

## Clinicohistopathological and Immunological Alterations in Egyptian Donkeys Infested by *Rhinoestrus* Spp. During the Winter Season

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**L**ITTLE information is available about the homeostasis and alterations appeared against infestation by nasal myiasis caused by *Rhinoestrus* spp. larvae in Egyptian donkeys. Therefore, the present work aimed to investigate the hematological and biochemical profiles associated with histopathological features and immunological response developed against *Rhinoestrus* spp. larvae infestation in donkeys in the winter season. The results revealed that 40 out of 80 examined donkeys were infested by nasal myiasis. IgG values were higher ( $P < 0.01$ ) in infested donkeys (IND) than non-infested ones all over the winter months while the highest mean larval burden was observed in December (11.6 larvae/animal). Neutrophils% in (IND) increased ( $P < 0.01$ ) during December and February. However, an elevation in lymphocytes% ( $P < 0.05$ ) was observed in (IND) during December and January while eosinophils% increased ( $P < 0.01$ ) in all winter months. These results paralleled to the nasal fluid cytological profile in which lymphocytes %, neutrophils% and eosinophils% in (IND) increased ( $P < 0.05$ ) in December, January and February. Glutathione peroxidase, superoxide dismutase and catalase activities in (IND) were declined ( $P < 0.01$ ) in all winter months. Interleukin-2 (IL-2) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) elevated ( $P < 0.05$ ) in December and January. The histopathological picture of turbinate bones, olfactory bulb and meninges reflected the blood and nasal fluid cytological picture as there were lymphocytes infiltration, meningitis and satellitosis. The results indicated that *Rhinoestrus* spp. infestation in donkeys might affect the vital and immunological processes. In addition, *Rhinoestrus* spp. infestation in donkeys could be linked to oxidative stress and shifting the immune response polarization towards Th2 cytokines.

**Keywords:** *Rhinoestrus* spp, Clinicohistopathological, Cytokines, Antioxidant, Donkeys.

### Introduction

Rhinoestrosis is a nasal bot myiasis caused by larvae of *Rhinoestrus* spp (Diptera: Oestridae) and occurs worldwide in both developing and developed countries [1]. Larvae are deposited by adult fly into host's nostrils. The first instar larvae developed into the second instar and then third instar larvae habituated at the nasal cavities, sinuses and pharynx of equids. Their maturation depends on the climatic conditions thus, it is almost impossible to generalize the timing of the life cycle of *Rhinoestrus* spp [2]. Pathogenesis of rhinoestrosis varied from asymptomatic to death of horses and donkeys. These larvae cause traumatic irritation to mucus membrane of nasal cavities and sinuses due to its creeping

and cuticle's spines. In addition, damages to olfactory nerve and encephalomyelitis occur due to penetration of the larvae in the ethmoid and meninges [3]. This affection may induce local inflammation and causes clinical signs of varying intensity and severity ranging from inflammation to dyspnea, sneezing and cough [2].

Infestations by these larvae stimulate defense immune mechanism strategies to survive with host's humoral and cell mediated immune responses. This immune response efficiency depends on health, physiological and nutritional status of animal beside site of parasitism and nature of antigenic determinants to recall immunological defense mechanisms [2]. Infiltration of inflammatory cells (mast cells,

eosinophils and macrophages) and secretions of immunoglobulin, T-helper1 (Th1) and T-helper2 (Th2) are characteristic of Oestridae nasal bot larvae infestations [4]. Diagnosis of such myiasis is very difficult in live animals. It usually depends on parasitological detection of infested larvae at post-mortem examination [5]. Previous literatures about life cycle, seasonal variations of *Rhinoestrus* spp [6] and characterization of different antigens of *Rhinoestrus* spp [1,7] were studied. However, little information is available about the host immune response and alterations appeared against *Rhinoestrus* spp. Larvae.

Hence, the present work aimed to investigate IgG and cytokines immune response combined with hematological profile and pathological features developed against *Rhinoestrus* spp larvae for understanding its chronobiology, which will help in planning the critical period for its diagnosis, treatment and control.

