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ABSTRACT

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ROLES OF TRANSFORMING GROWTH FACTOR BETA AND INTERLEUKIN-32 IN CYSTIC ECHINOCOCCOSIS

Introduction. The biologically different subspecific strain *Echinococcus granulosus* may infect domestic animals and people to varying degrees. Cystic echinococcosis (CE) is a severe zoonotic parasite that poses a significant threat to humans and animals. Epidemic echinococcosis diseases threaten people and animals, slow animal husbandry, and hurt the economy. This study evaluated the significance of immunological markers, including transforming growth factor beta (TGF-β) and interleukin-32 (IL-32), in CE.

Methods. Fifty CE patients and 50 healthy controls were recruited for the study. Blood samples were collected from all participants to determine their serum TGF-β and IL-32 levels using the enzyme-linked immunosorbent assay (ELISA) technique.

Results. The demographics of the study groups showed that most patients infected with CE were males (78.0%) within the age group 16–25 (30.0%), most had cysts in the liver (58.0%) with the fertile type (74.0%), and finally, most lived in rural areas (82.0%). There was a significant ($p \leq 0.0001$) increase in the levels of TGF-β and IL-32 in patients (17.82±7.34 and 21.70±7.12 pg/mL, respectively) compared with the controls (5.13±1.45 and 3.12±1.69 pg/mL, respectively). No significant differences ($p > 0.05$) between the levels of TGF-β and IL-32 were observed based on the site of *Echinococcus granulosus* cysts, type of cysts among patients, and residence of patients.

Conclusion. The liver had more hydatidosis than the lung. The study's findings revealed that the *Echinococcus granulosus* cyst site, kind, and residence did not significantly impact TGF-β and IL-32 levels in patients.

Keywords: cystic echinococcosis, TGF-β, IL-32, *Echinococcus granulosus*, fertile**.**

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ABBREVIATIONS

CE – cystic echinococcosis
IL-32 – interleukin-32
NK – natural killer
TGF-β – transforming growth factor beta
rpm – revolutions per minute
p – probability
ELISA – enzyme-linked immunosorbent assay
Th1 – T-helper 1
Th2 – T-helper 2
Th17 – T helper 17 cells
Treg – regulatory T cells
IgG – immunoglobulin G
Breg cells – regulatory β cells
HSCs – hepatic stellate cells
SE – standard error
IL-10 – interleukin-10
PRRs – pattern recognition receptors**.**
CD – cluster of differentiation
FoxP3 – forkhead box P3
IL-1Ra – interleukin-1 receptor antagonist
H.S. – highly significant
Sig – significance
N.S. – not significant

INTRODUCTION / ВСТУП

Cystic echinococcosis (CE) is one of the most important and commonly found parasitic zoonoses in humans and other animals [1]. It is caused by the larval stages of *Echinococcus granulosus* [2]. The echinococcosis epidemic poses a major threat to human and animal health, hindering animal husbandry output and national economic growth [3]. The mature worms lay eggs in the small intestines of their ultimate hosts, which include dogs and wild predators. After ingesting the worm's eggs, larvae grow in the internal organs of intermediate hosts, which are often herbivorous. Humans are accidentally infected, resulting in CE [4]. Parasitic *E. granulosus* is an exceptionally complex multicellular organism. Given that the human immune system is sensitive to several illnesses, host immunity plays a major role among the host-parasite interaction factors in human liver echinococcosis.

Parasite secretions and waste affect human immune cells, particularly immunologically competent cells. In addition, they activate antigen-presenting cells in humans, such as T cells, and humoral and proinflammatory cell-mediated immune responses, which lead to the release of large amounts of antibodies [5]. Therefore, comprehending immune mechanisms is critical for identifying fundamental protective mechanisms in humans with hydatidosis. Undeniably, protective antibodies are indispensable for producing novel, more potent vaccinations against *E. granulosus* and other parasites. To lessen the severity of undesirable immune and autoimmune reactions, such as several auto-inflammatory diseases and allergies, it may be useful to understand the immunological processes that occur in response to helminth parasitic infection [6]. Interestingly, immune and nonimmune cells in higher animals do not produce the cytokine interleukin-32 (IL-32) in rodents. Since IL-32 and other well-known cytokines have very few similarities, they cannot be classified under any cytokine family. Interleukin-32 is expressed by natural killer (NK) cells, monocytes/macrophages, T-lymphocytes, endothelial cells, fibroblasts, hepatocytes, and epithelial cells [7, 8]. On the other hand, different IL-32 isoforms have shown that IL-32 possesses regulatory properties in addition to its pro- or anti-inflammatory activities [9, 10]. Thirty-three genes that encode either homodimeric or heterodimeric secreted cytokines are part of the human transforming growth factor beta (TGF-β) family [11]. Transforming growth factor beta is a multifunctional cytokine that affects the ability of macrophages to remove parasites and mend tissues [12].

