

***Original Article***

## **Prognosis of Hepatic Amyloidosis Status and Insufficiency in Snake Antivenom Producing Horses**

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### **ABSTRACT**

Hyperimmunization of horses with specific antigens to harvest antisera is associated with high incidence of liver amyloidosis. The histopathologic examination is the gold standard method for diagnosis of the hepatic amyloidosis. The aim of the present study was to determine whether the clinical manifestations and serum values of appropriate chemical constituents due to liver injury, provide the prognostic criteria for liver amyloidosis and insufficiency. It was endeavored to determine the usage duration and survival in the horses, applied for polyvalent snake antivenom products. Thirty-seven Iranian, mixed breed male horses used for snake antivenom production were recruited in this study. All of the clinical characteristics of liver disease were assessed and the appropriate serum biochemical parameters were screened between two hyperimmunization stages. The results revealed that weakness, progressive weight-loss and chronic diarrhea were the warning signs for end of the period of using a horse in snake antivenom production. However, moderate to severe colic, often, was the sign of imminent death due to acute hepatic swelling and rupture. However, Serum biochemical findings were not suitable and useful for the assessment of outcome compared with the clinical manifestations. In this study, the authors have attempted to introduce the clinical and serum biochemical findings that are useful for prediction of the liver amyloidosis and insufficiency and, to determine the usage duration and survival in these horses. Consequently, a preliminary scoring guideline for assessing the issue was suggested.

**Keywords:** Biochemical parameters, Clinical signs, Hepatic amyloidosis, Horse, Hyperimmunization

### **Prévision de l'état d'Amyloidose et l'insuffisance hépatique chez les chevaux fabricants de sérum anti-venin pour serpent**

**Résumé:** Hyper immunisation des chevaux par les antigènes spécifiques afin de pouvoir produire les antisérums utilisable et exploitable est toujours accompagnée de l'émergence de sévère amyloidose hépatique chez les chevaux. L'examen histopathologique est un signe certain de l'Amyloidose hépatique. Le but de cette étude est d'évaluer la valeur diagnostique des symptômes cliniques et les résultats biochimique de sérum (qui sont utilisés dans le diagnostic des lésions et maladies du foie) en prévision de la gravité de l'insuffisance hépatique et l'Amyloidose, déterminer la durée de l'exploitation et aussi les chances de survie chez les chevaux fabricants sérum anti venin polyvalents pour serpent. Pour ce faire, dans cette étude, 37 chevaux mâles iraniens (métis) utilisés dans la production de sérum anti-venin de serpent ont été étudiés. Tous les signes vitaux et les paramètres cliniques et biochimiques de tous les chevaux entre deux tours de vaccination

ont été étudiés. Les résultats ont démontré que la faiblesse, la perte du poids progressive et la diarrhée chronique sont des signes clairs de la fin de la durée de vie utile de l'exploitation des chevaux utilisés et les douleurs modérées et sévères dans le ventre indiquent la morte imminente due à un gonflement grave du foie et la déchirure de ceci. Alors que les résultats biochimiques de sérum ne sont pas aussi utiles que les signes cliniques en prévision des animaux dans l'état de charge. Dans cet article, les auteurs ont essayé de prévoir la situation de l'insuffisance hépatique et l'Amyloïdose, la durée de l'exploitation et aussi les chances de survie chez les animaux utilisés tout en utilisant les signes cliniques et les résultats biochimiques de sérum. Pour ce faire, une table de guidage d'introduction (taux) a été préparée et proposée.

