluid of the *E. granulosus* on the oxidative damage in the host by measuring the changes in MDA and TAC levels.

The serum MDA levels exhibited a highly significant elevation in all the infected groups which come in agreement with Fidan et al. (33). However, TAC serum levels showed a highly significant elevation in the low and the high protoscoleces groups after 9 weeks of the *E. granulosus* infection that showed a highly significant reduction in the other weeks of investigation as compared to the control group. Moreover, the semi-Calc. group showed steady decrease levels all over the study period. This agreed with those investigations of El Sayed et al. and Kıvanç et al. (34, 35) who reported that the calcareous stage was responsible for many tough biochemical, serological, and histological changes. The intense peroxidation process of cell lipids and oxidative stress are associated with the MDA elevation and TAC depression values, which interfere with the antioxidant action of the host as a result of the parasitic infection.

Echinococcosis mediated- active immunomodulation of the host immune system, is based on clinical and experimental studies (36). The TNF-α and IL-10 accompanied by histological examination of the splenic tissues were evaluated to investigate the effect of different viability statuses of the hydatid cyst fluid of the *E. granulosus* on the inflammatory response and immunomodulation mechanism.

The TNF-α increased concentrations exhibited at all intervals of the experiment except in the 12th week in all the infected groups might be attributed to the apoptosis of monocytes, which may inhibit the host immunological function as reported by Ali and Hussain (37). Meanwhile, Vatankhah et al. (38) revealed that crude hydatid cyst fluid

causes significant production in the TNF-α and the IL-10 that may signify their role in chronic inflammation.

The current study demonstrated a highly significant elevation in the IL-10 concentrations in different infected groups that were more pronounced on the 12th week post-infection with reverse correlation to the TNF values (Table 3). This comes in agreement with Li et al. (16) findings that serum cytokine levels of T2-type (IL-10) were elevated in cystic echinococcosis patients during the active stage of the disease decreasing significantly after treatment.

Generally, the increase in the TNF-alpha is equilibrated by a simultaneous synthesis of the anti-inflammatory cytokine IL-10, which suppresses the production of various inflammatory stimuli and regulates pro-inflammatory cytokine activity (39). In the current study, increasing IL-10 production is contributed to inverse proportion with the TNF concentrations on the 11th and 12th week of infection. These results come in agreement with Mondragón et al. (40) who reported that the progressive reduction in the TNF-α expression on the eighth week after infection and elevation in the IL-10 suggest local immunosuppression response by the *E. granulosus* mediated parasite protective reaction where the inhibition of the pathogenesis and protective immunity is a key regulatory function of the IL-10 in the parasitic infection.

The relationship of host-parasite not only stimulates host cytokines, but also stimulates parasite cytotoxins that may have induced histocyte proliferation or necrosis and apoptosis. Furthermore, in the present study the inflammatory reaction to splenic tissue of rats infected with the *E. granulosus* protoscoleces visualized by histopathological changes at different infection stages, marked with necrotic areas with the debris of degenerated cells, hemolyzed blood cells, fibrosis, and granulomatous inflammation, that are logically incidence to oxidant, inflammatory mediators' levels elevation and antioxidant suppression. Increased proliferation of lymphocytes coexist within the splenic tissues.

Moreover, Hidalgo et al. (11) demonstrated that hydatid cysts that often contain viable protoscoleces have adventitial layers with scar tissue, which means that the immune-regulating molecules secreted by the parasite are most likely intended to stimulate inflammation resolution in the symptomatic layer.

CONCLUSION

Taking all results together, the Echinococcosis induced severe oxidative stress that led to a decrease of total antioxidant capacity accompanied by an elevation in malondialdehyde levels. Furthermore, this elevation was induced by the three different viability statuses of the *Echinococcus granulosus* (G6) camel strain and was more pronounced in the semi-calcareous stage. Also, the inflammatory response involved in the *E. granulosus* at different viable stages of infection will be mediated through the activation of pro and anti-inflammatory cytokines pathway, including the TNF-α and the IL-10 respectively that is at least in part responsible for the reactive species-mediated NO overproduction and further oxidative stress overall. The determination of these pathways involved in valuable diagnostic markers of the parasite evasion and host protection responses could help to mention unprecedented biomarkers for laboratory diagnosis of the disease without the risk of contamination. However, this study alone cannot confirm the above hypothesis, therefore, further studies are required on this issue.

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Conflicts of Interest: None declared.

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