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RESEARCH ARTICLE

Immunohistochemical Investigation of TNF-α and IFN-γ Expressions in Sheep Fetuses with Brucellosis

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ABSTRACT

The purpose of the study is to evaluate the expression of TNF-α and IFN-gamma (IFN-γ) in the lung and liver tissues of aborted sheep fetuses using immunohistochemical methods and determine, which type of cells specifically secrete TNF-α and IFN-γ. Moreover, the roles of these cytokines in the immune response and inflammatory reaction associated with the disease were evaluated. The materials used in the study consisted of abomasum contents and lung and liver tissue samples from 114 aborted sheep fetuses. Of the 114 fetuses brought to the Microbiology and Pathology Department, Faculty of Veterinary Medicine for routine histopathological and microbiological diagnoses. As a result of cultural and phenotypic analysis, *Brucella* spp. were isolated from 100 (87.71%) of 114 fetal tissue samples and all of them were identified as *B. melitensis*. These isolates identified as *B. melitensis* were also confirmed by the Bruce-ladder PCR in the presence of relevant amplified products representing specific gene regions. Bronchopneumonia and hepatitis were the most important pathological lesions. TNF-α and IFN-γ immunoreactivities were significantly increased in the brucellosis group compared to the control group. In conclusion, it is supported by the data obtained from this study that cytokines such as TNF-α and IFN-γ were highly effective in the cellular immune response (Th1) developed against ovine brucellosis.

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INTRODUCTION

Brucellosis, caused by the bacteria *Brucella*, affects animals (sheep, cattle, pigs, dogs, etc.), wildlife and humans. As one of the most prevalent zoonotic diseases worldwide, it is responsible for significant economic losses due to abortion, stillbirth, infertility, and reduced milk production in livestock (Dorneles *et al.*, 2015). The Brucellosis Expert Committee of the World Health Organization divided *Brucella* into 6 species and 19 types: *Brucella melitensis* (3 subtypes), *Brucella abortus* (8 subtypes), *Brucella suis* (5 subtypes), *Brucella ovis* (1 subtype), *Brucella neotomae* (1 subtype), and *Brucella canis* (1 subtype) (Zhou *et al.*, 2020). *B. melitensis* and *B. ovis* are the primary agents of ovine brucellosis such as sheep and goats (Bayu, 2018). Isolation, molecular techniques (PCR), histopathology and immunohistochemical techniques are methods frequently used to diagnose brucellosis (Unver *et al.*, 2006; Yesilmen *et al.*, 2018).

Brucella spp. are facultative intracellular bacteria whose disease-causing capacity depends on invasion, long-term survival, and proliferation within host cells (Galindo *et al.*, 2009; Poester *et al.*, 2013; Adem and Duguma, 2020). The cellular immune response to many intracellular bacterial infections, including brucellosis, listeriosis, and tuberculosis, plays a critical role in the host defense (Salas-Téllez *et al.*, 2005; Sözmen *et al.*, 2010; Priyanka *et al.*, 2021a). The course of brucellosis is determined by the different T cell subtypes (Th1 and Th2) that results from the production of specific cytokines (Dorneles *et al.*, 2015; Priyanka *et al.*, 2021b) those play a key role in the pathogenesis of *Brucella*. The Th1/Th2 ratio can be involved in resistance or susceptibility to *Brucella*

infection (Priyanka *et al.*, 2021a). Macrophages have the role of stimulating and shaping adaptive immune response through cytokine production and antigen presentation. Bactericidal activity of macrophages increases with the activation of cytokines such as tumor necrosis factor-alpha (TNF-α) and interferon (IFN), which, together with lipopolysaccharides, act as key players in brucellosis (Adem and Duguma, 2020; Priyanka *et al.*, 2021a; Priyanka *et al.*, 2021b).

The purpose of the study is to evaluate the expression of TNF-α and IFN-gamma (IFN-γ) in the lung and liver tissues of aborted sheep fetuses using immuno-histochemical methods and determine which type of cells specifically secrete TNF-α and IFN-γ. Moreover, the roles of these cytokines in the immune response and inflammatory reaction associated with the disease were evaluated.

