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Clinical, inflammatory, immunological and oxidative stress alterations in donkeys with Equine Herpesvirus-1 Myeloencephalopathy in Gharbia governorate, Egypt

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ABSTRACT

Equine Herpesvirus-1 Myeloencephalopathy (EHM) is a highly contagious neurological disease that primarily affects horses but can also affect donkeys and other equids. This study aimed to evaluate clinical, inflammatory, immunological, and oxidative stress alterations in donkeys with EHM in Egypt. The study was conducted on 25 clinical cases of donkeys (20 females and 5 males) suffering from signs suggestive of EHM, and five apparently healthy donkeys served as control. All affected donkeys were examined clinically. Nasal swabs were collected from all donkeys for molecular diagnosis of EHV-1 using PCR. Serum biochemical analysis was carried out for hepatic functions (ALP, ALT, and AST), renal functions (blood urea nitrogen (BUN) and creatinine), cardiac function (Cardiac troponin I (cTnI)), inflammatory markers (CRP and IL-6), immunological markers (IgM, IgG and IgA) and oxidative stress markers (CAT, TAC, GPX, and MDA). The PCR results revealed that all samples taken from affected donkeys were positive for EHV-1. Clinical examination revealed that affected donkeys suffered from fever, mild depression, and inappetence preceded neurologic signs such as hindlimb weakness, ataxia, urine dribbling, loss of tail tone, recumbency followed by death of all affected donkeys. Significant increases were noted in CRP, IL-6, IgG, IgM, IgA and MDA. It was therefore concluded that EHM in donkeys is associated with significant oxidative stress, immune dysregulation, and CNS damage. Accordingly, targeting inflammatory, immunological, and oxidative stress alterations in EHM cases could provide valuable diagnostic approaches to improve the prognosis of the disease.

1. INTRODUCTION

Equine Herpes viruses (EHV) infect single-toed ungulates and cause respiratory system infections besides neurological disorders which are often called equine herpesvirus myeloencephalopathy (EHM). EHM causes considerable economic losses in equine due to the occurrence of sporadic and epidemic outbreaks (Harless and Pusterla, 2006). Equine Herpes virus infection may disrupt the immune system, causing oxidative stress and an inflammatory reaction in donkeys which is a potential threat to their health (Costantini et al., 2018). EHM can occur as a single sporadic case caused by the reactivation of a latent virus or as an outbreak caused by the lateral spread of sources of infection EHV-1. It is difficult to define the incubation period for EHM, because the primary infection with EHV-1 might have developed from a long period before EHV-1 reactivation that led to the occurrence of EHM (Dunowska, 2014).

Susceptibility to certain infectious diseases and clinical signs are different between donkeys and horses. Although studies conducted on donkeys affected with EHM are limited, previous reports stated that EHV-1 infections were endemic in the donkey population (Vengust et al., 2008; Thiemann, 2012; Yildirim et al., 2015).EHM outbreaks in donkeys and mules were investigated in Ethiopia between 2011 and 2013 causing deaths in donkeys without apparent clinical signs (Negussie et al., 2017). EHM may result from a respiratory form of EHV-1 but can occur preceding respiratory signs (Slater, 2014).

Clinical manifestations of EHM are variable in degree, and include ataxia of the hind limbs, urine dribbling, and loss of tail muscle tone. Affected horses became recumbent in the late stages (Pusterla and Hussey, 2014). Donkeys with EHM show severe neurological signs prior to death.

There are limited reports on pathogenesis and viral immunity (Burden and Thiemann, 2015). Early diagnosis of the disease in the donkey is challenging because dullness and anorexia may be the only observed symptoms. Therefore, the donkey may be undetected until the late stages of the disease (Rickards and Thiemann, 2019).

The post-mortem examination of the brain of horses with EHM shows mild edema of the meninges. Neuropathologic lesions seen include vasculitis, degeneration of axons, and thick cuffs of lymphocytes and histiocytes surrounding small blood vessels in the spinal cord and meninges (Pusterla et al., 2022; Wang et al., 2022; Soboll-Hussey et al., 2024).

Following viral infections, an increased production of reactive oxygen species (ROS) occurs with the depletions of antioxidants (Adıgüzel and Oğuzoğlu, 2022). EHV-1 infection may be correlated with high levels of ROS, molecular oxidative damage, and alterations in antioxidant defenses. The replication of the virus is generally only detected with the onset of clinical signs, but clinical signs might not appear if the virus rapidly mounts an immune

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defense and controls virus replication (Sebastiano et al., 2016).