**Mots clés:** paramètres biochimiques, les symptômes cliniques, Amyloïdose du foie, cheval, hyper immunisation

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## INTRODUCTION

Amyloidosis is a term used for various diseases, lead to the deposition of proteins that are composed of  $\beta$ -pleated sheets of non-branching fibrils in various tissues (McGavin and Zachary, 2006). The Amyloid deposits of the reactive amyloidosis are seen in horses recruited for serum production. These deposits display a typical distribution, predominantly affecting liver, spleen and more variably the adrenal and kidneys. Within the liver, the amyloid deposits causes impairment of the normal access of plasma to the hepatocytes led to pressure atrophy, ischemic degeneration, and necrosis of the hepatic parenchyma, and varying degree of hepatomegaly. In severe cases, the affected animals may present the clinical manifestations of either hepatic dysfunction or failure. As the liver is the most sensitive organ among the affected ones, it frequently ruptures in which lead to severe intraperitoneal hemorrhage and death of horse (McGavin and Zachary, 2006). Due to high reserve capacity, the liver is capable of remaining functional albeit large sum of tissue damage (Ambrojo et al., 2013). Approximately, seventy percent or more number of the hepatocytes must be affected before the hepatic disease become symptomatic and alteration of the hepatic function become detectable by serum biochemical tests (Pearson, 1999; Latimer et al., 2003; Barton, 2007). Subsequently, the evidence of liver disease in horse is often identified when the animal's blood is tested for a reason other than liver disease, typically due to non-specific clinical signs such as mild

weight loss, lethargy, mild colic or poor performance (Ambrojo et al., 2013). The prognosis of hepatic insufficiency in horses depends upon the severity and type of the underlying disease. However, in the chronic liver disease with severe hepatic fibrosis and despite the reduction of functional hepatocytes, the serum levels of liver enzymes might remain in the normal range (Arslan and Sahal, 2009; Ambrojo et al., 2013). The aim of the present study was to determine whether the clinical signs and serum values of appropriate chemical constituents, representing the liver damage, provides prognostic information on liver amyloidosis and insufficiency; and determining the usage duration and survival in horses, used for polyvalent snake antivenom production.

## MATERIALS AND METHODS

**Human care guidelines.** All the animals used in the present study were treated in compliance with world health organization (WHO) technical report series, No. 964; fifty- ninth reports (WHO expert committee on biological standardization).

**Animals.** Thirty-seven Iranian, mixed breed male horses, which their ages during the test period ranging from four to eighteen years and weighted between 300-450kg, were used in the present study. They had been used for polyvalent snake antivenom production from 1-12 years in Razi vaccine and serum research institute, Karaj-Iran. During each stage of hyperimmunization, these horses had been subcutaneously injected, once a week up to five times with an increasing concentration gradient of mixture of venoms of six native snakes of

Iran, consisted of five snakes from Viperidae family and one from Elapidae. Besides, ten horses, which were not formerly used for antiserum production, were used as control group. All the horses were feed properly by alfalfa hay, wheat straw, grass, grinded barely and some beet pulp. The experimental group horses were kept often in stalls with minimum effort or exercise program. These horses were divided into four groups, based on the antiserum production duration. Groups I, II, III and IV were consisted of 12, 7, 6 and 12 horses and used for snake antivenom production for more than 9 years, 6-9 years, 3-6 years and less than 3 years, respectively. During the test period that was between two hyperimmunization stages, the clinical examination was done and a single blood sample was collected from each animal for serum biochemical analysis. During the test period another six horses, which were not included in this study, but were used for snake antivenom production died or euthanized because of poor prognosis. Liver of these horses were examined macroscopically and histopathologically. The formalin-fixed specimens were embedded in paraffin following the routine procedures and sections stained with haematoxylin and eosine (H&E) and finally examined with a light microscope.

**Clinical examination.** The information of all horses such as age, usage duration for snake antivenom production and clinical manifestations including: body temperature, pulse and respiratory rates, weakness and weight loss, decrease or loss of appetite, abdominal pain (colic), photosensitivity, congested or icteric mucous membranes, signs of central nervous system (CNS) associated to hepatic encephalopathy (HE) were collected. The target CNS involvement signs were behavioral change, depression, sensitivity, ataxia, stretching of the face and lips, and leaning head on barrier.

**Serum biochemical analysis.** Serum activities of gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanin amino transferase (ALT), and serum concentration levels of total, direct and indirect

bilirubins (tB, dB, iB), blood urea nitrogen (BUN) and triglyceride (TG) were analyzed using an autoanalyzer (Hitachi 912, Japan). The analysis was performed by using the commercial kits (Pars azmun, Iran) according to the manufacturer's instructions.

**Statistical analysis.** The results were expressed as mean  $\pm$  standard deviation. The statistical significance of difference among the groups was analyzed by using the one-way analysis of variance (ANOVA). Differences were considered as significant when p-value was equal or less than 0.05.

## RESULTS

**Clinical findings.** Mean body temperature, pulse and respiratory rates of the experimental and control groups are shown in Table 1.

