Vol: 2, No: 1, (2023)

Antakya Veteriner Bilimleri Dergisi
The Journal of Antakya Veterinary Science
https://dergipark.org.tr/tr/pub/antakyavet

Research article / Araştırma makalesi

Immunohistochemical evaluation of IFN-γ levels in sheep with cystic echinococcosis

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MAKALE BİLGİSİ:
ARTICLE INFORMATION:

Geliş / Received:
04.05.2023
Revizyon/Revised:
25.05.2023
Kabul / Accepted:
10.06.2023

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Abstract:

In the present study, with an aim to demonstrate in detail the cellular immune response given to the parasite in the lungs of sheep infected with cystic echinococcosis (CE), a comparative assessment was made of the interferon-gamma (IFN-γ) levels of healthy and infected animals using immunohistochemical methods. The material of this study consisted of lung tissue samples taken from an average of 10 dead sheep brought to Kafkas University Faculty of Veterinary Medicine Department of Pathology for routine diagnosis. Lung tissue samples taken from 10 healthy sheep without any pathological lesions were also used for control purposes. Hematoxylin & Eosin staining was applied to the sections in order to evaluate the histopathological changes in the tissues. Immunohistochemical staining was performed for IFN-γ immunopositivity in lung samples determined to be control and cystic echinococcosis after histopathological examination. Macroscopic examination revealed large and small oval-shaped cystic structures that spread to almost all lobes of the lung. In the histopathological examination of the lungs, it was observed that the cysts were surrounded by a fibrous capsule and there was a concentric lamellated cyst wall just below the fibrous capsule. It was determined that IFN-γ immunopositivity increased significantly in the CE group compared to the control group. This increase in IFN-γ levels reveals that it is an important cytokine in the diagnosis of parasitic diseases such as CE. The fact that IFN-γ immunopositivity is much more severe in phagocytes such as giant cells and macrophages, which play a dominant role in the cellular immune response developed against parasitic infection, shows that this pro-inflammatory cytokine may be effective in anti-echinococcal therapy.

Keywords: Cellular Defense, Interferon-γ, Cystic Echinococcosis, Sheep, Cytokine

Kistik ekinokoklu koyunlarda IFN-γ seviyelerinin immunohistokimyasal olarak değerlendirilmesi

Özet:

Güncel çalışmada kistik ekinokokkozisli (KE) koyunların akciğerlerinde parazite karşı gelişen hücresel immun yanıtın detaylandırılması amacıyla sağlıklı hayvanlara kıyasla enfekte hayvanlardaki interferon-gamma (IFN-γ) düzeyleri immunohistokimyasal yöntemlerle değerlendirilmiştir. Bu çalışmanın materyalini Kafkas Üniversitesi Veteriner Fakültesi Patoloji Anabilim Dalı'na rutin tanı amacıyla ölü olarak getirilen ortalama 10 adet koyundan alınan akciğer dokusu örnekleri oluşturmuştur. 10 adet sağlıklı ve herhangi bir patolojik lezyon sergilemeyen koyunlardan alınan akciğer dokusu örnekleri de kontrol amacıyla kullanılmıştır. Dokulardaki histopatolojik değişikliklerin değerlendirilebilmesi amacıyla kesitlere Hematoksilen & Eozin boyaması yapıldı. Histopatolojik incelemeden sonra kontrol ve kistik ekinokok olduğu belirlenen akciğer örneklerinde IFN-γ immunpozitifliği için immunhistokimyasal boyama yapılmıştır. Makroskobik muayenede akciğerin hemen hemen tüm loplarına yayılmış, irili ufaklı oval şekilli kistik yapılar tespit edildi. Akciğerlerin histopatolojik incelemelerinde kistlerin fibröz bir kapsülle çevrelendiği ve fibröz kapsülün hemen alt kısmında konsantirik lamellasyon gösteren kist çeperinin olduğu gözlendi. Kontrol grubuna kıyasla IFN-γ immunpozitifliğinin KE grubunda anlamlı düzeyde artış gösterdiği belirlendi. IFN-γ düzeylerindeki bu artışın KE gibi paraziter hastalıkların teşhisinde önemli bir sitokin olduğunu ortaya koymaktadır. Paraziter enfeksiyona karşı geliştirilen hücresel immun yanıtta baskın bir rol oynayan dev hücreleri ve makrofajlar gibi fagositlerde IFN-γ immunpozitifliğinin çok daha şiddetli olması da bu pro-inflamatuar sitokinin anti-ekinokok tedavisinde etkili olabileceğini göstermektedir.