Knowledge about the impact of oxidative stress on the nervous system's viral infections may lead to improved therapeutic approaches to reduce tissue damage without hindering the host antiviral immune response (Valyi-Nagy and Dermody, 2005). The current study aimed to investigate clinical signs, inflammatory, immunological, and oxidative stress biomarker alterations in donkeys with EHM, and their potential use for early diagnosis and better disease prognosis.

2. MATERIAL AND METHODS

Ethical approval

The protocol of this study was approved by the ethical and animal care and use committee of the Faculty of Veterinary Medicine, Benha University, Egypt with the approval number (BUFVTM 03-02-29).

2.1. Animals

This study was applied in Gharbia Governorate (Kafr El Zayat and Basyoun) in Egypt in the period from July to September 2023. During an outbreak of EHM, 25 clinical cases of donkeys (20 females and 5 males) suffered from nervous signs and aged from 1-5 years with average body weight (70-150) kg were examined. Five apparently healthy donkeys served as control. Clinical examinations were carried out on all donkeys according to Constable et al. (2016).

2.2. Samples

2.2.1. Blood samples

Five ml of blood was withdrawn from the jugular vein of each donkey in sterile vacuum tubes without anticoagulant. Then, samples were allowed to clot at room temperature and then centrifuged for ten minutes at 3000 rpm to extract clear non-hemolyzed serum. The serum samples were then transferred into clean, dry, and sterilized Eppendorf tubes and stored at -20 °C until they were needed for biochemical analysis.

2.2.2. Nasal swab samples

The nasal swabs were collected from donkeys with fever and during the early stage of disease showing signs suggestive of EHM. Nasal swabs were collected on transport media for molecular diagnosis of EHV-1 using PCR.

2.3. Molecular diagnosis of equine herpes viruses-1 using PCR

Extraction of the viral DNA from the nasal swabs was carried out using QIAamp DNA Mini kit (Qiagen, Germany) according to the guidelines of the manufacturer. The DNA was finally eluted with 100 µl of the kit's elution buffer. The resultant DNA was ready for PCR.

For PCR amplification, the following oligonucleotides forward and reverse primers were used targeting the glycoprotein H (gH) of EHV-1. The primers sequences were: forward F5'AAGAGGAGCACGTGTTGGAT-3', and reverse R5'TTGAAGGACGAATAGGACGC-3' yielding an expected amplicon of 636 bp length (Varrasso et al., 2001).

The target product was amplified in 25 µl reaction mix containing 12.5 µl of PCR Master Mix (Thermo Fisher Scientific, Germany), 1 µl of each forward and reverse primers (2.0 µM concentration), 4.5 µl RNase-free water, and 6 µl of DNA template. Thermocycler (applied biosystem 2720) was used for the amplification reaction as follows: initial denaturation 95°C for 5 min and 35 cycles of denaturation 95°C for 30 sec, annealing 60°C for 30 sec, extension 72°C for 1 min, then final extension at 72°C for 5 min to complete all extensions and hold at 4C°. Electrophoresis of the PCR products was carried out using 1.5 % agarose gel plates containing GelRed nucleic acid stain and visualized using ultraviolet light (Varrasso et al., 2001).

2.4. Serum Biochemical analysis

The spectrophotometric determination of serum concentrations of alkaline phosphatase (ALP), aspartate aminotransferase (AST), Alanine transaminase (ALT), blood urea nitrogen (BUN), creatinine, Cardiac troponin I (cTnI), C-reactive protein (CRP) immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), and Interleukin- 6 (IL-6) by using commercial kits of the Spectrum (Egyptian Company for Biotechnology (S.A.E.), Obour City Industrial Area, Cairo, Egypt). Using commercially accessible test kits (Bio diagnostics, Cairo, Egypt), standard procedures were followed to detect catalase (CAT), Total Antioxidant Capacity (TAC), glutathione peroxidase (GPX) and Malondialdehyde (MDA).

2.5. Statistical analysis

The findings of the current study were presented as mean ± SE and were evaluated using independent student *t*-test (SPSS Statistics for Windows, version 25.0. Armonk, NY: IBM Corp). Differences were determined significantly when $P< 0.05$.