## **Material and Methods**

### *Animals and sampling*

This study was conducted in the winter season, starting from December 2015 to March 2016 at temperature ranged from 12- 18°C and relative humidity (RH) varied from 40-60%. The samples were collected from 80 working donkeys after slaughtering at zoo abattoir (Giza, Egypt) in winter season (December 2015 to March 2016) with average 3 visits/month. The donkeys were brought to the Zoo from Fayoum, Beni-Suef and Monofia governorates according to the general administration of the Zoo. Before slaughtering, the nasal fluid was collected from each animal according to Zhang *et al.* [8]. Briefly, a syringe containing 10 ml PBS (pH 7.2) was flushed into the nasal cavity and quickly suctioned or “re-aspirated” back into the syringe. The re-aspirated fluid contains lining cells, secretions and other material that has been “washed off” by the flushing action to be exposed to the cytological examination. Blood samples of slaughtered donkeys were collected immediately after slaughter. Half of the blood sample was taken into EDTA containing vacutainer tubes for hematological study. The other half was maintained in plain tubes and allowed to clot for serum extraction then divided in small aliquots and stored at -20°C for immunological and biochemical assays. The corresponding heads of the slaughtered donkeys were longitudinally cut and examined by naked eyes for the presence of

*R.purpureus* larvae. Larvae of each head were collected and separately placed in vial and then transported to the laboratory for identification. Corresponding tissue specimens from turbinate bones, olfactory bulb, meninges and brains were collected from all slaughtered donkeys for histopathological examination.

### *Identification of Rhinoestrus spp larvae*

All collected larvae were washed in phosphate buffer saline (PBS, 7.2 pH) and microscopically identified into L1, L2 and L3 of *Rhinoestrus* spp larvae [9].

### *Specific IgG response*

The specific IgG humoral immune response developed against *Rhinoestrus* spp was estimated in collected sera by using indirect ELISA according to Angulo-Valadez *et al.* [10]. Crude 2<sup>nd</sup> larva of *Rhinoestrus* spp antigen (CL2A) was prepared as described by Tabouret *et al.* [11]. The protein concentration was measured [12]. A concentration of 6µg/well of obtained CL2A was used. The optical densities (OD) were read at 450nm using an ELISA reader (BIO-TEK, INC., ELx, 800UV). Ten positive and eight negative control sera were obtained from infested donkeys and non-infested foals, respectively. The cut-off value was calculated from mean OD values of negative control sera plus three-fold standard deviations. Sensitivity and specificity of CL2A were determined [11].

### *Hematological studies*

Total erythrocytes and total leukocytes (TLC) were counted. The blood samples were analyzed for hemoglobin (Hb) and packed cell volume (PCV) determination [13]. Neutrophils, lymphocytes and eosinophils were estimated as cross sectional method [14].

### *Cytological examination of nasal fluid*

To count the inflammatory cells in the nasal fluid, the method of Zhang *et al.* [7] was performed with some modifications. The collected nasal fluids were centrifuged at 3000 rpm for 5 min then the precipitate was resuspended. The cell pellet was gently suspended in 200 µL of PBS, and the suspension was enumerated microscopically in a Neubauer's chamber, discriminating leukocytes. Neutrophils, lymphocytes and eosinophils were estimated as cross sectional method.

### *Biochemical examination*

Total protein was determined by colometric

method (Biuret reagent) [15] using SPECTRUM kits (BioMerieux, SA). Albumin was determined by modified bromocresol green colorimetric method [16] using SPECTRUM kits (BioMerieux, SA). Globulin and albumin globulin (A/G) ratio were calculated.

#### *Interleukin-2 (IL-2) and Tumor necrosis factor alpha (TNF- $\alpha$ )*

IL-2 and TNF- $\alpha$  concentrations in serum samples were measured by an equine-specific ELISA kits (WKEA MED Supplies) according to the manufacturer's instructions using purified IL-2 and TNF- $\alpha$  antibodies respectively. The color change was measured spectrophotometrically at a wavelength of 450 nm and the concentration of IL-2 and TNF- $\alpha$  in the samples were determined by comparing the OD of the samples to the standard curve.

#### *Antioxidant activity*

Glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) and catalase (CAT) activities in serum samples were measured using SPECTRUM kits (BioMerieux, SA). Absorbencies were measured at 340 nm, 560 nm and 520 nm respectively by spectrophotometer.

#### *Histopathological examination*

The collected tissue specimens from turbinate bones, olfactory bulb, meninges and brains were rapidly fixed in 10% neutral buffered formalin for at least 24 hours. Fixed specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylene, embedded in paraffin wax, sectioned at 4-5 $\mu$ m thickness then stained with hematoxylin and eosin (H&E) [17].

#### *Statistical analysis*

The statistical analysis was conducted to determine the significance between infested donkeys by *R. larvae* and uninfested for all tested parameters. Therefore, the data were statistically analyzed by Student's t-test at level  $P < 0.05$  [18] using Statistical Package for Social Science (SPSS) for Windows version 15 computer program.