AIM OF THE STUDY

The present study aimed to assess the roles of two immunological markers, specifically TGF-β and IL-32, in cystic echinococcosis.

MATERIALS AND METHODS

Subjects

A case-control investigation was conducted on fifty (50) patients, diagnosed with echinococcosis and fifty (50) healthy control humans. The Iraqi Ministry of Health and Environment's Ethics Committee authorized the study procedure. The nature of the study was revealed to all participants, who gave their written consent. The study precluded any individual undergoing any form of therapy or having a disease other than hydatid.

Blood collection and preparation

Blood was collected from all the participants. An aliquot of 5 mL of venous blood was separated by centrifugation at 5,000 revolutions per minute (rpm) for 5 minutes. From each blood sample, 2 mL of serum were transferred into a sterilized flat-bottomed tube and stored at -20ºC until they were used to analyze serum markers.

Protoscoleces collection and preservation

Protoscoleces of *Echinococcus granulosus* were obtained from fertile hydatid cysts of infected patients. They were examined at low power without staining to test their vitality. Flame cell activity was observed, and a vital dye (0.1% eosin) was used for staining. The germinal layer was viewed under a microscope to reveal the cyst's fertility.

Biochemical analysis

The enzyme-linked immunosorbent assay (ELISA; Cusabio Technology, LLC, USA) kit was used to measure the levels of TGF-β and IL-32 indicators in the serum, and the procedures were conducted following the manufacturer's instructions.

Statistical analysis

The Statistical Package for Social Sciences (SPSS, version 29) software was employed to perform the statistical analysis. The correlation between different parameters was detected using the Pearson correlation analysis. The results were considered significant when the p-value was ≤ 0.05. The independent t-test was employed to compare the means.

Ethics statements

The present study was approved by the Ethics Committee of the College of Health and Medical Techniques Research (Ref. No.: CSEC/3/205). All of the participants were allowed to provide the researchers with the specimens. Informed consent was obtained from all participants according to the Declaration of Helsinki.

RESULTS

The current study showed that most patients infected with *Echinococcus granulosus* were males (78.0%) within the age group 16-25 (30.0%). Most have cysts in the liver (58.0%) with fertile type (74.0%). Finally, most participants lived in rural areas (82.0%), as depicted in Table 1. The results (Table 2) of the TGF-β and IL-32 levels in the serum revealed a significant ($p \leq 0.0001$) increase in the levels of TGF-β and IL-32 in patients (17.82±7.34 and 21.70±7.12 pg/mL, respectively) compared with the controls (5.13±1.45 and 3.12±1.69 pg/mL, respectively). According to the site of cysts among patients, there were no statistically significant ($p > 0.05$) differences in IL-32 and TGF-β levels (Table 3). As shown in Table 4, the study's results demonstrated no statistically significant ($p > 0.05$) differences in TGF-β and IL-32 levels across patients based on the kind of cyst. Also, there were no statistically significant ($p > 0.05$) correlations between the levels of IL-32 and TGF-β and patient residence (Table 5).