3. RESULTS

3.1. Clinical findings

Clinical examination of donkeys with EHM (Figure 1) revealed the occurrence of fever (39±0.30°C), mild depression, and inappetence followed by neurologic signs. The duration of fever varied from 2 to 8 days (mean 5 days). Hind limbs are more severely affected than forelimbs, and signs were symmetric. Donkeys showed ataxia, urine dribbling, loss of tail tone, and weakness of hind limbs. Donkeys showed toe dragging in the hindlimbs and knuckling of the hind fetlocks. Donkeys were leaning against a wall or fence to maintain balance. There were multiple wounds and traumatic abrasions on the head and at the level of the bony prominences of the affected donkeys. All donkeys showed lethargy and recumbency followed by death of all affected donkeys.

Figure 1 Clinical signs of equine herpes viruses -1 myeloencephalopathy in donkeys. A- Donkey showed ataxia, loss of tail tone, and hind limb weakness. B- Donkey showed leaning against a fence to maintain balance with injuries in the head. C- Donkey had multiple injuries on their bodies, including pressure sores and traumatic abrasions. D- Recumbent donkey (unable to rise) prior to death.

3.2. Molecular Detection of equine herpes viruses -1 using PCR

The PCR results revealed that all samples taken from affected donkeys were positive for EHV-1 and showed clear positive bands at the target size '636 bp' (Figure 2).

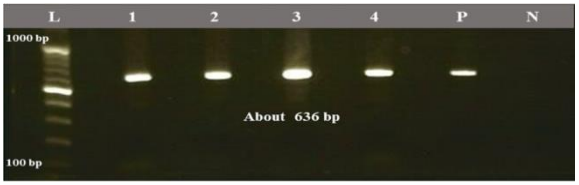

Figure 2 Agarose gel electrophoresis using primers pair for equine herpes viruses -1 targeting gH gene, (L) 1000bp DNA ladder, (P) positive control lane, (N) negative control lane, lanes (1-4) positive samples and specific bands at 636 pb.

3.3. Biochemical findings

3.3.1. Hepatic, renal, and cardiac function in donkeys with equine herpes viruses -1:

ALP, ALT, AST, BUN, creatinine, and cTnI levels did not show significant differences between donkeys with EHM and control donkeys (Table 1)

3.3.2. Inflammatory, immunological and oxidative stress markers in donkeys with equine herpes viruses -1:

CRP, IL-6, IgG, IgM, IgA and MDA were significantly increased ($P< 0.05$) while CAT, TAC and GPX were significantly decreased ($P< 0.05$) in donkeys with EHM compared to healthy control donkeys (Table 2).

Table 1 Hepatic, renal, and cardiac functions of donkeys with equine herpes viruses -1myeloencephalopathy (Means ± SE)

Parameter	Control	Diseased
ALP(U/L)	247.00±60.40 [a]	250.00 ±70.40[a]
AST(U/L)	190.20±3.22 [a]	195.40±3.80[a]
ALT(U/L)	36.30±0.47 [a]	39.69±0.98[a]
BUN (mg/dl)	38.20±0.57[a]	38.10±0.49[a]
Creatinine (mg/dl)	1.72±0.29 [a]	1.81±0.04[a]
cTnI (mg/dl)	0.020±0.001 [a]	0.025±0.001[a]

Table 2 Inflammatory, immunological, and oxidative stress markers in donkeys with equine herpes viruses -1myeloencephalopathy

Parameter	Control	Diseased
CRP (g/L)	5.43±0.26 [b]	16.58±0.40 [a]
IL6 (pg/ml)	3.32±0.10 [b]	5.25±0.21[a]
IgG (mg/dl)	690.00±1.56 [b]	798.30±1.13[a]
IgM (mg/dl)	75.70±1.10 [b]	152.00±2.78[a]
IgA (mg/dl)	9.41±.59 [b]	16.95±0.54[a]
CAT (mmol/L)	9.17±0.85 [a]	5.23±0.21[b]
TAC (mmol/L)	3.26±0.04[a]	1.76±0.02[b]
GPX (U/ml)	52.9±1.77 [a]	21.19±1.37 [b]
MDA (nmol/ml)	11.17±0.34[b]	22.57±0.74 [a]

Means (± SE) with different superscript letters within the same row indicate significant difference at $P< 0.05$.

4. DISCUSSION

Equine herpes viruses -1 is a highly contagious neurological disease that primarily affects horses but can also affect donkeys and other equids. Early detection of EHM through PCR and monitoring of febrile animals during the prodromal phase of the disease enhances the diagnostic success of the disease. PCR is particularly useful during the prodromal febrile phase, as virus shedding often ceases before nervous symptoms appear (Burgess et al., 2012).