**Table 1.** Body temperature, respiratory and pulse rates of the control and test groups (Mean $\pm$ standard deviation)

Groups	Body temperature (C°)	Respiratory rate (min)	Pulse rate (min)
<b>Control (n=10)</b>	37.66 $\pm$ 0.52 (37.1-38)	13.5 $\pm$ 2.97 (11-18)	41.6 $\pm$ 8.89 (34-47)
<b>I (n=12)</b>	37.6 $\pm$ 0.4 (37-38.2)	11.5 $\pm$ 2 <sup>a</sup> (10-15)	37.94 $\pm$ 7.02 <sup>bc</sup> (26-50)
<b>II (n=7)</b>	38.24 $\pm$ 0.52 <sup>a</sup> (37.7-39)	13.4 $\pm$ 3.73 (10-20)	36 $\pm$ 5.12 <sup>a</sup> (29-47)
<b>III (n=6)</b>	37.38 $\pm$ 0.22 <sup>b</sup> (37-37.5)	13.5 $\pm$ 2.6 (10-15)	44.8 $\pm$ 3.39 <sup>b</sup> (44-50)
<b>IV (n=12)</b>	37.46 $\pm$ 0.46 <sup>b</sup> (36.8-38.1)	14.69 $\pm$ 2.9 <sup>b</sup> (15-20)	48.2 $\pm$ 10.14 <sup>b</sup> (26-66)
<b>Kahn and line 2010</b>	37.6(stallion)	10-14	28-40

<sup>abc</sup> = Mean with different superscript in the same column differ significant (p $\leq$ 0.05)

These parameters were detected nearly within normal range in the control group. However, body temperatures were above the normal value in 3 (25%), 5 (71%), and 3 (50%) horses in groups I, II, and IV, respectively. In groups I, II, III and IV, the respiratory rate was above the reference limits in 1 (8.3%), 2 (28.6%), 2 (33.3%) and 4 (33.3%) horses, respectively. Finally, the pulse rates were above reference limits in 2 (16.6%), 1 (14.3%), 4 (66.7%) and 6 (50%) horses in groups I, II, III and IV, respectively. Appetite and body

**Table 2.** Serum enzymes activities of the control and experimental groups (Mean  $\pm$  standard deviation)

Groups	GGT (U/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
<b>Control (n=10)</b>	14.6 $\pm$ 5.3 (5-23)	375 $\pm$ 72 (277-484)	18.3 $\pm$ 4.9 (12-27)	365 $\pm$ 59 (271-469)
<b>I (n=12)</b>	14.2 $\pm$ 3.2 (9-21)	337 $\pm$ 114 (173-506)	14.3 $\pm$ 7.1 <sup>a</sup> (4-28)	243 $\pm$ 43.5 <sup>*a</sup> (183-311)
<b>II (n=7)</b>	19.3 $\pm$ 13 (9-47)	291.4 $\pm$ 87.8 <sup>*</sup> (217-476)	15 $\pm$ 6.3 <sup>ab</sup> (8-26)	240.3 $\pm$ 27.2 <sup>*</sup> (200-269)
<b>III (n=6)</b>	14.2 $\pm$ 5.7 (9-25)	277 $\pm$ 179 (39-550)	17.5 $\pm$ 8.2 (13-34)	253 $\pm$ 28 <sup>*</sup> (217-296)
<b>IV (n=12)</b>	13.4 $\pm$ 6 (5-26)	326 $\pm$ 89 (230-460)	21.6 $\pm$ 7.3 <sup>c</sup> (12-36)	281 $\pm$ 54 <sup>*b</sup> (213-411)
<b>Kahn and Line 2010</b>	6-32	-	-	160-412
<b>Latimer et al 2003</b>	2.7-22.4	70.1-226.8	2.7-20.5	115.7-287
<b>Kaneko et al 2008</b>	5-20	120-250	-	150-300

<sup>abc</sup> Mean with different superscript in same column differ significant ( $p \leq 0.05$ ). \* There was a significant differences with the control group ( $p \leq 0.05$ )