Table 1 – Demographics of study groups

Variable		Case	Control	Total	P-value
Age (years)	(M±SD)	35.70±12.35	38.52±16.15	-	0.42
Age range (years)	(16-25)	15 (30.0%)	14 (28.0%)	29 (29.0%)	0.37
	(26-35)	12 (24.0%)	9 (18.0%)	21 (21.0%)	
	(36-45)	12 (24.0%)	9 (18.0%)	21 (21.0%)	
	(46-55)	6 (12.0%)	14 (28.0%)	20 (20.0%)	
	(56-65)	5 (10.0%)	4 (8.0%)	9 (9.0%)	
	Total	50 (100.0%)	50 (100.0%)	100 (100.0%)	
Gender	Male	39 (78.0%)	39 (78.0%)	78 (78.0%)	1.0
	Female	11 (22.0%)	11 (22.0%)	22 (22.0%)	
	Total	50 (100.0%)	50 (100.0%)	100 (100.0%)	
Site of cyst	Liver	29 (58.0%)	-	29 (58.0%)	-
	Lung	21 (42.0%)	-	21 (42.0%)	
	Total	50 (10.0%)			
Type of cyst	Fertile	37 (74.0%)	-	37 (74.0%)	-
	Sterile	13 (26.0%)	-	13 (26.0%)	-
	Total	50 (100.0%)		50 (100.0%)	-
Residency	Urban	9 (18.0%)	29 (58.0%)	38 (38.0%)	≤0.0001
	Rural	41 (82.0%)	21 (42.0%)	62 (62.0%)	
	Total	50 (100.0%)	50 (100.0%)	100 (100.0%)	

Table 2 – Comparing the mean levels of TGF-β and IL-32 between cases and control groups

Marker	Mean ± SD	SE	Min-Max	T-test	P-value	Sig
TGF-β pg/mL	17.82±7.34	1.03	1.9-38.95	11.97	≤0.0001	H.S
	5.13±1.45	0.20	2.73-7.91			
IL-32 pg/mL	21.70±7.12	1.43	0.66-48.30	12.53	≤0.0001	H.S
	3.12±1.69	0.38	0.1-7.91			

H.S: Highly significant; Case n = 50; Control n = 50

Table 3 – Comparing the mean levels of TGF-β and IL-32 based on the site of cysts among patients

Marker	Site of cyst	N	Mean ± SD	SE	T-test	P-value	Sig.
TGF-β pg/mL	Liver	29	18.30±7.21	1.33	0.53	0.59	N.S
	Lung	21	17.16±7.65	1.67			
IL-32 pg/mL	Liver	29	22.99±10.53	1.95	1.07	0.28	N.S
	Lung	21	19.92±9.49	2.07			

Sig.: Significance; N.S: Not significant; SE: Standard error

Table 4 – Comparing the mean levels of IL-32 and TGF-β based on the type of cysts among patients

Marker	Type	N	Mean ± SD	SE	T-test	P-value	Sig.
TGF-β pg/mL	Fertile	37	18.33±7.54	1.24	0.87	0.39	N.S
	Sterile	13	16.35±6.81	1.88			
IL-32 pg/mL	Fertile	37	21.97±10.17	1.67	0.38	0.76	N.S
	Sterile	13	20.94±10.38	2.87			

Sig.: Significance; N.S: Not significant; SE: Standard error

Table 5 – Levels of biomarkers in study groups based on residency

Marker	Residency	N	Mean ± SD	SE	T-test	P-value
TGF-β pg/mL	Urban	9	16.64±8.12	2.70	0.48	0.63
	Rural	41	18.08±7.24	1.13		
IL-32 pg/mL	Urban	9	21.72±12.72	4.24	0.07	0.99
	Rural	41	21.69±9.65	1.50		

SD: Standard deviation

DISCUSSION

As a complex parasitic disorder, CE is considered a widespread health concern in developing countries [13]. Several social factors can impact its conditions, including medical awareness, living style, and the efficiency of veterinary treatment. Moreover, the number of dogs, parasite egg availability, intermediate host infection rate, and definitive-intermediary host transmission potential affect the disease's prevalence [14]. Cystic echinococcosis transmission is perpetuated by illegal animal slaughter, poor abattoir conditions in rural areas, and slaughterhouse personnel who feed stray dogs with tainted viscera [15]. It was previously shown in a study that the presence of children in a household

with a dog is significantly associated with CE, regardless of whether the dog is currently affected or was owned by the household up to a decade ago. Additionally, the likelihood of CE tends to rise in proportion to the quantity of canines owned [16]. Aregawi [17] showed that most patients infected with *Echinococcus granulosus* were males (55.3%) within the age group 1-40 (71.4. %), most of them had cysts in the lung (53.0%), and finally, most lived in a rural area (67.0%). These results are consistent with the present study's findings (Table 1). In contrast, Paduraru [18] showed that most patients infected with *Echinococcus granulosus* were female (51.2%) within the age group 10-20 (82.4%), and most of them had cysts in the liver (65.0%).