Anahtar kelimeler: Hücresel Savunma, İnterferon-γ, Kistik Ekinokokkozis, Koyun, Sitokin

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How to cite this article: Karakurt E, Kızıltepe Ş & Yıldız A (2023). Immunohistochemical evaluation of IFN-γ levels in sheep with cystic echinococcosis. *J. Antakya Vet. Sci.*, 2(1), 37-42.

Introduction

Cystic hydatid disease, also known as cystic echinococcosis (CE), is a chronic granulomatous disease of zoonotic nature, which commonly affects sheep and is caused by the development of the intermediary larval stage (metacestode) of the tapeworm *Echinococcus granulosus* in various organs, including primarily the liver and lungs and occasionally the mesentery in the intermediate host (Al Malki and Ahmed, 2022). The indirect life cycle of the parasite *E. granulosus* involves members of the family Canidae (dogs, jackals, wolves, etc.) as the definitive hosts, and herbivores and omnivores (cattle, pigs, buffaloes, camels, sheep and goats, etc.) as the intermediate hosts (Kesik et al., 2019; Abo-Aziza et al., 2020). Humans, as aberrant dead-end hosts, are not part of the natural life cycle of the parasite and become infected accidentally (Atmaca, 2022). Intermediate hosts become infected by ingesting the eggs of *E. granulosus*, which are expelled in the feces of the definitive hosts and contain infective oncospheres (Brik et al., 2018). Once transmitted to the intermediate host through the fecal-oral route, the oncospheres are activated by digestive enzymes, penetrate the intestinal wall, and after reaching the bloodstream, migrate to parenchymatous organs, primarily the liver and lungs (Yildiz et al., 2022). The hydatid cysts of *E. granulosus* develop in the form of fluid-filled unilocular cysts in the visceral organs of humans and other intermediate hosts (Tarladaçalışır et al., 2022). Within a period of one year, these cysts reach a size of 1 to 5 cm in diameter (Abo-Aziza et al., 2020). The cysts are composed of a parasitic inner germinal layer (GL), an outer laminated layer (LL), and an adventitial layer (AL), the latter of which is formed by the host immune response. The adventitial layer is enclosed by a fibrous capsule produced by the host. The germinal layer is a cellular layer, which produces the clear cyst fluid as well as the brood capsules from which infective protoscolices (PSCs) are released. The laminated layer is a modified extracellular matrix also synthesized by the germinal layer. The adventitial layer is composed of innate and adaptive immune cells and the tissue of the organ to which the cyst is localized. Some cysts do not contain any PSCs, and thus, are considered to be non-fertile/sterile as they are incapable of continuing the life cycle of the parasite (Hidalgo et al., 2021, Atmaca, 2022).

Parasitic antigenic stimuli trigger the selective differentiation of pure T cells into T1 helper (Th1) cells or T2 helper (Th2) cells. Th1 cells play a major role in the defense of the body against intracellular parasitic infections by producing cytokines such as gamma interferon (IFN-Gamma/IFN-γ) (Ma et al., 2014). Cytokines, which are a group of low molecular weight-proteins, are responsible for the regulation of immunity (Biranvand et al., 2020). Mostly antigen-presenting cells, T lymphocytes and natural killer cells (NK) produce IFN-γ (Rahdar et al., 2020). IFN-γ is a major Th-1 cytokine and is involved in the activation of immune cells, the increase of chemokine release and the release of nitric oxide (NO) from M1 macrophages. All these functions of this particular cytokine enable the control of the parasite (Horta et al., 2020).

In the present study, with an aim to demonstrate in detail the cellular immune response given to the parasite in the lungs of sheep infected with cystic echinococcosis, a comparative assessment was made of the IFN-γ levels of healthy and infected animals using immunohistochemical methods.

Material and Method

Animals

The study material comprised of pulmonary tissue samples taken from 10 dead sheep that were admitted to the Pathology Department of Kafkas University Faculty of Veterinary Medicine for routine diagnosis. Pulmonary tissue samples taken from 10 healthy sheep with no pathological lesion were used for control purposes. A total of 20 animals were used.