### **Results**

#### *Intensity of the larval instars and serum IgG response*

During winter season, 40 out of 80 (50%) examined donkeys were infested with one or more equine nasal bot (*Rhinoestrusspp*) larvae. A total number of 346 larvae were collected. The percentages of 1<sup>st</sup> larva (L1), 2<sup>nd</sup> larva (L2) and 3<sup>rd</sup> larva (L3) were notably varied as they were 95.45%, 23.4% and 3.75%, respectively. The number of L1 and L2 recorded its highest intensity in January and March respectively. However, the number of L3 was very small and its highest value was observed in March (Table 1). ELISA detected IgG humoral immune response against *Rhinoestrusspp* using CL2A and the existence of larval instar of *Rhinoestrus spp* in the examined donkeys. The sensitivity of ELISA and specificity of CL2A were 97.5% and 47.5%, respectively. IgG mean optical densities values of infested donkeys were higher all over the winter months ( $P < 0.01$ ). The highest mean larval burden was recorded in December (11.6) while the lowest mean larval burden was detected in February (7.06) (Table 1).

**TABLE 1. Infestation intensity of larval stages of *Rhinoestrus spp* in donkeys during winter months combined with IgG optical densities (OD).**

Parameters \ Months	December		January		February		March	
	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested
No. of examined donkeys	15	5	9	11	2	16	14	8
L1 Intensity of larval stages	-	56	-	110	-	82	-	4
L2 Intensity of larval stages	-	2	-	7	-	29	-	43
L3 Intensity of larval stages	-	0	-	0	-	2	-	11
Mean larval burden	-	11.6	-	10.6	-	7.06	-	7.25
IgG OD	0.300 $\pm 0.01$	0.476** $\pm 0.02$	0.270 $\pm 0.06$	0.473** $\pm 0.03$	0.283 $\pm 0.03$	0.631** $\pm 0.09$	0.314 $\pm 0.05$	0.449** $\pm 0.04$

Values have \*\* were significantly differ than the corresponding non-infested group at  $P < 0.01$ .

*Haemogram and Leukogram*

Total erythrocytes of infested donkeys significantly decreased ( $P < 0.05$ ) in December and January, while PCV% significantly decreased ( $P < 0.05$ ) in December comparing with non-infested donkeys (Table 2). However, neutrophils% of infested donkeys recorded a significant elevation during December ( $P < 0.01$ ) and February ( $P < 0.05$ ) while lymphocytes% increased ( $P < 0.05$ ) in December and January. As well as, eosinophils% of infested donkeys showed marked significant elevation ( $P < 0.01$ ) in all winter months (Table 2).

*Cytological parameters of nasal fluid*

Table 3 shows the alteration in some cytological parameters of nasal fluid of *Rhinoestrus* spp infested donkeys comparing with non-infested donkeys during winter months. A significant decline ( $P < 0.05$ ) in neutrophils% in nasal fluid of infested donkeys was observed in December and significantly increased ( $P < 0.05$ ) in February. Lymphocytes% in nasal fluid of infested donkeys decreased ( $P < 0.05$ ) in December while increased ( $P < 0.05$ ) in January and March. However, eosinophils % in nasal fluid of infested donkeys increased significantly ( $P < 0.01$ ) in December, February and March.

**TABLE 2.** The effect of *Rhinoestrus* spp infestation on blood haemogram and leukogram of donkeys during winter months.

Parameters	December		January		February		March	
	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested
Rbcs ( $10^6$ cells/ mm <sup>3</sup> )	5.64 ±0.82	3.18* ±0.15	5.92 ±1.37	3.72* ±0.21	4.96 ±1.12	4.37 ±1.08	5.52 ±1.08	5.47 ±1.14
Hb (gm/dl)	12.48 ±2.97	9.85 ±1.68	11.63 ±1.46	9.38 ±2.10	12.49 ±2.83	11.93 ±1.57	12.96 ±2.02	11.19 ±2.82
PCV%	39.23 ±2.51	30.44* ±2.81	40.07 ±3.81	34.74 ±2.46	35.67 ±4.11	34.10 ±5.23	39.67 ±4.84	38.25 ±4.62
Total leukocyte	7.43 ±1.98	8.65 ±1.65	7.26 ±1.76	8.34 ±2.22	8.11 ±2.55	7.39 ±1.87	7.40 ±1.09	7.01 ±0.92
Neutrophils%	18.14 ±1.023	24.42** ±2.015	17.50 ±2.41	19.72 ±1.038	19.72 ±2.17	24.11* ±1.02	15.66 ±3.030	19.53 ±1.34
Lymphocytes%	61.22 ±1.04	69.51* ±2.08	55.15 ±1.11	64.60* ±2.12	58.18 ±4.022	61.71 ±3.121	59.61 ±1.021	66.41 ±2.01
Eosinophils%	4.25 ±0.93	9.10** ±1.034	4.45 ±1.43	8.32* ±1.033	4.2 ±1.11	8.12** ±1.87	4.76 ±0.96	9.02* ±1.33

Values have \*, \*\* were significantly differ than the corresponding non-infested group at  $P < 0.05$  and  $P < 0.01$  respectively.

