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ORIGINAL ARTICLE

Evaluation of tumor necrosis factor-alpha (TNF-α); gamma interferon (IFN-γ) genes and oxidative stress in sheep: immunological responses induced by *Oestrus ovis* (Diptera: Oestridae) infestation

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Abstract This study aimed to evaluate the cell mediated immune responses against *Oestrus ovis* (*O. ovis*) in sheep through measurement of the changes in mRNA expression of the tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) cytokines using quantitative Real time-PCR (qRt-PCR). Also; to detect the role of *Oestrus ovis* infestation in the oxidative stress markers in sheep. Fifty sheep head were examined in Cairo abattoir from the period of May to August 2019. Sera were separated and collected for measurement of nitric oxide, zinc and malondialdehyde (MDA). While TNF-α and IFN-γ mRNA were extracted from nasal mucosa. Levels of IFN-γ and TNF-α were significantly higher in infested sheep than that in non-infested one. Also, oxidative stresses were indicated by high level of nitric oxide as one of reactive oxygen species (ROS) and serum MDA as oxidative stress marker and low antioxidant capacity (zinc concentration in serum) in infested sheep. The obtained results indicated that measurements of TNF-α and IFN-γ cytokines using *q*RT-PCR could be used as an association and reproducible quantitative method for the diagnosis of *O. ovis* infestation in sheep.

Keywords *Oestrus ovis* · Tumor necrosis factor alpha (TNFα) · Gamma interferon (IFN-γ) · Sheep nasal fly · Oxidative stress in sheep

Introduction

Oestrosis is the specific nasal myiasis that infests sheep and goat livestocks (Caracappa et al. 2000; Alcaide et al. 2003; Attia et al. 2019). It causes different clinical problems in infested animals as sneezing and nasal discharges which may lead to significant economic losses (Scala et al. 2001 and El-Tahawy 2010). It is caused by *O. ovis* larvae which are commonly present in Mediterranean, tropical and desert environments due to their ability to adjust their development to different climatic conditions in such areas (Alcaide et al. 2005). Molecules secreted and excreted by the larvae are the main cause of oestrosis pathogenesis through induction of a hypersensitivity immune reaction. Such stimuli are responsible for pathological damage and local systemic immune-stimulation (Tabouret et al. 2003; Angulo-Valadez et al. 2011). There is increasing interest to define the role of host immune response and the immune factors that help in control of infection and disease development (Donahoe et al. 2017). So, many researches focused on illustration how host immune system attack the larvae and how the larvae modulate their immune reactions to survive and develop inside the host. Previous studies recorded by Sandeman 1996 and Attia et al. 2019 on *O. ovis* larvae which are obligatory parasites that harbored and feed on the host tissues for long time, so they greatly stimulate cellular and humoral immune response. Also, Alcaide et al. 2005, Angulo-Valadez et al. 2011, Silva et al. 2012 and Attia et al. 2019 studied the humeral immune responses against *O. ovis.* While cellular immune response in sheep which were naturally infested with *O. ovis* needs

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further studies. Dubey and Schares 2011 reported that the efficient cell-mediated Th1 immune response that affected mostly by the cytokine gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) is very important for controlling disease by restriction of parasite replication and induction of chronic latent infections with *Neospora caninum* (*N. caninum*) and *Toxoplasma gondii* (*T. gondii*) through cyst formation.

Host immune system increases the generation of reactive oxygen species (ROS) as nitric oxide, superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) which used as defense mechanism which is very important in the host resistance to infection with parasite as in case of liver fluke (Kolodziejczyk et al. 2005), but high reactivity of ROS causes oxidative damage to host tissues causing what is called oxidative stress. So, the host cells to neutralize ROS harmful effects produce antioxidant enzymes, nutritional antioxidants and trace elements as zinc, copper, and iron. Oxidative stress was observed in several parasitic infections as the infections of sheep with *D. dendriticum* (Şimşek et al. 2006), *Fasciola hepatica* (Saleh 2008) and *Echinococcus granulosus* (Heidarpour et al. 2013). So, in the present study, the cell mediated immune responses against *O. ovis* in sheep was evaluated through measurements of the changes in mRNA expression of the tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) cytokines using Real time-PCR (*q*RT-PCR). Also, to measure the nitric oxide level and evaluate the relationship between malondialdehyde (MDA) as one of the oxidative stress markers due to infestation with *O. ovis* larvae in the nostrils, and to compare the trace elements (Zinc) in parasitized and healthy animals to evaluate the immunological response of the animals.