Histopathological Examinations

The pulmonary tissue samples taken from the sheep were fixed in 10% formaldehyde solution. After routine tissue processing, 5-micrometer-thick serial sections were cut from the paraffin blocks. For the assessment of any histopathological alterations in the tissues, the sections were stained with hematoxylin and eosin (H&E). At least two different pathologists examined the preparations under a light microscope in detail and any pathological finding detected was imaged with a camera.

Immunohistochemical Examinations

Four-micrometer-thick serial sections cut from the paraffin blocks of the pulmonary tissue samples were stained with the avidin-biotin-peroxidase complex (ABC) technique, using commercial IFN-γ antibodies, according to the manufacturer's instructions. Detailed information on the primary antibody used in the study is presented in Table 1. All immunostainings were performed using a Thermo Scientific Histostain IHC Kit (HRP, broad spectrum, REF: TP-125-HL). In order to prevent endogenous peroxidase activity, the sections were treated with 3% hydrogen peroxide solution in Phosphate Buffered Saline (PBS) for 15 minutes. For antigen retrieval, the sections were boiled in Citrat Buffer Solution (pH 6) for 25 min in the

microwave oven (at 800 watt). 3.3-diaminobenzidine tetra hydrochloride (DAB) solution (Thermo Scientific, REF:TA-125-HD) was used as a chromogenic substrate and incubated with the sections for 15 minutes. After being washed in distilled water for 5 minutes, the sections were stained with Mayer's hematoxylin and embedded in an immune mounting medium.

After being mounted, the preparations were examined under a light microscope (Olympus Bx53) and images were taken using the Cell ^P software (Olympus Soft Imaging Solutions GmbH, 3,4). A detailed analysis was made of the images taken using the Image J software (1.51j8).

Table 1. Information on the primary antibody used in the immunohistochemical examinations.

Primary Antibody	Pretreatment	Company and Catalogue Numbers	Dilution	Incubation Conditions
IFN-γ	Microwave oven	MyBioSource, MBS2091397, Polyclonal	1/100	Overnight, 4°C

The results of the IFN-γ stainings were assessed with a scoring system based on the number of positive cells in the areas with the strongest staining as determined by the examination of immunopositive reactions. For each case, five different microscopic areas were examined at X50 magnification. The number of cells that had stained positively were separately recorded for each case and the mean number of three areas were determined as the mean number of positive cells for a given case. Scoring was performed as follows: (-) no immunoreactivity; (+) weak, 1-10% positivity; (++) moderate, 11-59% positivity and (+++) severe with >60% positivity (Karakurt et al., 2023).

Statistical Analysis

The statistical analysis of the study results was performed with the SPSS® software (SPSS 26.0, Chicago, IL, USA). The pairwise comparison of the control group and the group infected with echinococcosis was made with the Mann-Whitney U test. The results are expressed as mean ± standard error of the mean (SE). Statistical significance was set at $P<0.05$.

Results

Macroscopic Findings

The macroscopic examination of the lungs demonstrated the presence of oval cystic structures, which were distributed throughout almost all of the pulmonary lobes and were of various dimensions. Upon palpation, the majority of the cysts were determined to be of a rather hard consistency, and some were determined to be of a very soft consistency.

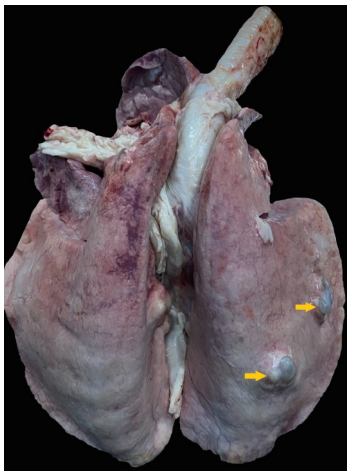

Figure 1. Lungs, macroscopic appearance of hydatid cysts filled with clear fluid.

Furthermore, while the large cysts were observed to be filled with a clear fluid, the small cysts displayed calcifications (Figure 1).