**Table 3.** Serum biochemical findings of the control and experimental groups (Mean  $\pm$  standard deviation)

Groups	Bilirubin Indirect (mg/dl)	Bilirubin Direct (mg/dl)	Bilirubin Total (mg/dl)	TG (mg/dl)	BUN (mg/dl)
<b>Control (n=10)</b>	0.55 $\pm$ 0.26 (0.2-1.1)	0.36 $\pm$ 0.07 (0.3-0.5)	0.91 $\pm$ 0.3 (0.5-1.4)	12.9 $\pm$ 4.5 (9-22)	41.2 $\pm$ 5.8 (33-53)
<b>I (n=12)</b>	0.63 $\pm$ 0.19 (0.5-1.1)	0.31 $\pm$ 0.09 <sup>a</sup> (0.2-0.4)	0.99 $\pm$ 0.24 (0.7-1.5)	21.2 $\pm$ 11 <sup>*a</sup> (8-45)	32.3 $\pm$ 7.3 <sup>*</sup> (22-45)
<b>II (n=7)</b>	0.73 $\pm$ 0.26 (0.4-1)	0.29 $\pm$ 0.07 <sup>*</sup> (0.2-0.4)	1.01 $\pm$ 0.29 (0.6-1.4)	20.4 $\pm$ 6.4 <sup>*a</sup> (13-31)	32.1 $\pm$ 17.4 (14-65)
<b>III (n=6)</b>	0.82 $\pm$ 0.515 (0.2-1.6)	0.28 $\pm$ 0.117 (0.1-0.4)	1.1 $\pm$ 0.54 (0.3-1.4)	16 $\pm$ 6.9 (5-24)	37.3 $\pm$ 3.9 <sup>a</sup> (33-44)
<b>IV (n=12)</b>	0.66 $\pm$ 0.58 (0.2-2.2)	0.23 $\pm$ 0.09 <sup>*b</sup> (0.1-0.4)	0.89 $\pm$ 0.58 (0.4-2.4)	12.8 $\pm$ 7.4 <sup>b</sup> (3-31)	30.1 $\pm$ 5.2 <sup>*b</sup> (23-41)
<b>Kahn and Line 2010</b>	-	0.0-0.4	0.0-3.2	6-54	11-27
<b>Latimer et al 2003</b>	-	-	0.3-3	-	10.4-24.7
<b>Kaneko et al 2008</b>	0.2-2.0	0.0-0.4	1.0-2.0	4.0-44	10.0-24.1

<sup>abc</sup> Mean with different superscript in same column differ significant ( $p \leq 0.05$ ). \*There was a significant difference with the control group ( $p \leq 0.05$ )

**Table 4.** Clinical, biochemical and pathological findings of died or euthanized horses

Horse No.	Usage duration (year)	Clinical findings	Pathological findings	Prominent biochemical findings <sup>*</sup>	Blood sampling (time)
1087	12	Colic; mild icterus	Severe hepatic amyloidosis and rupture	Mild decreased AST, ALP and BUN	1 month before death
1248	7.5	Colic	Severe hepatic amyloidosis and rupture	Mild to moderate decreased BUN, TG, dB and ALP; highly increased AST and ALT	Before death
1176	>10	Weakness; diarrhea	larval migration tract in the liver with hepatitis; enteritis; pneumonia	Moderate increased TG; highly increased ALP and GGT	Before death
1173	10	Weakness; diarrhea	larval migration tract in the liver ; hepatitis; enteritis	Moderate increased tB, iB and highly increased TG, ALP and GGT	Before death
976	>10	Weakness; weight loss	Aortic aneurism with moderate hemorrhage; Heart insufficiency	Nil	1 month before death
967	>10	Weakness; severe weight loss	Heart insufficiency	Mild increased TG, decreased AST, ALP and slightly BUN	1 month before death

\* Compared with the control group

weight were, almost, normal in the majority of horses in the experimental groups, except, two horses in group I (17%) and one horse in groups III (17%) and IV (8%). Mild to moderate colic was observed in four horses in group I (34%). Diarrhea was observed in three horses in group I (25%), but, defecation of other horses was normal. Photosensitization was not observed in any of the experimental animals. Sclera was found moderately icteric in one dead horse (No.1087) and slightly congested in some of the experimental horses, in all groups. Neurological symptoms in the form of restless, aggressive and/or violent behaviors (e.g. biting and kicking), were observed in four horses in each of group III (67%) and IV (33%).