**TABLE 3.** The effect of *Rhinoestrus* spp larvae infestation on some cytological parameters of nasal fluid of donkeys during winter months.

Parameters	December		January		February		March	
	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested
Neutrophils%	21.15 ±1.02	15.42* ±2.01	16.60 ±2.42	14.72 ±1.04	15.72 ±2.17	20.11* ±1.02	16.66 ±3.03	14.53 ±1.14
Lymphocytes%	63.22 ±1.02	57.59* ±2.033	53.12 ±1.13	63.50* ±2.041	58.13 ±4.06	60.72 ±3.141	57.65 ±1.02	68.44* ±2.035
Eosinophils%	4.21 ±0.85	8.12** ±1.01	4.33 ±1.03	7.37 ±1.03	4.61 ±1.02	9.15** ±1.07	3.99 ±0.81	7.22* ±1.03
Monocytes%	3.12 ±0.84	3.42 ±0.63	3.17 ±0.94	4.01 ±0.98	3.22 ±0.27	4.72 ±0.52	3.55 ±0.13	4.33 ±1.41

Values have \*, \*\* were significantly differ than the corresponding non-infested group at  $P < 0.05$  and  $P < 0.01$  respectively.

*Biochemical studies*

Total protein concentrations in the serum of *Rhinoestrus* spp infested donkeys significantly decreased ( $P < 0.05$ ) in February and March. However, albumin concentrations of the infested donkeys significantly decreased ( $P < 0.05$ ) in February only while globulin concentrations significantly decreased ( $P < 0.01$ ) in February and March. Sodium levels in serum of *Rhinoestrus* spp infested donkeys significantly decreased ( $P < 0.05$ ) in January, February and March ( $P < 0.01$ ). On the other hand, serum potassium levels of infested donkeys significantly increased ( $P < 0.05$ ) in December and February. However,

calcium concentration in serum of *Rhinoestrus* spp infested donkeys significantly decreased ( $P < 0.05$ ) in January only comparing with the non-infested group (Table 4).

*Antioxidant activity*

Table 4 shows the changes in antioxidant activities of *Rhinoestrus* spp. infested donkeys during winter months. GSH -PX serum concentrations of infested donkeys significantly decreased ( $P < 0.01$ ) in January, February and March. As well as, SOD significantly decreased ( $P < 0.05$ ) in December, January and February. However, CAT significantly decreased ( $P < 0.05$ ) in all the months of the study.

**TABLE 4. The effect of *Rhinoestrus* spp larvae infestation on serum protein parameters, some electrolytes and glutathione peroxidase (GSH -PX -U/ml), superoxide dismutase (SOD- U/ml) and catalase (CAT - U/ml) activity of donkeys during winter months.**

Parameters	December		January		February		March	
	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested
Total protein g/dl)	7.68 ±2.93	5.45 ±1.19	6.22 ±1.38	4.09 ±1.03	6.57 ±1.56	3.26* ±0.07	7.41 ±2.01	3.18* ±0.05
Albumin g/dl	4.12 ±1.31	4.15 ±1.08	4.21 ±1.33	3.98 ±1.02	5.03 ±1.06	3.77* ±0.42	4.05 ±1.10	4.14 ±1.37
Globulin g/dl	3.91 ± 0.72	2.40 ±0.08	3.51 ±0.07	2.07 ±0.03	4.54 ±1.28	2.16** ±0.07	4.46 ±1.18	1.85** ±0.03
Sodium mmol/l	170.14 ±8.56	143.10 ±9.47	168.73 ±9.64	138.56* ±7.06	153.87 ±5.42	132.54* ±7.38	164.48 ±7.29	127.38** ±9.13
Potassium mmol/l	6.42 ±0.07	9.77* ±0.61	7.12 ±0.13	9.63 ±1.02	6.13 ±1.04	9.62* ±0.82	6.65 ±1.15	9.33 ±0.09
Chloride mmol/l	111.67 ± 6.53	11.81 ± 14.95	111.86 ±10.13	109.47 ±9.24	110.30 ±11.61	109.02 ±10.07	111.45 ±9.84	109.82 ±10.03
Calcium mg/dl	12.41 ±1.26	9.63 ±1.18	13.71 ±1.44	9.38* ±0.59	11.22 ±1.27	8.72 ±1.20	11.37 ±1.05	9.33 ±0.09
GSH -PX (U/ml)	8.91 ±2.05	5.68 ±1.37	8.67 ±1.58	4.25* ±1.33	7.36 ±1.94	3.71** ±0.14	7.98 ±2.23	3.85** ±0.23
SOD (U/ml)	110.26 ±9.31	82.45* ±8.10	101.33 ±5.13	78.17** ±5.02	103.58 ±6.84	87.12* ±4.29	98.59 ±6.30	84.77 ±7.03
CAT (U/ml)	156.37 ±11.53	115.42* ±10.09	163.12 ±9.28	130.60* ±8.13	157.93 ±9.46	126.39* ±7.32	147.90 ±8.13	118.66* ±7.55