Most cases of CE (52.7%) were females, as indicated by the results of a previous study. Overall, especially in rural parts of Iran, it seems that women are more vulnerable than men to infection causes (like polluted soil and fresh vegetables). Furthermore, women tend to be more sensitive to CE than men [19]. The domestic population had the greatest infection incidence (28.10%), and most patients had no dog interaction. In contrast, rural Mazandaran women feed on vegetables. As a result, individuals have a higher chance of developing CE in the region under investigation [19], demonstrating that most patients aged 40 to 60 were infected even though the disease can affect individuals of all ages. However, Kabiri [20] showed that teenagers account for about 10–20% of all CE cases. Some have hypothesized that areas with a high adult CE incidence also have a high childhood CE prevalence [21]. Children are often seen playing in the fields and touching contaminated dirt because the homes of farm workers are close to the fields. This kind of exposure increases the risk of infection in young people because of carelessness during play or pica disorder, which causes the virus to spread directly from the hands to the mouth [16]. Additionally, the results presented in the present study indicated that rural regions exhibited a higher prevalence. As previously reported, CE is predominantly prevalent in rural areas [21]. People with this disease are more likely to live in rural places with lots of farming, a regional temperature, low socioeconomic levels, and bad animal slaughter. Vegetables contaminated with dog excrement, vegetables irrigated with contaminated water, or contaminated water supplies may be the primary sources of infection in rural areas. Furthermore, the proportion of cases from urban areas may be overstated due to patients' frequent travel to rural high-risk zones or their origins in peri-urban regions [18].

Liver involvement was observed in over 50% of the patients, while lung involvement was less prevalent. Aygün [22] reported comparable findings. Other research has shown that the lung is the most prevalent site for hydatid lesions in minors, as opposed to the liver [23]. The development of cysts in the lung appears to be facilitated by the elasticity of the tissue, negative pressure, and vascularization. The earlier and more severe signs of lung cysts in children (e.g., wheezing, chest pain) may lead parents to seek medical assistance more quickly, increasing pulmonary cases [18]. Two types of hydatid cysts may be found in naturally infected intermediate hosts. The first type is fertile, with protoscoleces either floating in the hydatid fluid or connected to the germinal layer. The second type is sterile, with no protoscoleces [24]. In a previous investigation, it was observed that the fertility of EC represented 82.81% and infertility recorded 17.19% [25]. This finding was almost similar to those of the current investigation. Accordingly, the possibility of secondary CE infections during surgical operations and the need to use albendazole as an adjuvant either before or after the operation were emphasized. The composition of hydatid cyst fluid in people infected with *Echinococcus granulosus* can be established based on the previous research findings [26]. Therefore, it is necessary to determine the chemical composition of *Echinococcus granulosus* to detect two types (fertile and infertile). The multicellular parasite *E. granulosus* is exceedingly intricate. Numerous pathogens are highly immunogenic to the human host. Thus, the host immune system plays a pivotal role in the host-parasite dynamic in human hepatic echinococcosis. The parasite's secretory and excretory substances affect the human host's immunological and immune-competent cells. In addition, they activate antigen-presenting cells in humans, such as T cells, and humoral and proinflammatory cell-mediated immune responses, which lead to the release of large amounts of antibodies [5]. Thus, understanding immunological systems is crucial to finding a key protective mechanism in hydatidosis patients. Protective antibodies are essential for developing new, more effective *E. granulosus* and parasite vaccines. Understanding immunological processes occurring after helminth parasite infection may lower the severity of auto-inflammatory disorders and allergies [6]. There is mounting evidence that parasite-released bioactive immunomodulatory molecules and chemical compounds mitigate inflammatory responses that exacerbate several auto-inflammatory diseases, either by activating an anti-worm immune response spectrum or acting directly on these responses [26].

Cystic echinococcosis induces cellular and humoral immunological responses in wild animals raised for agriculture. A previous investigation displayed that