**Biochemical findings.** The serum biochemical parameters are shown in tables 2 and 3

The serum TG and tB concentrations, and GGT activity of all horses in the control group were determined within reference limits. While, the serum BUN concentration and ALP activity were, prominently, above the reference limits. In addition, in two horses serum concentrations of dB and AST, as well as ALT activity were above the reference limits. In experimental groups, serum tB and dB concentration levels and AST activity were within the reference limits. While, increased serum BUN concentration levels were detected in 9 (75%), 3 (43%), 6 (100%) and 8 (67%) horses in group I, II, III and IV, respectively. Mild decreased TG level were detected in one horse in group III (17%) and two horses in group IV (17%). The increased serum ALP and ALT activities in 9 (75%), 5 (71%), 4 (67%) and 12 (100%); 2 (17%), 1 (14%), 1 (17%) and 5 (42%) horses were detected in group I, II, III and IV, respectively. Moreover, moderate increased GGT activity was noticed in one (14%) horse in group II. However, the average serum activity of AST, ALP, ALT (except group IV), and the serum concentration level of BUN and dB, in all experimental groups, were lower than the control group. Whereas, the serum concentration level of tB and TG showed raise in all experimental groups (except group IV). There was no prominent difference in the average serum GGT

activity between the experimental and control groups. During the test period, six horses, which were not included in this study and used for variable duration in snake antivenom production, were died or euthanized, because of poor prognosis due to weakness, weight loss and disability. The clinical findings, gross and histopathological examinations and the serum biochemical data of these horses are summarized in table 4.

**Table 5.** Score for prediction of survival and usage duration in snake antivenom producer horses with hepatic amyloidosis

Category	1	2	3
Usage duration	<6 year	6-9 year	>9 year
Weight loss (Carroll and Huntington, 1988)	Moderate to good	Poor	emaciated
Diarrhea (persistence) (Kahn and Line 2010)	Absent or transient	<1month	>1month
Abdominal pain (Colic)	Absent or mild	Moderate	severe
Decreased BUN (Barton 2007)	>11mg/dl	9-11mg/dl	<9mg/dl
Bilirubin (dB/tB %) (Barton 2007)	0-13%	25-30%	>30%

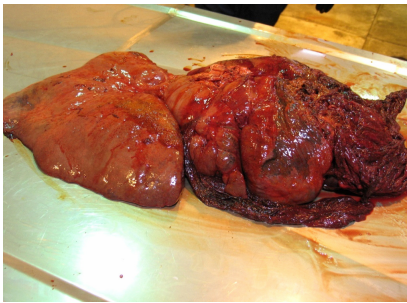
- 12 or more, severe hepatic amyloidosis; death is imminent.
- 8-11, Moderate to high hepatic amyloidosis; final stages of efficiently usage for antiserum production.
- 7 or less; good predictive.

In two horses (No. 1087 and 1248), severe colic was the most important clinical finding. Gross and histopathological examinations revealed severe hepatic amyloidosis and rupture as the probable cause of death (Figures 1, 2). The highly increased serum AST and ALT activities before death were the most prominent serum biochemical findings, in one of these horses (No. 1248). In two other horses (No. 1173 and 1176), diarrhea and weakness were detected in the clinical examination. The increased serum TG concentration level and highly increased GGT and ALP activities were the most outstanding serum biochemical findings. In these two horses, hepatitis and enteritis were detected in gross and histopathological examinations. However, in another two horses (No. 967 and 976) weakness, weight loss and cardiac insufficiency were

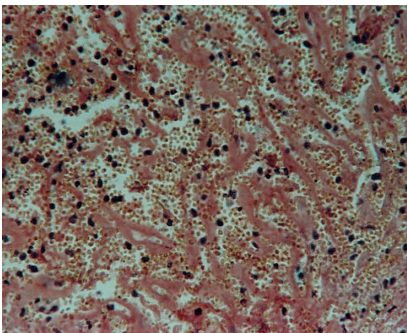
detected in clinical and pathological examinations. On the other hand, interestingly despite long time duration of usage (>10 years) for serum production, severe hepatic and/or splenic amyloidosis was not detected in these animals and no obvious change in serum biochemical parameters was found in the serum analysis.

## DISCUSSION

Amyloidosis is a common cause of death in horses aggressively immunized for antiserum production (Kahn and Line, 2010). These horses may die suddenly because of hepatic insufficiency without any clinical manifestations; that is due to usage in serum production for long-term period (Abdelkader et al., 1991; Ambrojo et al., 2013).