Values have \*, \*\* were significantly differ than the corresponding non-infested group at  $P < 0.05$  and  $P < 0.01$  respectively.

*Interleukin-2 (IL-2) and Tumor necrosis factor alpha (TNF-α)*

Table 5 showed an increase ( $P < 0.05$ ) in IL-2 in December, January ( $P < 0.01$ ) and March while TNF- $\alpha$  increased ( $P < 0.05$ ) in December and January.

*Histopathological results*

The turbinates' mucosae of *Rhinoestrus*-

infested donkeys showed variable degrees of degenerative, necrotic and inflammatory changes. The picture of catarrhal rhinitis was evident. Hence, the mucosal epithelium was greatly thickened with moderate to severe goblet cell hyperplasia (Fig. 1A). Sometimes, there were atrophy and thinning of the mucosal epithelium with underlying inflammatory cell infiltrates and congestion (Fig. 1B). Focal to diffuse inflammatory

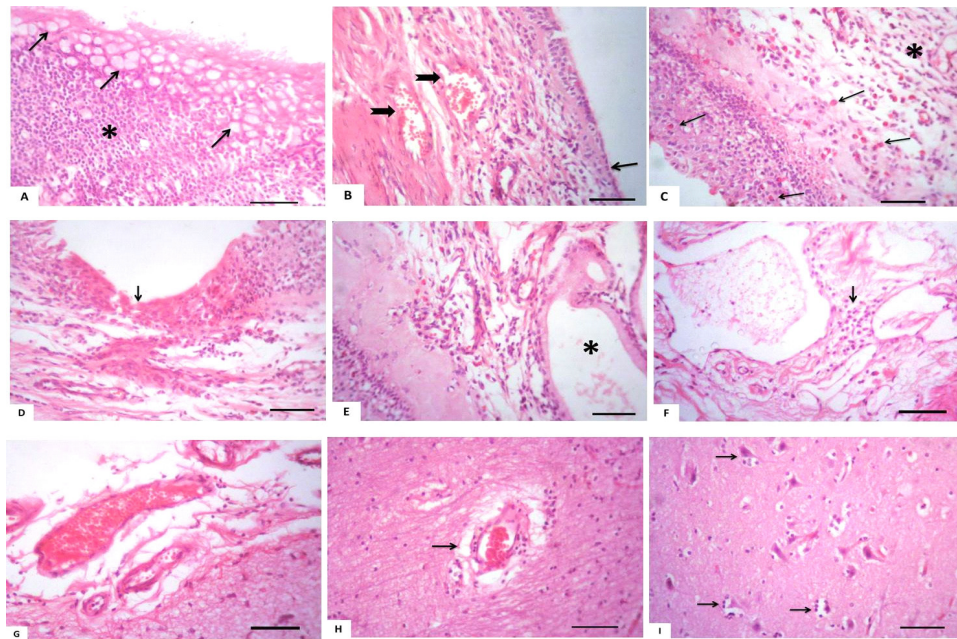
cells infiltration, particularly macrophages, lymphocytes and eosinophils were evident in the mucosa and submucosa (Fig. 1C). Also, there was necrosis of mucosal epithelium besides mucosal ulceration. Chronic ulcers were covered by areas of squamous metaplasia with severe inflammatory cell infiltrates in the underlying mucosa and submucosa (Fig. 1D). Regarding to submucosal glands, there were epithelial degeneration and hyperplasia as well some glands were cystically dilated with presence of eosinophilic secretions

(Fig. 1E). Olfactory mucosa showed mononuclear cell infiltrates mainly lymphocytes in lamina propria (Fig. 1F). Moreover, mild to severe meningoencephalitis was recorded. As there were congestion and lymphocytic cell infiltrates in the meninges (Fig. 1G) and the cerebral cortex giving the characteristic lesion of perivascular cuffing (Fig. 1H). There were neuronal degenerative and necrotic changes with different glial cell reaction, particularly satellitosis and neuronophagia (Fig. 1I).