while T-helper 1 (Th1) cytokines increased, T-helper 2 (Th2) cytokines decreased accordingly. There should be a new focus on CE and the Th1 response to *E. granulosus* [27]. The current investigation's results (Table 2) are consistent with those discovered by De Biase [28], who observed that TGF-β levels were higher in CE-infected groups than in control groups. This increase is associated with the pro-inflammatory impacts of TGF-β. Previous studies have shown both fertile and sterile hydatid cysts in the liver have an inflammatory response to parasites. This reaction is marked by higher TGF-β levels and TGF-β-positive macrophages [28]. Cystic echinococcosis-infected hosts show elevated TGF-β expression in leukocytes, especially surrounding the parasite, indicating that immunomodulation plays a vital role in parasite survival [29]. Another study suggested that TGF-β impacted macrophages' parasite removal and tissue healing abilities [30]. In peripheral blood, patients with CE showed an imbalance between the T helper 17 cells (Th17) and regulatory T cells (Treg) response, which might impact the immunological evasion of cestode larvae, as shown by increased regulatory T cells and cytokines (IL-10 and TGF-β) and reduced inflammatory Th17 cells [31]. Experimental treatments for colitis and airway inflammation in mice have been achieved by the *E. granulosus* infection, known for its significant immunomodulatory capabilities [32]. *Echinococcus granulosus* hydatid-laminated layer extracts decreased mucosal damage and inflammatory responses in mice with dextran sulfate sodium-induced colitis [6].

Previous studies using the ELISA technique found no significant difference in serum TGF-β1 levels in CE-infected animals throughout the early phases. However, it was significantly ($p < 0.05$) increased during the middle and end stages of infection. At 90 days following infection, mice infected with *E. granulosus* had more CD1dhiCD5+CD19hiBreg cells and IL-10+Breg cells in the spleen, showing that Breg cells expanded late in the infection. One of the reasons for the immunosuppression of the *E. granulosus* infection may be the CD1dhiCD5+CD19hi regulatory B cell. Bregs' inhibitory action is thought to be involved via controlling cytokine expression and promoting the release of inhibitory TGF-β1 [33]. New research evidence of immune suppression in the parasite vicinity arose from the observation of an expansion in FoxP3+ CD4+ regulatory T cells and increases in the local concentrations of the anti-inflammatory cytokines TGF-β and IL-1Ra [34]. Additionally, CE develops into a bladder in the liver of the intermediate host. The collagen capsule surrounding this structure comprises activated hepatic stellate cells (HSCs) derived from the host. More research is needed to determine the effects of CE on NK cells in the liver. More so, whether the impairment of TGF-β signaling will alleviate hepatic damage associated with CE requires further study. Wang [35] suggested targeting TGF-β1 for treating liver fibrosis linked to CE. The immune response to hydatid disease depends on Th1 and Th2 cytokines [36]. Researchers have found that Th1 cytokines are connected to protective immunity, and Th2 cytokines are connected to the chronic stage, clinical problems, and more episodes [37].

The current investigation demonstrated that CE patients exhibited elevated levels of IL-32 compared to controls (Table 2). This is attributed to the pro-inflammatory effects of this cytokine in these diseases. Interleukin-32 is a cytokine of both immunological and non-immunological nature. It was formerly known as the NK cell transcript (NK4). It was originally recognized as a pro-inflammatory cytokine. Nonetheless, the availability of different isoforms of IL-32 has proven that, besides its pro- or anti-inflammatory effects, IL-32 has regulatory features [10]. The role of IL-32 in several inflammatory and infectious diseases, including different types of leishmaniases, has been studied so far [38]. Interleukin-32 is a complex cytokine linked to various illnesses and inflammatory disorders. It involves numerous biological processes, including antimicrobial responses and inflammation, immunological activation, immune suppression, mitogenic signaling, migration, adhesion and angiogenesis, metabolism, oxidative stress, cell death, and bone homeostasis [39]. Importantly, the current study is considered the first to investigate the association between IL-32 and CE patients because no previous research has been published on the subject. Therefore, further research is required to study the role of IL-32 in the pathogenesis of CE. The present study's findings showed no differences in cellular (IL-32 and TGF-β) responses in the liver and lung of patients infected with *E. granulosus* parasites (Table 3). This suggests similar inflammatory responses in different organs of patients infected with *E. granulosus* parasites. Cystic echinococcosis is usually observed in the liver and lungs. However, they may also occur in the bones, kidneys, spleen, muscles, and central nervous system in humans infected with *E. granulosus* [40]. The liver represents the fundamental organ of frontline immune tissue. It would seem that the liver's purpose is to detect, trap, and eliminate macromolecules, viruses, and bacteria that enter the body via the intestines [41]. Consistent with the findings of the current investigation, Al-Nuaimi and Al-Hassan [42] demonstrated that liver cyst injuries have a major impact on immunity and result in raised immunoglobulin G (IgG) levels when

compared to injuries in other parts of the body, such as the lungs.