MATERIALS AND METHODS

Ethical Approval: This study was approved by the Kafkas University Animal Experiments Local Ethics Committee (KAU-HADYEK-2019/125).

Animals: The materials used in the study consisted of abomasum contents and lung and liver tissue samples from 114 aborted sheep fetuses. Of the 114 fetuses brought to the Microbiology and Pathology Department, Faculty of Veterinary Medicine for routine histopathological and microbiological diagnoses between 2013-2021, 86 (75.43%) were at 3-5 months of gestation, 18 (15.78%) were at 1-3 months of gestation, and 10 (8.77%) lived between 1 and 16 hours. Lung and liver tissues were evaluated because macroscopic and microscopic findings related to brucellosis are known to be more severe in these tissues. Placenta tissues could not be assessed as they were not provided. Metritis, high fever, weight loss, diarrhea, mastitis, and lameness in pregnant sheep were recorded according to the animal owner's information. In addition, 10 lung and liver samples obtained from fetuses aborted due to non-infectious causes (trauma, etc.) were evaluated as controls.

Isolation and identification by culture: Investigation of *Brucella* spp. from fetal tissue samples was performed by in vitro culture (Büyük and Şahin, 2011). Tissue samples were inoculated directly onto blood agar (Thermo Fischer Sci, CM0271B) containing 7% sheep blood (SBA) and *Brucella*-selective agar medium base (Oxoid CM0169) with a *Brucella*-selective supplement (Oxoid SR0083) and left to incubate aerobically and microaerobically at 37°C for 5–7 days. *Brucella* spp. identification was performed by phenotypically evaluating growth characteristics, macroscopic and microscopic morphologies, hydrogen sulfide activity, and agglutination reactions with A and M antigen monospecific sera (Alton *et al.*, 1988).

Identification by bruce-ladder PCR: Identification of *Brucella* spp. was conducted using the Bruce-ladder PCR method, which allows differentiation of all identified classic *Brucella* spp. (López-Goñi *et al.*, 2008). Genomic DNA extraction from fresh *Brucella* cultures on SBA was conducted with single colony lysis buffer solution supplemented with heat treatment (Marmur, 1961). Bruce-ladder PCR was adjusted to 25µl per sample with 3µl template DNA, 5µl 5XLongAmp™ Taq (Mg-free) Reaction Buffer, 1µl $MgCl_2$ (20 mM), 0.75µl dNTP mix (0.2mM), 1µl of each primer (12 pieces) (20pmol), 1µl LongAmp® Taq DNA Polymerase (5U), and 2.25µl nuclease-free water. The thermal cycle of the Bruce-ladder PCR was carried out by initial denaturation at 95°C for 3 min, followed by 30 cycles with denaturation at 95°C for 35 sec, primer attachment at 62°C for 45 sec, elongation at 65°C for 3 min, and finally elongation at 65°C for 10 min. The amplified products were analyzed using 1.5% agarose gel electrophoresis, and the simultaneous presence of products of 1682, 1071, 794, 587, 450, and 152bp for *B. melitensis* were evaluated. *B. melitensis* biotype 1 (16M) and *B. abortus* biotype 1 (544) were used as controls.

Macroscopy: Detailed photographs were taken of the fetuses before and after necropsies. The condition of vital organs, such as lungs (pneumonia, etc.) and liver (hepatitis, etc.), presence of any fluid in the abdominal cavity, abomasum contents, and fetal edema were evaluated. Macroscopic lesions observed were recorded for each case separately.

Histopathology: Lung and liver tissue samples from both *Brucella*-infected (pneumonic) and healthy animals were processed routinely. Fixated in 10% neutral buffered formalin, embedded using paraffin wax, 5µm, sections were taken, stained with Hematoxylin-Eosin (H&E). Examinations were performed under a light microscope (Olympus Bx53) and detected histopathological changes were photographed.