Materials and methods

Animal and sampling

Fifty sheep head (aged from 10 months to 2 years old) were examined in Cairo abattoir from the period of May to August 2019. Blood was collected from each examined sheep during slaughtering from jugular vein in two tubes; one tube containing 0.5 mg/ml EDTA (ethylene diamine tetra acetic acid) as an anticoagulant and other tube without EDTA for sera collection. The clotted blood was centrifuged at 3500 rpm for 15 min, and the sera were then collected and preserved at − 20 °C until used. Sagittal section of each examined sheep head was done for the detection of *O. ovis* larvae (all the collected larvae were 3rd stage larvae). All the collected larvae were identified according to Zumpt 1965 and Attia et al. 2019. Mucous from nasal passages and nasal sinuses were collected for oxidative stress markers measurement. All samples (sera, whole blood, feces, mucous from nasal passages and nasal sinuses) were transferred into Faculty of Veterinary Medicine; Cairo University for further analysis. *All Institutional and National Guidelines for the care and use of animals were followed.*

Hematological and parasitological examination

The anti-coagulated blood was used to prepare thin blood film from each anti-coagulated blood sample and stained with Giemsa stain to be examined for presence of any blood parasites. Fecal samples were examined for detection of any parasitic infestation using concentration techniques as described by (Soulsby 1986).

Biochemical analysis

Concentration of zinc in the collected serum samples (fifty sera samples were collected in which 30 sera were positive for *O. ovis* and twenty were negative for *O. ovis*); these sera were measured using ionized coupled plasma using mass spectrometry method (Page et al. 2018).

Measurement of oxidative stress markers

The level of malondialdehyde (MDA) in sera (the positive and negative sera); all the sera were measured according to Aktas et al. 2017. While nitric oxide level in positive and negative sera was detected according to Aytekin and Unubol Aypak (2011).

Evaluation of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) activity

Mucous samples from the infested nasal sinuses and its passages; which had the 3rd larval stages with a number of 3rd stage larvae ranged from 5 to 35 larvae; were aseptically dissected. Samples (mucous samples in the nasal sinuses and its passages) from 5 uninfected sheep were collected in a similar manner and used as negative controls. All the mucous were aseptically preserved in − 20 °C.

RNA isolation

Isolation of RNA from 100 mg of mucous and nasal sinuses were performed by total RNA kit (Ambion, Applied Biosystems), following the manufacturer's instructions. Homogenization of the tissues were applied in Lysing Matrix D tubes (MP Biomedicals) using a FastPrep-24 homogenizer (MP Biomedicals, 2 cycles of 30 s at 6 m/s). Nanodrop (Thermo Scientific) were used to measure the RNA purity and quantity. A 500 ng of RNA were made

with DNaseI amplification grade (Invitrogen) following the manufacturer's instructions. The reverse transcription of treated RNA was performed by High-Capacity cDNA Archive Kit (Applied Biosystems) (Picard-Sánchez et al. 2019).

Quantitive real-time PCR protocol (qRT-PCR)

PCR primer sets specific for tumor necrosis factor alpha (TNFα) and gamma interferon (IFN-γ) specific for sheep were designed and based on sequences deposited in the GeneBank (Table 1). β-actin was used as a reference gene and for sample normalization. The genes expression included in this study was tested on a separate pool of cDNA, generated from five un infested sheep previously examined for presence of any parasites.