Previous research found that the laminated layer thickness differed between fertile and infertile hydatid cysts and liver and lung tissues [43]. There is some evidence that the parenchyma of these organs limits how quickly hydatid cysts grow. For example, because lungs are less dense than livers, it stands to reason that cysts in the fertile hydatid would have a thicker laminated layer than cysts in the infertile hydatid. However, there was no statistically significant difference between small and large cysts [43]. The study found no significant differences in cellular (IL-32 and TGF-β) responses to fertile and sterile *E. granulosus* parasites. This indicates a similar activity of fertile and sterile types of *E. granulosus* parasites in inducing immune responses. The adventitial layer characteristics were detected in many small hydatid cyst samples, including infertile and poor viability cysts [43]. It has been shown that cysts of the same parasite strain in the same host and organ may differ in size, viability, and fertility [44]. The findings from the present study indicated that tiny hydatid cysts characterized by adventitial layers and robust immune responses are indicative of fertilization. Infertile cysts lack characteristics like palisading foamy macrophages, lymphocytes in follicles, multinucleated giant cells, a thin lamination layer (<50 μm), and host immune cells inside the germinal layer. Such qualities are necessary for a healthy pregnancy. The immune response significantly influences cyst viability, as evidenced by the increased presence of inflammatory infiltrates, particularly lymphocytes and multinucleated giant cells, in both small and infertile cysts [28]. Previous research has revealed that live cysts from sheep consistently exhibit fibrotic resolution near the laminated layer and cattle respond in a mixed pattern, including granulomatous reactivity and fibrotic resolution in direct contact with the laminated layer. Cattle non-fertile cysts exhibit a granulomatous response when they are close to the laminated layer. However, this reaction is observed in sheep when the cysts are close to the fibrotic resolution. This demonstrates that the local immune response patterns of sheep and cow CE cysts differ and are related to the fertility of metacestodes [45]**.** Scar tissue in the adventitial layers of fertile hydatid cysts with mostly living protoscoleces implies that the adventitial layer is likely the target of the immune regulatory molecules released by the parasite to trigger the resolution of inflammation. Rarely, calves have granulomatous immunological responses with reduced protoscolex viability, laminated layer disruption, and immune cell infiltration [43].

The findings (Table 5) demonstrated that, in patients with *Echinococcus granulosus*, residency had no impact on the cellular immune response. According to several studies, environmental influences are the main determinants of human immunological characteristics. Factors unique to rural and urban environments are linked to some of the most significant early life exposures that shape the functional programming of the newborn immune system. Children's innate (e.g., pattern recognition receptors; PRRs) and adaptive immune responses have been demonstrated to be altered by rural or traditional agricultural lifestyles [46]. Variations in early life immune system development have been linked to food patterns, pollutant-generating cooking or heating techniques, animal exposure, and socioeconomic level [47]. In the previous study population, the immune response of individuals residing in an urban environment was more frequently associated with producing the immune regulatory cytokine, IL-10. Elevated IL-10 production was linked to factors associated with inadequate hygiene and living conditions in urban and rural populations [48]. Molecular techniques can be recommended for the accurate and rapid diagnosis of CE infection. The molecular technique known as polymerase chain reaction (PCR) has been utilized in various areas of medical study [49–56].

CONCLUSIONS

The age group (16-25 years) showed a higher percentage of infection by CE than other age groups. Males had a higher percentage than females, and the fertile type showed a higher percentage than sterile. Furthermore, rural residents outperformed urban residents in terms of proportion. The number of cases of liver hydatidosis was higher than that of the lung. Immunological parameters included in the present study (TGF-β and IL-32) showed high levels among patients with CE diseases compared to healthy controls. No significant differences were observed in the levels of TGF-β and IL-32 across the following three categories: the site of the cyst of *Echinococcus granulosus*, the type of cyst among patients, and the residence of patients.

AUTHOR CONTRIBUTIONS / ВКЛАД АВТОРІВ

Riyadh Resan Saad: sample collection and analysis, data collection, statistical analysis, and manuscript writing. Dr. Maysara Samer khalaf and Dr. Amal Hasan Atiyah: conceptualization of the research, research design, and manuscript proofreading.

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CONFLICT OF INTEREST / КОНФЛІКТ ІНТЕРЕСІВ

The authors declare no conflict of interest.

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