**Figure 1.** Liver ruptures due to sever amyloidosis in a polyvalent snake antivenom producing horse. The liver is highly inflamed and fragile.



**Figure 2.** Severe hepatic amyloidosis in a polyvalent snake antivenom producing horse. There is diffused hemorrhage between necrotic hepatocytes. Pink color cords are Amyloid deposits. H&E(×400).

Based on the authors' previous experiences, the liver amyloidosis -extracellular deposition of insoluble beta-fibrillar proteins- occurs mostly in left lateral, medial, and quadrate lobes. Therefore, in the majority of cases

the lethal liver rupture, associated with hepatic amyloidosis, occurs in these anatomical sections. In the present study, the most common clinical signs of liver disease were detected in group I, which were used for more than nine years in snake antivenom production. The symptoms such as hepatic encephalopathy (HE), icterus and photosensitivity were not detected in any experimental horses. However, the clinical signs of colic (34%), diarrhea (25%) and weight loss (17%) were recorded in some horses in group I. In the other experimental groups weakness and weight loss (8-17%) were the only common clinical findings. It should be noticed that clinical signs of hepatic insufficiency in the horses are variable and the appearance of specific clinical signs of hepatic disease often reflects the type of alteration in their function (Reed et al., 2010). Induction of colic, most probably, was related to the acute hepatic swelling associated to severe hepatic amyloidosis, but alteration in the intestinal microflora and/or deficiency of bile acids might be the etiology of diarrhea. However, weakness and weight loss, most likely, was provoked by decreased appetite and loss of normal hepatocellular metabolic activities (Barton, 2007). These data and the necropsy and histopathological examination findings of the six horses revealed that the aforementioned clinical signs (colic, diarrhea and weight loss) were appeared, mostly, after seven years after being used for immunization; which was coincided with the incidence of severe hepatic amyloidosis. Thus, the clinical signs and usage duration for snake antiveom production may serve as a practical method for evaluation of liver amyloidosis status and insufficiency in the horses applied for hyper-immune serum. Consequently, it can be concluded that the weakness, progressive weight loss, and chronic diarrhea are the warning signs for end of efficient usage of horses in the process of snake antivenom production, due to the severe hepatic amyloidosis. Furthermore, in the present study, death due to severe colic in two horses (No. 1087 and 248) showed that the sever colic is the hint of imminent death due to acute hepatic swelling which, in most instances is accompanied with

the rupture of this organ. Based on the authors' previous experience in these situations, usually there is a positive history of abdominal trauma. According to gross and histopathological findings, some challenges were raised, which the authors have found no justification for them. Whether there were no obvious signs of hepatic amyloidosis (highly enlarged and fragile liver) in the necropsy of two horses (No. 967 and 976) despite long-time duration of usage for snake antivenom production (Durham et al., 2003a). As the severity of clinical signs is the most useful non-invasive prognostic test for diagnosis of the suspected liver disease cases in the mature horses. In the current study, despite presence of restlessness, aggressive, and violent behaviors in four horses in each of the groups III (67%) and IV (33%), these symptoms were not related to HE, but were most likely associated to less adaptation to pain and discomfort, due to repeated antigen injection and bleeding, of the horses of the groups I and II during the hyperimmunization process. Lack of progressive signs of depression, head pressing, circling, ataxia and persistent yawning in affected the horses, agreed to this conclusion. Despite, significant difference in the average heart and respiratory rates of the experimental groups in the present study, relevant to the horses' age or associated to usage duration, the excitement levels of horses during examination might be the reason of alterations. Some researchers reported the normal body temperature of the horses with hepatic insufficiency, in the absence of secondary infection (Smith, 2015). Conversely, according to Amory et al. (2005) the most frequently presented clinical signs in the equine suffering from hepatic diseases are fever, tachycardia, tachypnea, depression, decreased appetite, icterus and the congested mucous membranes. However, the results of the current study and Arslan and Sahal (2009) revealed the statistically significant change in average body temperature between the experimental groups. These variations, probably, indicated a direct association between the body temperature and usage duration of horses for snake antivenom production. Nevertheless, further