PCR cycling conditions

Amplification was carried out for 40 cycles as follows: denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C and extension for 45 s at 72 °C. Real-time PCR protocol run according to Puech et al. (2015).

Statistical analysis

Results were analyzed using Predictive Analytics Software (PASW) Statistics, Version 18.0 software (SPSS Inc., Chicago, IL, USA). Blood parameters in diseases and control groups were compared by independent sample *T* test or Wilcoxon-Mann–Whitney test according to the normality of data. A *P*-value < 0.05 was considered statistically significant.

Results

Thirty examined sheep were positive for *O. ovis* larvae by postmortem examination of the heads with 60% prevalence rate during May to August 2019, the collected larvae were full mature 3rd stage larvae. The larvae were collected from the nasal passages; its sinuses and on the base of the two horns. The 3rd stage larvae were 15–25 (20 ± 0.5) mm in length and yellow to brown in colour. On the dorsal surface; the larvae had broad transverse brown to blackish bands which supported with several small denticles. On the ventral surface, each segment supported by 3 to 4 rows of irregularly placed strong spines.

Table 2 The intensity of infestation with *O. ovis* in sheep

Intensity of infestation	No. of sheep	% of sheep
5–10 larvae	15	50
11–20	11	36.6
21–35	4	13.3

The intensity of infestation with the total number of the 3rd instar of *O. ovis* larvae was recorded in 30 (60%) of the infested sheep during the period from May to August 2019. A total of 15 sheep had 5–10 larvae while 11 sheep had 11–20 larvae and 4 sheep harbored 21–35 larvae (Table 2).

Examined sheep showed significantly higher in nitric oxide [mean difference = 14.30 (± 10.44, ± 95% C.I.); t (4) = 3.802, $p = 0.019$] and serum MDA [18.00 (± 5.65, ± 95% C.I.); $t(4) = 8.848$, $p = 0.001$] levels than that in non-infected sheep (Table 2). Whereas we found that Zinc levels in infested sheep were -62.33 (± 8.38, ± 95% C.I.) lower than that of non-infested sheep (t (4) = − 20.651, $p < 0.0001$); Table 3.

Infested sheep showed significantly higher in INF-γ [mean difference = 16.00 (± 8.43, ± 95% C.I.); $t(4) = 5.269$, $p = 0.006$] and TNFα [17.50 (± 8.96, ± 95% C.I.); $t(4) = 5.422$, $p = 0.006$] levels than that in non-infested sheep (Table 3; Figs. 1;2).

Discussion

In Egypt, Sheep breeding is very important as most peoples in village and bed wins in the desert breed variable flocks of sheep due to its high fertility rate and low raising cost. Ovine parasitic infestations cause an important problem of economic losses (Ramadan et al. 2013). *O. ovis* larva is one of these parasites that affect the hosts performance, growth and animal production. In this study infestation rate of 3rd stage larvae of *O. ovis* during summer was found to be

Table 1 The sequences of the forward and reverse primer used in the quantitative real-time PCR

Gene	Sequence [5-3]	Accession number	References
INF-γ	F-CAGAGCCAAATTGTCTCCTTC R-ATCCACCGGAATTTGAATCAG	NM_001009803	Puech et al. (2015)
TNFα	F-CCAGAGGGAAGAGCAGTCC R-GGCTACAACGTGGGCTACC	NM_001024860	Puech et al. (2015)
β-Actin	F-TGGGCATGGAATCCTG R-GGCGCGATGATCTTGAT	NM_001009784	Puech et al. (2015)

Table 3 Oxidative stress markers, trace elements levels in healthy and *O. ovis* infested sheep (Mean ± SE)