Immunohistochemistry: The routine streptavidin-biotin peroxidase complex method was used for immuno-histochemistry (Thermo Scientific Histostain-Plus IHC Kit, HRP, broad-spectrum, REF: TP-125-HL). Anti-*Brucella* (Becton Dickinson, Cat: 240934, polyclonal, 1/1000 dilution ratio), anti-TNF-α (MyBioSource, MBS2025729, monoclonal, 1/100 dilution ratio), and anti-IFN-γ (MyBioSource, MBS2091397, polyclonal, 1/100 dilution ratio) antibodies were used after antigen retrieval and nonspecific protein blocking. The reactions were detected with aminoethyl carbazole (AEC) chromogen (Thermo Scientific, TA-125-HA). Counterstaining was conducted using hematoxylin. Glass slides were mounted with AEC special adhesive and a coverslip. Instead of the primary antibodies, drops of PBS were applied to the control lung tissue sections. Same microscope (Olympus Bx53) was used for examining slides and Cell^P software (Olympus Soft Imaging Solutions GmbH, 3,4) was used for photographing. Images were analyzed with the Image J Program (1.51j8). Immunohistochemical analysis of TNF-α and IFN-γ was performed using a grading system for positively stained cell numbers on sites with intensive staining characteristics. A total of 5 different areas on each slide were examined at 40X magnification. The rating system was as follows: (−) no immunoreactivity; (+) low = 1%–10% positive cells; (++) moderate = 11%–59% positive cells; and (+++) intense = more than 60% positive cells.

Statistical analyses: SPSS® software (SPSS 26.0, Chicago, IL, USA) was used for statistical analysis. A Mann-Whitney U test was used for pairwise comparisons of TNF-α and IFN-γ expressions according to positive cell scoring. P<0.05 was accepted as statistically significant.

RESULTS

Microbiology: As a result of culture and phenotypic analyses, *Brucella* spp. Were isolated from 100 of 114 (87.71%) fetal tissue samples, and all were identified as *B. melitensis.* These isolates identified as *B. melitensis* were also confirmed by Bruce-ladder PCR with the presence of relevant amplified products representing specific gene regions (Fig. 1).

Macroscopy: Varying degrees of subcutaneous edema were detected in the fetuses infected with *B. melitensis*. There were hemorrhagic fluid accumulations in the abdominal and thoracic cavities. Inflammation was observed in the pleura. In the lungs, pneumonia was the most striking macroscopic finding. The color of the lungs became darker, and the tissue consistency hardened. Rib impressions were evident on the lung. The liver was enlarged, and pale, whitish foci were detected on the cross-sectional surface. In addition, the content of the abomasum was quite dense and coagulated with a lemon yellow color which was considered abnormal. There were no remarkable macroscopic findings in other organs (Fig. 2).

Histopathology: No pathologic lesions were detected in the lungs of the control animals as normal histologic structure was observed. In fetuses with brucellosis, interstitial and bronchopneumonia were dominant findings in the lungs. There was inflammatory cell infiltration, mainly in the bronchioles, where macrophages, lymphocytes, and neutrophils made up the majority of cells. In addition to these findings, edema with interalveolar septal hyperemia was detected. Similar to the bronchiolar lumens, there were varying amounts of the same type of inflammatory cell infiltration in the alveolar lumens, primarily macrophages, lymphocytes, and neutrophils. In addition to these cells, dense cellular debris and fibrin had accumulated in the alveolar lumens (Fig. 3 a-b-c). It was determined that the livers of the control animals had a normal structure and did not exhibit any pathologic changes. At the same time, severe degeneration and necrosis of hepatocytes were recorded in the livers of *Brucella*-positive fetuses. Areas of mild and intense inflammatory cell infiltration were found, especially around the vena centralis or portal triad. While most of these cells were mononuclear, small numbers of neutrophils were detected (Fig. 3 d-e-f).

Immunohistochemistry: Group immune positivity scores are shown in Table 1. The control group was negative for *Brucella* spp., TNF-α, and IFN-γ reactions. TNF-α and IFN-γ immunoreactivities were significantly increased in the brucellosis group compared to the control group. *Brucella* spp. Immune-positive reactions were detected in the cytoplasm of inflammatory cells (alveolar macrophages, lymphohistiocytic cells, and neutrophils), mainly in the exudates of the alveoli and bronchioles, in dark red and granular form. Inflammatory cell infiltration