Histopathological Findings

Histopathological examination revealed that the cysts were enclosed by a fibrous capsule. Just beneath this capsule, a cystic membrane displaying concentric lamellation was observed. Inflammatory cell infiltration, mostly composed of lymphocytes, histiocytes and plasma cells was observed in the periphery of the cysts. Apart from these cells, although at a small number, eosinophil leukocytes were also present. In close proximity to the cyst membrane, foreign body giant cells, which were of various shapes and had nuclei facing the opposite side of the cysts, were detected. Some cases presented with mild calcification. Each case was evaluated for the presence of protoscolices in the cyst membrane. The presence of viable protoscolices, in other words the presence of fertile cysts, was detected in only one case. Thus, the remaining cases were all recorded as involving the development of infertile/sterile cysts (Figure 2).

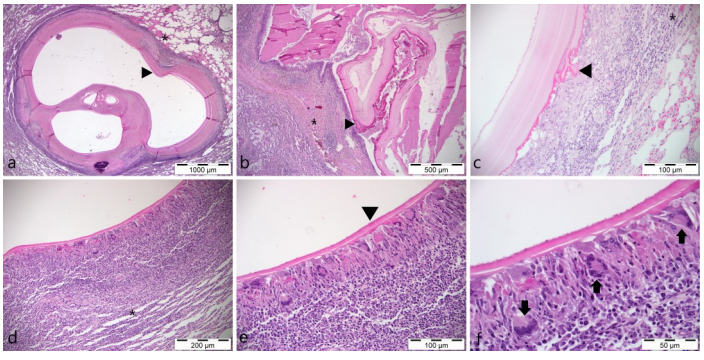

Figure 2. Lung, H&E, **a-f:** Cystic granuloma (*), cyst wall (arrowheads) and multinucleated giant cells localized to the pericystic region (arrows).

Immunohistochemical Findings

The immunopositivity scores of the control and infected (CE) groups are presented in Table 2. When compared to the control group, the infected group displayed significantly higher IFN-γ levels. IFN-γ expression was intracytoplasmic and localized mostly to the multinucleated giant cells in the periphery of the parasite cysts and to the inflammatory cells surrounding the giant cells. IFN-γ expression was of a fine granular appearance. Furthermore, IFN-γ-positive stainings were also observed in the alveolar macrophages found outside the parasitic granulomas (Figure 3).

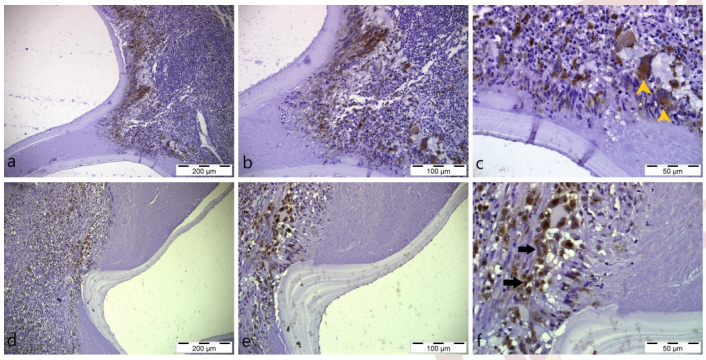

Figure 3. Lung, IHC, IFN-γ, **a-e:** Intracytoplasmic granular IFN-γ-positive stainings observed in the giant cells surrounding the parasitic granuloma (yellow arrowheads) and in the mononuclear cell infiltration (black arrows).

Discussion

As is the case with several other helminthic parasites, the causative agent of CE is known to have developed complex mechanisms to evade the cytotoxic effects of the host immune system (Al-Qaoud and Abdel-Hafez, 2008). Thus, CE causes immune imbalance. This parasitic infection is characterized by prolonged interplay with the host. Th1/Th2-type immune responses play a major role in this interplay (Xian et al., 2022). The parasite disrupts the Th1- and Th2-mediated immune balance of the host by means of certain antigens and produces various inhibitory cytokines to evade host immunity, and thereby, may survive for relatively long periods (Shirgholami et al., 2021). It is reported that while Th1-type immune responses increase in the early phases of CE, the late phases of the disease are associated with Th2-type immune responses (Atmaca, 2022). The early phase of CE has been indicated to be associated with increased Th1-type responses and particularly increased IFN-γ levels (Zhang et al., 2021). Cytokines, including IFN-γ, play a critical role in the inhibition of parasitic growth and the development of resistance against parasitic infection (Shirgholami et al., 2021). Nevertheless, only very limited information is available on immune reactions that occur during the early phases of parasitic development (Yildiz et al., 2022). Th-1 and Th-2 cytokines fight against parasitic infection through different immune pathways (Abo-Aziza et al., 2020). While Th-1 cytokines coordinate the cellular immune response, Th-2 cytokines coordinate the humoral immune response (Wang et al., 2016). The cellular immune response results in the granulomatous infiltration of the periparasitic tissue (Dvoroznáková et al., 2009), and this response depends on the interaction between macrophages and T lymphocytes (Dvoroznáková et al., 2008). In the present study, it was determined that IFN-γ levels had significantly increased in the animals infected with CE, compared to the healthy controls. This confirms that, as a major Th1-type cytokine involved in the immune response against parasitic infection, IFN-γ indeed plays a central role.