Although scientific studies of EHV infection in donkeys are limited, EHV greatly impacts donkeys' health. Horses are significantly different from donkeys in their physical and behavioral characteristics despite the genetic relationship between them. Donkeys exhibit distinct susceptibilities to certain infections and display unique clinical signs compared to horses (Burden and Thiemann, 2015). Clinical manifestations of EHM in horses are well-established, and associated with substantial morbidity and death (Nielsen et al., 2022).

Equine herpes viruses -1 has a per acute onset and early signs of ataxia, paresis, and urinary dribbling, the involvement of multiple animals in the village, and a recent history of fever and lethargy in affected donkeys are characteristic features, although the outbreaks are variable within their clinical and epidemiological findings (Wilson, 1997; Dunowska, 2014). The clinical signs may be attributed to the neuropathologic lesions that include vasculitis, degeneration of the axons, and thick cuffs of lymphocytes and histiocytes surrounding small blood vessels in the nervous system (Pusterla et al., 2022). No respiratory signs were observed in diseased donkeys which coincided with Slater (2014) those who reported that EHM can develop as a complication of the respiratory form of EHV-1, but it may arise without preceding respiratory symptoms.

In the current study, serum biochemical analysis revealed that liver, kidney, and cardiac functions (ALP, ALT, AST, BUN, creatinine, and cTnI) of the diseased donkeys were not significantly elevated which coincided with previous reports (Perkins et al., 1999; Fararh et al., 2016).

A significant increase was observed in CRP in the serum of donkeys with EHM. CRP is an acute-phase protein synthesized during the inflammatory state that binds to microbial surfaces, particularly phosphorylcholine, serves as a binding site for complement proteins, and stimulates macrophage-mediated phagocytosis. The increase in CRP might be attributed to inflammatory response and tissue damage of the nervous system caused by EHV1 infection (Nunokawa et al., 1993).

IgA, IgG, IgM, and IL-6 are important inflammatory and immunological markers. Our results revealed that the levels of IgA, IgG, IgM, and IL-6 were significantly higher in all donkeys with EHM, and this may be attributed to EHV infection stimulation of the antiviral response, and the host improved resistance to the virus through elevated antibody secretions (Foote et al., 2005). Upper respiratory infection with EHV-1 in horses stimulated the production of high levels of IgG, IgA, and IgM (Bannai et al., 2011; Wagner et al., 2015). IL-6 was significantly higher in donkeys with EHM as EHV infection caused an inflammatory response and improved the expression of pro-inflammatory factors (Costantini et al., 2018). EHV-1 infects equine endothelial cells, leading to a consistent increase in the levels of IL-6 and inflammatory markers regulated by IL-6 (Johnstone et al., 2016).

Oxidative stress is an important factor in the pathogenesis of EHM. Viral replication causes elevated production of ROS, causing molecular damage and overwhelming antioxidant defenses (Sebastiano et al., 2016; Costantini et al., 2018). Markers such as MDA highlight lipid peroxidation, while decreased levels of CAT and GPX reflect compromised antioxidant capacity in infected animals (Costantini et al., 2018). EHV infection may lead to elevated production of ROS, causing oxidative molecular damage to antioxidant defenses. Viral replication is typically only detected when clinical signs emerge, although signs may not be evident if the immune system quickly responds and controls the virus (Sebastiano et al., 2016). These findings suggest that EHV infection can disrupt the activity of antioxidant enzymes and trigger oxidative stress in donkeys (Costantini et al., 2018). A significant reduction in TAC may be linked to more severe neurological symptoms, as the CNS damage due to oxidative stress is more pronounced in donkeys with reduced antioxidant defenses. Lower TAC levels have been linked with more severe CNS damage, potentially explaining symptoms like incoordination, muscle weakness, and recumbency. The oxidation and antioxidant markers in donkeys with EHM were similar to previous reports in equine (Costantini et al., 2018).

5. CONCLUSIONS

Equine herpes viruses -1 infection and subsequent EHM in donkeys are associated with significant oxidative stress, immune dysregulation, and CNS damage. Targeting oxidative stress and inflammation could provide effective diagnostic approaches and improve the prognosis for

affected donkeys. The Control measures should be applied during outbreaks to minimize the spread of infection and reduce stress-related EHV-1 reactivation that leads to the occurrence of EHM.

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