Table 1: Immune positivity scores of groups

Groups		TNF-α	IFN-γ
Control (n=10)	Mean	0[a]	0[a]
	Median	0	0
	Minimum	0	0
	Maximum	0	0
	Percentiles25	0	0
	Percentiles75	0	0
Brucellosis (n=100)	Mean	2.7[b]	2.8[b]
	Median	3	3
	Minimum	2	2
	Maximum	3	3
	Percentiles25	2	3
	Percentiles75	3	3

Footnote: [a-b]: Expresses the statistical difference between groups (P<0.001). Positive cell scoring is shown in the table as negative (-) = 0, low (+) = 1, moderate (++) = 2, and severe (+++) = 3

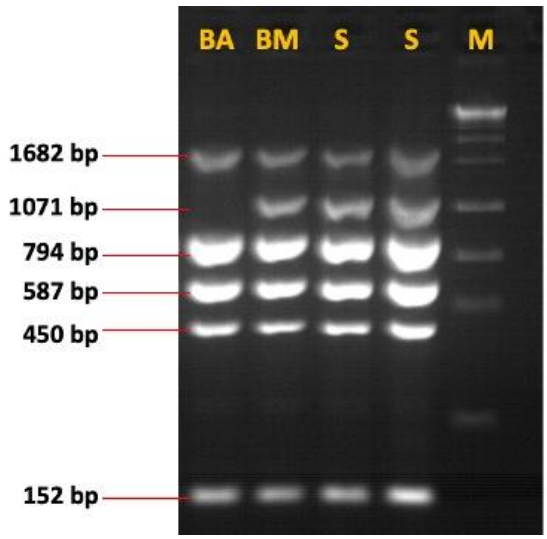

Fig. 1: 1.5% agarose gel electrophoresis images of Bruce-ladder PCR products. BA: *B. abortus* biotype 1 (544), BM: *B. melitensis* biotype 1 (16M), S: Field sample (*B. melitensis*), M: HyperLadder™ 100bp Plus DNA ladder (BIOLINE, 33071).

in the bronchioles and alveolar lumens, cellular debris, and dense *Brucella* antigens in the cytoplasm of glandular epithelial cells around the bronchi and bronchioles were observed. In particular, TNF-α and IFN-γ positive stains were detected in alveolar macrophages, neutrophils, and lymphohistiocytic cells in the alveolar and bronchiole lumens of the lung. The reactions were dark red in color and granular in the cytoplasm of the aforementioned cells. The intensity of immune-positive expressions was more pronounced, particularly in areas where inflammatory cell infiltration was intense (Fig. 4 a-b-c). In the cytoplasm of hepatocytes and Kupffer stellate cells in the liver, *Brucella* spp. Positive reactions were detected. *Brucella* spp., TNF-α, and IFN-γ immunoreactivities were primarily observed in inflammatory cell infiltration, especially mononuclear cells around the vessels and portal triad. Immune positivity was detected mainly in the cytoplasm of lymphohistiocytic cells, and plasma cells were more remarkable in regions where the inflammatory reaction was severe (Fig. 4 d-e-f).

DISCUSSION

Bacteriological isolation, PCR, histopathology, and immunohistochemistry are frequently used reliable methods to definitively diagnose *Brucella* agents (Unver *et al.*, 2006; Büyük *et al.*, 2011; Yesilmen *et al.*, 2018).

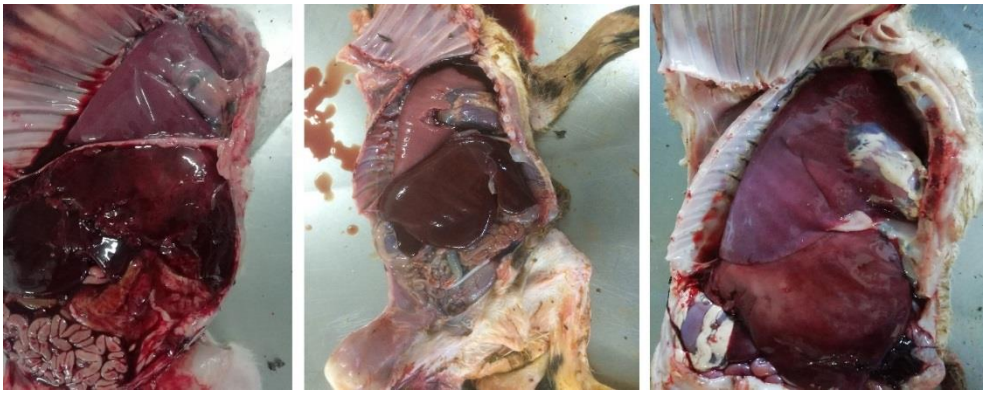

Fig. 2: Pneumonia in the lung, enlargement of the liver and the pale areas on the cross-section, hemorrhagic fluid accumulation in the abdominal and thoracic cavities.