**TABLE 5. Interleukin-2 (IL-2) and tumor necrosis factor alpha (TNF- $\alpha$ ) measurement in *Rhinoestrus* spp infected donkeys.**

Parameters	December		January		February		March	
	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested	Non-infested	Infested
IL-2	8.84	13.57*	8.57	15.76**	9.46	12.38	10.16	14.98*
(ng/ml)	1.05	1.18	1.93	1.27	1.03	1.93	0.75	$\pm 1.66$
TNF- $\alpha$ (ng/ml)	9.14	15.78*	10.35	16.05*	9.86	12.29	11.55	14.97
	1.58	1.15	1.31	1.41	1.27	$\pm 1.81$	1.07	$\pm 1.26$

Values have \*, \*\* were significantly differ than the corresponding non-infested group at  $P < 0.05$  and  $P < 0.01$  respectively.



**Fig. 1.** (A-E) Turbinate bones mucosa of a *Rhinoestrus*-infested donkey stained with HE. Bar=50 $\mu$ m. (A) Marked thickening of nasal mucosa (asterisk) with goblet cell hyperplasia (arrows). (B) Thinning of mucosal epithelium (arrow) with mucosal and submucosal congestion (notched arrows). (C) Marked thickening of mucosa and submucosa with inflammatory cells (asterisk) and infiltration of numerous eosinophils (arrows). (D) Mucosal ulceration covered by areas of squamous metaplasia (arrow) with mucosal and submucosal inflammatory cells infiltration. (E) Submucosal inflammatory cells infiltration with cystic dilatation of submucosal glands. (F) Olfactory mucosa of a *Rhinoestrus*-infested donkey stained with HE. Bar=50 $\mu$ m. Submucosal perivascular lymphocytic cell infiltration (arrow) with presence of fibrin admixed with inflammatory cells inside dilated blood vessel. (G) Meninges of a *Rhinoestrus*-infested donkey stained with HE. Bar=50 $\mu$ m. congestion of meningeal blood vessels with few lymphocytic cell infiltration. (H-I) Cerebral cortex of a *Rhinoestrus*-infested donkey stained with HE. Bar=50 $\mu$ m. (H) Lymphocytic perivascular cuffing (arrow). (I) Shrunken neurons with glial cell reaction, particularly satellitosis (arrows).

## Discussion

Rhinoestrosis is a parasitic disease that impairs equids' welfare and performance of infested animals, resulting in significant economic losses [3]. Studies on *Rhinoestrus* spp. infesting donkeys in Egypt are scanty [6], without any documentation of its hematological, oxidative stress and pathological features. Therefore, the present work aimed to investigate IgG and cytokines response combined with hematological profile, antioxidant defense and histopathological features developed in donkeys against *Rhinoestrus* spp. larvae during winter season. Some parameters were recorded previously in *Oestrusovis* infected sheep such as blood eosinophils [19], lymphocytes and leukocytes [20], IgG [10,21] and Th2 cytokines [22]. In this study, the percent of *Rhinoestrus* spp. existence was 50%. This higher percentage was recorded in previous investigation as it could be due to the changes in the locality and hence the epidemiological environment prevailing around the sampled donkeys [2,23]. The present study indicated that the intensity and the existence of larval *Rhinoestrus* spp. were varied during winter season. The first larval instar (L1) was dominantly found with a higher percentage with a peak value in January 2016 then the second larval instar (L2) and the third larval instar (L3) stage larvae with a peak value in March 2016. These results could be attributed to chronobiology of rhinoestrosis in this season where the climatic temperature was suitable for emerging of adult fly and deposition of their larvae into hosts' nostrils beside that this low temperature in winter season delaying maturation of the L1 into L2 and L3 [6]. The IgG response in examined donkey's sera was estimated along the four months. Depending on IgG optical densities' values combined with the existence of larval stage, the results showed that infested and non-infested animal cases could be distinguished from each other all over the winter season as the infested cases were constantly detected. This finding might confirm previous results [3,6] where the larval stage was established and the optical densities' values were increasing gradually during the winter season. This result was likeminded with activity of adult fly which began in December so, specific IgG levels were found to be sharply increased and positively interact with developing L2 and/or L3 larvae. This result was compatible to chronological generations of *Rhinoestrus* spp. in Egypt, as well as, the larvae might stimulate the immune response to the higher level needed to complete their nutritional requirements and growth [4,6,24].