investigations are essential to confirm this finding. Totally, it seems that clinical application of vital signs in assessment of the prognosis of hepatic amyloidosis and/or hepatic insufficiency is not very practical. The laboratory diagnosis of hepatic disease of horses can be challenging because the various liver functions are variably altered by the diseases and the majority of hepatic tissue involvement must be present (more than 70%) before the alterations be detected by the laboratory tests (Barton, 2007). According to Pearson (1999), the serum GGT activity is a useful screening test for detecting subclinical liver disease in the horses exposed to pyrrolizidin alkaloids. Amory et al. (2005) reported a significant difference in the serum GGT and ALP activities, between the surviving and non-surviving equine from acute hepatic insufficiency. Similarly, in the present study, the highly increased serum GGT and ALP activities in two horses (No. 1173 and 1176) with fatal outcome from hepatitis were consistent to the aforementioned evidence. However, the decreased serum GGT activity to less than 20U/L was detected in most of the horses in the experimental groups in the present study. In contrast, Abdelkader et al. (1991) reported that during a five year period in 27 horses, used for hyperimmune serum production; in the majority of horses GGT activity had increased to more than 10-fold greater than the upper reference limit within 6-7 years of first immunization. Decreased serum GGT activity below 20U/L, as AST in the present study, was probably the sign of an underactive liver, which, is commonly seen in horses after long spells or after grazing for periods on poor quality and high weed pasture (McGregor, 2015). In the present study keeping the horses, with minimum effort or exercise program inside the stalls, perhaps, caused underactive hepatic status. Low serum AST activity in 26 horses (70%) and presence of a statistically significant difference in the average serum AST activity between the control group and four experimental groups ( $P \leq 0.05$ ) was an interesting finding in the present study. The serum AST activity below 300 U/L is associated with either under work or

under effort and may explain a disappointing performance (McGregor, 2015). This hypothesis can be accepted because all the experimental horses were rarely removed out of the stables. Thus, it should be considered that the reduced vitamin B6 level which, rarely occurs in animal with liver disease or on certain drugs, can result in decreased aminotransferases. It happens, because both AST and ALT activities require active metabolite of vitamin B6 (pyridoxal 5' phosphate (P5P)) as an essential cofactor for maximum enzyme activity (Keller et al., 1985; McGregor, 2015). On the other hand, the serum biochemical analysis and necropsy findings of two dead horses due to liver rupture revealed that the AST activity remained unchanged in one month before death (case No. 1087). However, it was highly increased just before death in the other horse (case No. 1248). These observations showed that at the final stages of severe liver amyloidosis, hepatocellular injury and necrosis was increased. However, increased AST activity can be attributed to muscle leakage during the severe straining in colic and/or concurrent infections (Saad et al., 2007). More investigation is needed to confirm such a declaration. Although, ALT is not a useful specific marker of liver disease in the large animals, due to low enzyme activity in the liver of these species (Keller et al., 1985; Pearson, 1999; Latimer et al., 2003). Nevertheless, in the current study the average serum ALT activity of the horses, in all experiment groups (except group IV), were found less than the control group, might be due to the reduced vitamin B6. Nevertheless, serum ALP activity of the horses in the experimental groups was detected to be increased to more than the reference level in the present study, but it was less than the control group. So, by using the reference values for assessment, the ALP activity in all experimental groups was measured higher, that might be related to biliary obstruction due to hepatic amyloidosis. Whereas, if the serum ALP activity limits of the control group be applied, the average serum ALP activity in all experiment groups would be considered high. The zinc deficiency, which led to various clinical

signs and decreased ALP activity in foals, may be involved in these conditions (Kahn and Line, 2010). Ellison and Jacobs (1990) and Muñoz et al. (2012) reported the decreased age-dependent serum ALP activity in foals. Alternatively, Abdelkader et al. (1991) reported moderately, less than two-fold reference limit increase of the serum ALP activity in the serum producing horses after 6 to 7 years being used for immunization. Additionally, Arslan and Sahal (2009) in similar study reported a significant increase in the serum ALP activity in the serum producing horses. Since, the decreased serum AST, ALT, ALP and to somewhat GGT activities, most probably, are attributed to under work or deficiencies of vitamins and minerals, therefore, the regular exercise programs and additional supplementary nutrient to diet should be considered in the management of snake antivenom producing horses. In horses, the decreased serum BUN concentration, below 9mg /dl, may be attributed to chronic hepatocellular disease (Barton, 2007). Although, in the present study the lowest measured level of serum BUN concentration was 14 mg/dl, but in twenty horses (54%) it was less than the lower limit (33 mg/dl) of the control group. Likewise, Arslan and Sahal (2009) reported the serum BUN concentration levels of the serum producing horses lower than the control group. Decreased serum urea, is associated with severe hepatopathies, therefore in these conditions, the measurement of serum BUN concentration level has a prognostic value and might be served as a useful screening test for detection of hepatic insufficiency in these horses (Ambrojo et al., 2013). The hepatic insufficiency increases the mobilization of TG from adipose tissue to support energy requiring processes, together with decreased clearance by the liver. This might cause the increment of the concentration of blood TG (Barton, 2007; Ambrojo et al., 2013). In the present study, the serum TG concentration levels were determined within physiological limits. However, the presence of a statistically significant difference in serum TG concentration levels between two experimental groups (group I and II) and the control