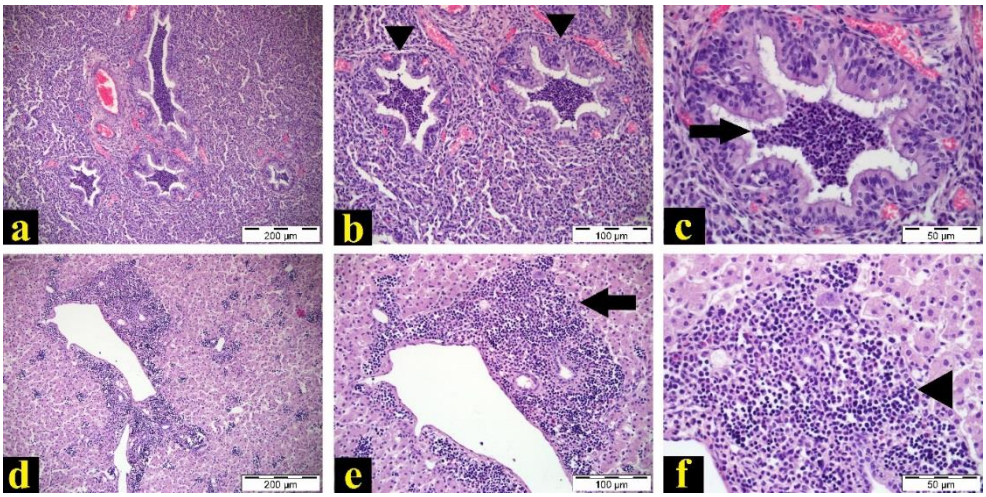

Fig. 3: a= Lung, bronchopneumonia, b= Inflammatory cell infiltration in bronchiole lumens (arrowheads), c= Higher magnification, cell infiltration mostly composed of neutrophil leukocytes (arrow), d= Liver, hepatitis, e= Intense cell infiltration around the vena centralis and portal triad (arrow), f= Inflammatory cell proliferation (arrowhead) mostly composed of mononuclear cells and few neutrophil leukocytes.