	Healthy	Infested	Change (%)[a]	*P*-value
Nitric oxide (nmol/mg protein)	48.37 ± 0.19	62.67 ± 3.76	29.6	0.019*
MDA (nmol/ml)	12.33 ± 0.17	30.33 ± 2.03	146.0	0.001*
Zinc (μg/dl)	118.00 ± 0.58	55.67 ± 2.96	− 52.8	<0.0001*
IFN-γ	5.33 ± 0.88	21.33 ± 2.91	300.2	0.006*
TNF-α (U/ml)	5.50 ± 0.29	23.00 ± 3.21	318.2	0.006*

[a]Percentage of change between the mean values of the infested group (*O. ovis* infested sheep) and the healthy control group (group of non-infested sheep; with no *O. ovis* infestation and no any parasites); *Indicate significant difference at *p*-value < 0.05; *SE* Standard error

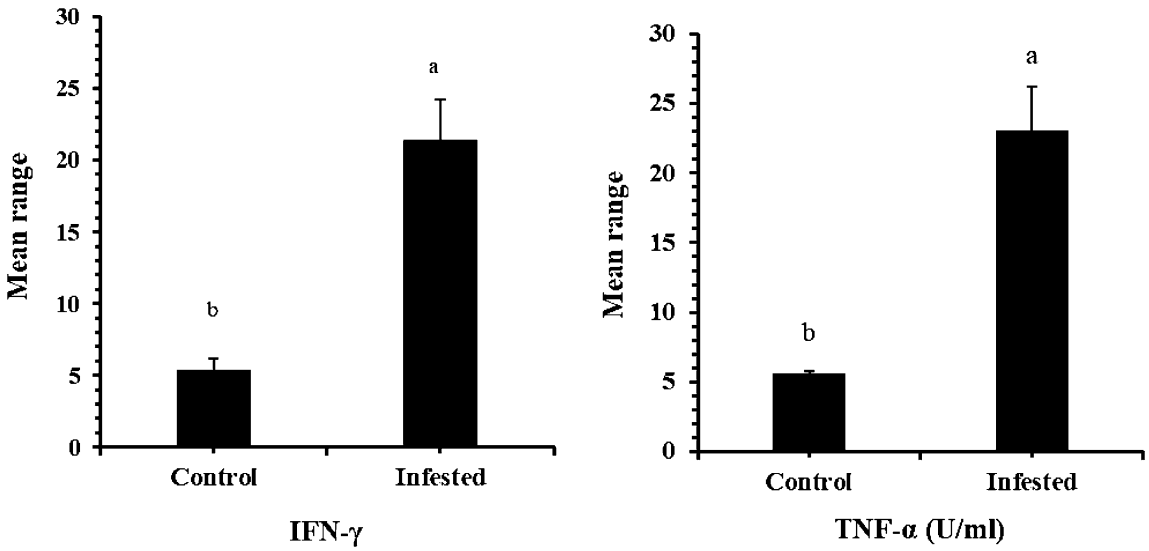

Fig. 1 Gamma interferon (IFN-γ) and tumor necrosis factor-alpha (TNF-α) activities in healthy and infested sheep with *O. ovis*. ([a,b]) The difference between healthy and infested group is significant ($P < 0.05$)

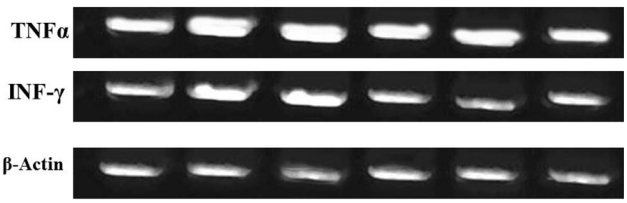

Fig. 2 Electrophoretic mobility of q RT-PCR products of TNFα and INF-γ versus β- actin as control (internal control) on 2% agarose gel

60%. This result was in agreement with Ramadan et al. 2013 who recorded that infestation rate was at peak during summer in Egypt. Also; Ahaduzzaman 2019 reported that *O. ovis* was a parasite of high prevalence rate in Africa and Europe and, Hidalgo et al., 2015 found that the prevalence of *O. ovis* in sheep slaughtered in Chile abattoirs was 60.9%. The high percentages of L3point out to the year-round development and that the flies present with at least three generations in year (Ramadan et al. 2013).