group may be reflection of the decreased clearance ability and insufficiency of the liver, in these two study groups. Assessment of serum dB and iB concentrations is useful for diagnosing liver dysfunction in horses but it is not a sensitive method (Durham et al., 2003b; Ambrojo et al., 2013). In the present study, serum tB, iB and dB concentration levels were within the physiological limits. But, average serum tB and iB concentration levels (except tB concentration in group IV) were found more than the control group. However, the average serum dB concentration levels showed decline, which was statistically significant between group II and IV, as well as between group I and IV ( $p \leq 0.05$ ). In the serum producing horses, due to pressure from amyloid deposition in the liver, the cholestasis is probable (McGavin and Zachary, 2006). However, the absence of hyperbilirubinemia in the present study might be due to the large reserve capacity of hepatic secretory and excretory functions. This is the reason, that albeit overall impairment of bile flow, it is not sufficient to influence the decline of serum bilirubin concentration (Abdelkader et al., 1991). According to Barton (2007), when the serum dB concentration level is greater than 25 percent of the tB value, hepatocellular disease should be considered in the differential diagnosis, and when greater than 30 percent of the total value, cholestasis should be considered as well. In the present study, the proportion of dB to tB was more than 25 percent in 5 (42%), 1 (14%), 1 (17%) and zero percent in group I, II, III and IV, respectively. However, it was more than 30% in 5 (42%), 3 (43%), 3 (50%) and 7 (58%) in groups I, II, III and IV, respectively. These data might represent a close relation between incidence of hepatocellular disease and the duration of usage for snake antivenom production. Moreover, cholestasis is a prominent complication in the hypeimmunized horses, even, without any changes in serum dB concentration. Although in the present study the transabdominal ultrasonography was not used, but it is a useful technique for prediction of the hepatic amyloidosis by evaluation of the liver size, changes in hepatic

parenchyma and dilated bile ducts or obstruction (Barton, 2007). However, in some medical literatures refers to the fact that the specificity of the imaging techniques in demonstration of the hepatic amyloidosis in humans is not satisfactory, even, when the clinical and radiologic evidences are present (Shin, 2011). In order to suggest the prognostic criteria to aid decision-making regarding the hepatic disease, Durham et al. (2003b) reported the reliability of clinical, ultrasound and single or combined clinic-pathological laboratory tests. However, in their study, the positive or negative measures of liver disease were not compared favorably with biopsy findings. In another study, Durham et al. (2003c) categorized the liver biopsies and provided a numerical score for statistical evaluation and assessment of the survivors and mortalities in mature horses with liver disease. Nevertheless, using liver biopsy for serum producing horses because of high risk of fatal exsanguinations and subsequent liver rupture is not recommended. In a case review study, statistical analysis of age, gender, and breed was applied to suggest prognostic criteria to develop an appropriate guideline for decision-making in the hepatic diseases (Smith et al., 2003). In this article, the authors have attempted to introduce some useful clinical signs and serum biochemical findings for prediction of the liver amyloidosis and insufficiency; determining the usage duration and survival in serum-producing horses (Table 5). Our results revealed that weakness, progressive weight loss, and chronic diarrhea were the warning signs for end of the period of using a horse for snake antivenom production. Nevertheless, commonly the moderate to severe colic was the sign of imminent death, due to acute hepatic swelling and rupture. However, the serum biochemistry results were not useful for assessment of the outcome compared with the clinical manifestations.

### **Ethics**

We hereby declare all ethical standards have been respected in preparation of the submitted article.

### Conflict of Interest

The authors declare that they have no conflict of interest.

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