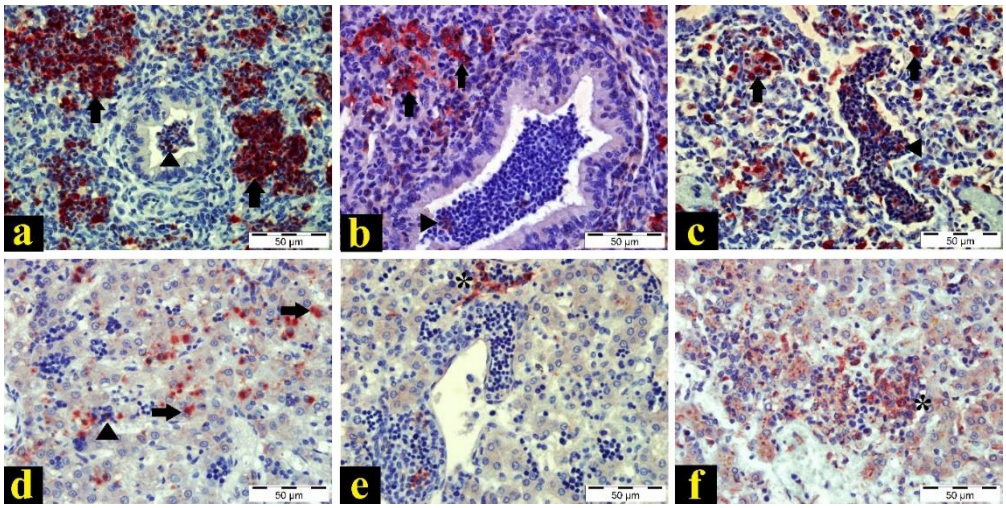

Fig. 4: a= *Brucella* spp. positive reactions (arrows) on macrophages and neutrophils in the alveolar lumens and inflammatory cells in the bronchiolar lumen (arrowhead), b= Immune positive TNF-α reactions in lympho-histiocyte cells and macrophages in alveolar lumens (arrows) and inflammatory cells in bronchiole lumen (arrowhead), c= IFN-γ positive staining in alveolar macrophages, lymphocytes (arrows) and inflammatory exudate in the bronchiole (arrowhead), d= In the cytoplasm of hepatocytes (arrows) and kupffer stellate cells (arrowhead) *Brucella* spp. existence, e= TNF-α immune positive reactions in inflammatory cells (*) around the vessel, f= Intracytoplasmic IFN-γ expressions in lympho-histiocyte cells around the vena centralis.

The macroscopic, histopathologic, and immunohisto-chemical findings observed in the lungs and livers of *Brucella*-positive sheep fetuses in the current study were consistent with the literature (Salas-Téllez E *et al.*, 2005; Poester *et al.*, 2013; Bayu, 2018; Yesilmen *et al.*, 2018).

Cytokines are key molecules that play significant roles in determining protective immune responses (Li *et al.*, 2021). *Brucella* spp. can stimulate the secretion of cytokines responsible for regulating immune and inflammatory responses (Eskandari-Nasab and

Moghadampour, 2018). In particular, proinflammatory cytokines enable the emergence of antimicrobial responses in monocytes and macrophages, which have a significant role in defending against intracellular pathogens such as *Brucella* spp. (Ritchie *et al.*, 2012).

TNF is a proinflammatory cytokine that regulates host responses to microbial challenges and initiates the expression of chemokines and adhesion molecules required for the recruitment of neutrophils to the site of infection, which is particularly important for the restriction of initial infection (Hop *et al.*, 2017). TNF-α increases the elimination of *Brucella* spp. by activating bactericidal activities in macrophages, thus regulating the protective cellular immune response against this infection (Antunes *et al.*, 2013; Zhu *et al.*, 2016; Adem and Duguma, 2020). TNF-α also induces CD8+ cytotoxic T lymphocyte-mediated cytotoxicity against macrophages infected with *Brucella* spp. (Priyanka *et al.*, 2021a). TNF-α, produced from many cells such as neutrophils, natural killer cells, macrophages, lymphocytes, and fibroblasts, is a vital mediator for clearance of brucellosis infection from a host (Eskandari-Nasab and Moghadampour, 2018). This study detected TNF-α positive reactions mostly in alveolar macrophages, neutrophils, and lymphohistiocytic cells in lung tissue. In the liver, TNF-α positive reactions were primarily observed in mononuclear cells around vessels and Kupffer stellate cells. Inhibition of the TNF-α response in human and mouse macrophages in the initial stages of brucellosis is associated with greater tolerance of these cells to bacterial proliferation and thus the ability of *B. abortus* to overcome the host's innate immune response and establish infection (Dorneles *et al.*, 2015). It is known that serum levels of proinflammatory cytokines such as TNF increase significantly in brucellosis cases (Li *et al.*, 2021). Hop *et al.*, 2017 reported that TNF expression and secretion were significantly induced due to *B. abortus* infection. Eskandari-Nasab and Moghadampour, 2018 demonstrated a link between polymorphism in the TNF-α gene and an increased risk of brucellosis. Priyanka *et al.*, 2021b revealed rhythmic induction of the cytokine profile by enhanced expression of genes encoding IFN-γ, IL-1β, IL-6, and iNOS, and reduced expression of TNF-α, IL-4, and IL-12p40, showing the transformation from a predominantly Th1-like model to a Th2-like model, which contributes to the development of chronic brucellosis of cattle. Consistent with the literature data (Li *et al.*, 2021), it was determined that TNF-α expression was significantly increased in cases with brucellosis compared to the control group. Although many studies evaluated TNF-α levels in association with human, mouse, or bovine brucellosis, there is no literature detailing the evaluation of brucellosis in sheep. Cells that showed positive staining for TNF-α expression were mostly alveolar macrophages, neutrophil leukocytes, Kupffer stellate cells and mononuclear cells. In the light of these findings, it was concluded that TNF-α triggered the infiltration of immune system cells into the inflammatory region in ovine brucellosis and played a role in the elimination of bacterial agents. In this study, the remarkable increase in TNF-α expression in sheep fetal tissues in parallel with the severity of the disease suggests that this proinflammatory cytokine is also active in the cellular immune defense associated with ovine brucellosis.