Concerning hematological study, there was a decrease in RBCs and PCV of infested donkeys besides decrease in Hb concentration. These results indicated that infestation by *Rhinoestrus* spp. induced anemia in donkeys as previously recorded [25]. The leukogram in both blood and nasal fluid of infested animals was investigated in this study. It was observed that TLC slightly increased in infested donkeys with an elevation in neutrophils% in December and February while lymphocytes% of infested donkeys elevated during December and January. These results might be considered as an indication for both inflammatory and immune responses against infestation [24]. Some alterations in cytological parameters of nasal fluid of *Rhinoestrus* spp. infested donkeys during winter months were recorded in this investigation. An increase in neutrophils% in nasal fluid of infested donkeys was recorded in February, as well as, lymphocytes% increase in January and March. These results showed a parallelism between the inflammatory and immune cells pattern in blood and nasal fluid. As well as, it was observed that both blood and nasal fluid eosinophils% increased in this work during all winter months. These results were parallel to the previous studies where the blood eosinophilia elevated to its peak in *Oestrusovis* experimentally infected sheep [19]. This elevation in blood and nasal fluid eosinophilia indicated that the immune system was strongly stimulated during the infestations [24]. In the present investigation, serum protein and globulin concentrations were declined in February and March while serum albumin was decreased in February only in *Rhinoestrus* spp. infested donkeys. These alterations might be due to blood loss and impaired appetite [25]. In the present investigation, low sodium and high potassium serum levels were observed in *Rhinoestrus* spp. infested donkeys. This result might be an indication for neuromuscular irritability and polarization [26,27]. As well as, serum calcium level decreased in infested animals and accompanied by oxidative stress which might lead to bone formation problem [28]. Collectively, the hematological and biochemical results in this study might indicate that the decline in serum biochemical parameters started later than the cellular alteration. Estimation of the enzymatic antioxidants activities, such as GSH-Px and SOD [29] as well as estimation of serum CAT [30] was a mean of evaluating oxidative stress. It was found that GSH-Px serum activity in infested donkeys

decreased in January, February and March, SOD decreased in December, January and February while CAT activity decreased in all the months of the study. This data reflected the oxidative stress in *Rhinoestrus* spp. infested donkeys in all winter months with increasing in December and January.

The shifting of IL-2 and TNF- $\alpha$  were measured in this study where the results revealed that IL-2 as referred to Th2 increased in the months of winter and continued to increase until March, while TNF- $\alpha$  increased in December and January only. This cellular profile suggested a Th2-type cytokine production by T lymphocytes [22] which indicated that the shifting was toward Th2 [31]. Clear evidence to support this hypothesis is still missing in *Rhinoestrus* spp. infection. Several proteins were secreted by the parasite that involved in the Th2 cytokines regulation such as IL-2 production. In addition, it was known that TNF- $\alpha$  was a main mediator which initiate an inflammatory cascade [32]. Moreover, parasitic infection provoked Th2 dominant immune responses that stimulate eosinophil and antibody switching [31].

The myiasis caused by the larvae of *Rhinoestrus* spp. induces severe respiratory diseases, impairs animals' health and leads to severe economic losses. The larvae localize in nasal cavities, sinuses and pharynx of the animal [33], inducing inflammation, difficult breathing, sneezing and cough. Our histopathological results revealed presence of catarrhal rhinitis, ulceration of nasal mucosa and meningoencephalitis. The latter might be associated with penetration of the ethmoid bone and the meninges by *Rhinoestrus* spp. [3]. Mechanical effect of oral hooks and spines of oestrids larvae besides the proteolytic enzymes found in the secretory and excretory products of these larvae were responsible for these changes in the brain [20]. Regarding to the inflammatory cells, there was a mixture of various types of cells such as macrophages, eosinophils and lymphocytes. Eosinophils are usually associated with the resistance to helminth infections [34]. The frequent recorded lesions in the sinus were disarrangement stratified epithelium, wide intercellular spaces, with loosing epithelia. Degenerative signs were prominent in some rounded cells as previously recorded [35]. This decay might be due to the effect of proteases that secreted by the larvae. These abnormal changes might lead to the diffusion of antigenic determents to be in direct contact

with the locally recruited immune cells [36]. The numbers and distribution of recruited eosinophils were previously observed within the mucous membrane and sub mucosa of naturally infected sheep [37]. These recruitment process might be resist the parasitic larval populations and sustain the hypersensitivity reaction at the site of tissue damage during infection [36].