Many researches were done on prevalence, morphology and serodiagnosis of oestrosis in Egypt. As, that the study recorded by Gaaboub 1978, Hassanin et al. 1989, Amin et al. 1997, Ramadan et al. 2013 and Attia et al. 2019. But no researches were done on the cell mediated immune response against *O. ovis* in sheep. Cytokines play a key role in the immune system response to infectious parasites. So, lately cytokine level measurement was used in many researches to detect immune response against parasitic infestation in ruminants (Puech et al. 2015). As, they control the activation, differentiation of different types of cells and antibodies secretion (Mosmann and Coffman, 1989). So, differential cytokine production and expression is commonly used method to characterize disease pathogenesis in cattle (Coussens et al. 2004 and Boddu-Jasmine et al. 2008), goats (Singh et al. 2013) and sheep (Smeed et al. 2007 and Channappanavar et al. 2012). The cytokine balance is analyzed by detection of the secreted cytokine proteins quantity or by the analysis of cytokine mRNA

expression (Giulietti et al. 2001). So, our study was performed to evaluate the cell mediated immune responses against *O. ovis* in sheep through measurement of the changes in mRNA expression of the tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) cytokines using Real time-PCR (qRT-PCR).

In this study, infested sheep showed significantly higher IFN-γ and TNF-α levels than that in non-infested sheep. These results agree with that recorded in different parasitic infections as (*Toxoplasma gondii*; *Neospora caninum*; *Demodex* mites and malaria infection) by Donahoe et al. 2017; Lacey et al. 2018 and Nasr et al. 2014 which recorded significant high IFN-γ and TNF-α levels. This data was considered as a beginning of further researches on the functional and genetic analysis of the TNF-α and IFN-γ genomic region in the oestrosis infestation of sheep.

The fecal and blood samples reveal no other associated parasites which prove that the risen in the gene expression was due to *O. ovis* infection.

Moreover, oxidative stress and changes in antioxidants capacity was studied. Infested sheep showed significantly higher nitric oxide level as one of ROS than that of non-infested sheep. High level of ROS causes the production of MDA by initiating the peroxidation of membrane un-saturated fatty acids (Uner et al. 2001). This explain the higher level of serum MDA in infested sheep than that of non-infested sheep. While antioxidant capacity (Zinc level) in infested sheep was lower than that of non-infested sheep. As the host body needs an adequate amount of trace elements for the structure and function of some antioxidant enzymes that is important in the cells protection against toxic effect of ROS (e.g., zinc and copper for superoxide dismutase and iron for catalase (Munoz et al. 2007). Also, Samadieh et al. 2017 reported decreased zinc and iron concentrations in the parasitized animals with dicrocoeliasis explaining that stress could result in losses of trace elements during parasitic infections. This result showed that oestrosis in sheep cause a case of oxidative stress indicated by high level of serum MDA and low antioxidant capacity (zinc concentration in serum). Other studies reported oxidative stress in sheep suffering from dicrocoeliasis (Şimşek et al. 2006 and Samadieh et al. 2017).

Conclusion

In this study, the cell mediated immune response against infestation with *O. ovis* larvae in sheep was studied. The higher of TNF-α and IFN-γ cytokines levels measured by *q*RT-PCR could be associated with positive oestrosis diagnosis even in low levels of cytokine mRNA. This study pointed out to the role of *O. ovis* larvae in the production of a case of oxidative stress indicated by high level of nitric oxide and MDA with low antioxidant capacity by zinc concentration in sera of infested sheep.

Authors Contribution All authors sharing in the aim of works; Marwa M. Attia: collect the samples and identify the clinical sign on the sheep; Marwa M. Attia and Sohila M. El-Gameel Identify the parasites; applying the clinical works; Elshaimaa Ismael applying of statistical analysis of the results. All authors sharing in writing and revised this manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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