IFN-γ is an important proinflammatory cytokine critical to the host's protective cellular immune response after *B. abortus* infection (Zhu *et al.*, 2016; Feng *et al.*, 2017; Zhou *et al.*, 2020). IFN-γ is produced mainly by $CD4^+$, $CD8^+$, and $CD5^+$ T lymphocytes (Pérez-Sancho *et al.*, 2014; Agnone *et al.*, 2019). IFN-γ functions to increase the antimicrobial capacity of macrophages (He, 2012; Eskandari-Nasab and Moghadampour, 2018). In vivo and in vitro experiments revealed that IFN-γ is the most potent inducer of anti-*Brucella* activity in monocytes and macrophages. Agnone *et al.*, 2019 reported that IFN-γ positive T lymphocytes increased in cattle samples infected with *B. abortus* compared to a control group. In a similar study, Feng *et al.*, 2017 found that IFN-γ levels in *Brucella*-infected samples were much higher than in vaccinated and negative control samples. Eskandari-Nasab and Moghadampour, 2018 suggested that polymorphism in the IFN-γ gene, similar to TNF-α, is an important parameter that increases the risk of brucellosis. Zhu *et al.*, 2016 found that IFN-γ and TNF-α levels increased remarkably in their *Brucella* vaccine study in mice. In another vaccine study in mice, Sancho *et al.*, 2014 observed that IFN-γ and TNF-α were at the highest level. Suraud *et al.*, 2008 revealed that IFN-γ is mainly produced by $CD4^+$, $CD8^+$, and $CD4^-$, $CD8^-$ γδ cells in sheep acutely infected with *B. melitensis*. Durán-Ferrer *et al.*, 2004 reported nonvaccinated diseased ewes dispersing the pathogen had antibody levels and antigen-specific IFN-γ profiles higher and notably different from those of vaccinated ewes that neither contracted *B. melitensis* nor excreted the pathogen. In this study, in parallel with the literature data, (Durán-Ferrer *et al.*, 2004; Feng *et al.*, 2017; Agnone *et al.*, 2019) it was determined that IFN-γ expression in the lung and liver tissues of fetuses naturally infected with *Brucella* spp. were increased statistically compared to the control group. In addition, IFN-γ was primarily produced from lymphocytes and macrophages, as previously reported (Durán-Ferrer *et al.*, 2004; Pérez-Sancho *et al.*, 2014; Agnone *et al.*, 2019). According to current study data, IFN-γ is also expressed by neutrophil leukocytes. This shows that this cytokine activates neutrophils as well as macrophages and lymphocytes. The similarity of TNF-α and IFN-γ results indicates that the two cytokines exert a synergistic effect especially in the cellular immunity acquired against this disease. Notably, IFN-γ expression increased especially in areas where inflammatory reactions were severe. These study data indicate that IFN-γ is a crucial cytokine involved in the cellular immune response of ovine brucellosis.

Conclusions: Cytokines such as TNF-α and IFN-γ are highly effective in the cellular immune response (Th1) developed against ovine brucellosis. No other study was found in the literature in which the levels of these cytokines and interleukins were evaluated in sheep fetal tissues. In addition, these proinflammatory cytokines increased in parallel with the severity of the inflammatory reaction. To better understand the cytokine profile associated with ovine brucellosis, different pro- and anti-inflammatory cytokines should be evaluated comparatively and in detail in future studies.

Authors contribution: EK designed and wrote the manuscript; EK, EB and SD performed immunohisto-

chemical and histopathological analysis; HN and AY performed immunohistochemical and H&E stainings. MK performed statistical analysis. ÖÇ, FB and DA performed the diagnosis of Brucella agents.

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Conflict of Interest: The authors declare that they have no conflicts of interest associated with this research.

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