### **Conclusion**

Finally, it could be concluded that the infestation by *Rhinoestrus* spp. might affect the health conditions leading to anemia and electrolytes disturbance. Specific IgG levels were found to be sharply increased and positively interact with developing L2 and/or L3 larvae where it stimulated the immune response. In addition, polarization of the immune response was shifted towards Th2 cytokines by *Rhinoestrus* spp. Down regulated or altered protective Th1 cytokine responses. The histopathological picture reflected the blood and nasal fluid cytological picture results as there was a picture of inflammation such as inflammatory cells elevation, lymphocytes infiltration and inflammation of meninges and cerebral cortex. The current results also confirmed that infestation by *Rhinoestrus* spp. could lead to oxidative stress and inflammation of central nervous system which attributed to the high irritability. This might indicate the risk of the infestation with *Rhinoestrus* spp. on the vital and immunological process in donkeys. *Rhinoestrus* spp. infection was linked with oxidative status, particularly during the winter period when energetic needs were high; hence suggesting that overload while dealing with an infestation could exaggerate oxidative stress.

### *Conflict of Interests*

The authors declare that they have no conflict of interests.

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## التغيرات الإكلينيكية النسيجية المرضية والمناعية في الحمير المصرية المصابة بالريנוاستروس اثناء فصل الشتاء

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تهدف هذه الدراسة إلى فحص التغيرات الكيميائية الحيوية والاستجابة المناعية للمحفزات الخلوية و الأجسام المناعية جى جنبا إلى جنب مع صورة الدم والخصائص النسيجية المرضية المتطورة نتيجة الإصابة ببرقات رينو استروس خلال فصل الشتاء. لذلك أجريت هذه الدراسة في فصل الشتاء حيث تراوحت درجة الحرارة بين ١٢ - ١٨ درجة مئوية، وكانت الرطوبة تتراوح بين ٤٠-٦٠٪. تم تجميع عدد ٨٠ عينة من الحمير المذبوحة في مجزر حديقة الحيوان بالجيزة (ديسمبر ٢٠١٥ إلى مارس ٢٠١٦). تم تجميع السائل الأنفي من كل حيوان قبل الذبح في سرجة تحتوي على ١٠ مللي محلول ملحي لاستخدامها في الفحص الخلوي. اما بعد الذبح مباشرة فقد تم تجميع عينات دم من الحمير في انابيب تحتوي هيبارين لفحص صورة الدم الكاملة وانابيب أخرى لا تحتوي على الهيبارين لفصل المصل لهذه العينات وحفظها عند درجة -٢٠م لحين الاستخدام لتقدير نسب بعض التغيرات المناعية والكيميائية الحيوية. تم أيضا تجميع البرقات من كل رأس لتعريفها. وأخيرا تم تجميع عينات أنسجة من البصيلات الشمية، والسحايا المخية لنفس الحمير المذبوحة وحفظها في الفورمالين ١٠٪ لعمل الفحوصات النسيجية المرضية. وظهرت النتائج زيادة في متوسط قيم الأجسام المناعية جى في مصل الحمير المصابة في جميع أشهر الشتاء. وكان أعلى متوسط لعيب البرقات في ديسمبر (١١,٦). اما فيما يتعلق بفحص صورة الدم، فكان هناك انخفاض في كرات الدم الحمراء و PCV في الحمير المصابة بالإضافة إلى الانخفاض في تركيز الهيموجلوبين (Hb). كما لوحظ ارتفاع في العدلات٪ و اللمفاويات٪ في الحمير المصابة. وظهرت التغيرات في القياسات الخلوية في السائل الأنفي من الحمير المصابة وبذلك تظهر وجود توازي بين خلايا الالتهابات والمناعة في الدم وسائل الأنف. ولوحظ انخفاض في تركيزات البروتين والألبومينوالجلوبولين بجانب انخفاض مستويات الصوديوم وارتفاع البوتاسيوم في مصل الحمير المصابة. وظهرت نتائج الفحص النسيجي ارتفاع خلايا الالتهابات ، والخلايا الليمفاوية، والتهاب السحايا. والخلاصة أن: الإصابة بالرينواستروس في الحمير يمكن أن ترتبط بالإجهاد التأكسدي والتحول نحو خلايا تى المساعدة الثانية (Th2) ويمكن أن تؤثر على العمليات الحيوية والمناعية.

**الكلمات الدالة:** رينو استروس، الكيميائية الحيوية، المحفزات الخلوية، مضادات الأكسدة، الحمير.