In sheep and humans, hydatid cysts are enclosed by a fibrous capsule, which is infiltrated by various types of cells, including macrophages and T and B lymphocytes. In some cases, eosinophil leukocytes may also be present. However, eosinophils are mostly localized far away from the parasite (Atmaca 2022). In agreement with previous research (Hashemnia et al., 2019; Moudgil et al., 2020; Al Malki and Ahmed, 2022; Gädicke et al., 2022), in the present study, the inflammatory reaction that had developed against the hydatid cysts were of a proliferative granulomatous nature. The pericystic tissue contained multinucleated giant cells, mononuclear cells and a few eosinophil granulocytes.

Macrophages are among the most important elements of natural immunity, and play a critical role in inflammation and host defense. Macrophages are classified under two groups. M1 macrophages eliminate pathogens by increasing the synthesis of nitric oxide synthase (NOS). M1 macrophages also trigger the proinflammatory and Th1 responses. On the other hand, M2 macrophages have immunoregulatory functions and are involved in tissue repair. M1 macrophages are generally activated by IFN-γ (Atmaca et al., 2022). The protective effect of IFN-γ in cases of parasitic infection is closely related to the activation of macrophages, monocytes, endothelial tissue cells and the increase of both the major histocompatibility complex (MHC) and reactive nitrogen compounds (Rahdar et al., 2020). IFN-γ controls intraparasitic infections by increasing the phagocytic capacity of macrophages (Ma et al., 2014; Ma et al., 2016). Literature review clearly demonstrates that cytokines such as IFN-γ can be effectively used in the

treatment of echinococcosis (Dvoroznáková et al., 2008; Dvoroznáková et al., 2009). In the present study, IFN-γ-positive stainings were mostly concentrated to multinucleated giant cells. In addition to giant cells, mononuclear inflammatory cells in the pericystic tissue also reacted positively for IFN-γ expression. Intracytoplasmic IFN-γ-stainings were also observed outside the parasitic cysts, particularly in the alveolar macrophages. The intensity and severity of the stainings were much stronger in the giant cells and macrophages, compared to the other inflammatory cells. All these immunohistochemical findings are in support of the opinion that IFN-γ contributes to the control of parasitic infection by increasing the phagocytic capacity of macrophages.

In conclusion, while many human medical studies have been published on the evaluation of immune responses against CE, there are only very few veterinary medical studies available on CE immunology in animals, in particular sheep. In this respect, the present study is expected to make a significant contribution to scientific literature in this field. Furthermore, the statistically significant increase detected in the IFN-γ levels of the infected animals, compared to the healthy control subjects, demonstrates the significance of this cytokine in the diagnosis of parasitic infections such as CE. The IFN-γ-stainings having been determined to be much stronger in giant cells and macrophages, which predominate the cellular immune response against parasitic infection, further demonstrates that this pro-inflammatory cytokine could be effective in anti-echinococcus treatment.

Acknowledgements: The authors thank Dr. Mushap Kuru for hiscontribution to the statistical analysis

Funding: This research is not funded by any organizations

Ethical approval: This study was approved by the Local Ethics Board for Animal Experiments of Kafkas University (KAÜ-HADYEK/2023-034).

Conflict of interest: Not applicable

Author contribution: E.K: Idea, concept, writing, histopathological and immunohistochemical analysis; S.K: Material collection; A.Y: histopathological and immunohistochemical staining

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