Serum Amyloid, and Haptoglobin Concentartion Changes After Experimentally Induced Inflammation in Caspian Horses (*Equus ferus caballus*)

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ABSTRACT

Introduction: The Caspian horse (*Equus ferus caballus*), is one of the rarest and oldest horse breeds worldwide, is native to northern Iran. Although it is not listed on the IUCN endangered species list, the breed is endangered due to its small population and limited distribution. Health monitoring is crucial for this vulnerable breed, particularly using biomarkers such as acute phase proteins (APPs) to detect inflammation and subclinical stress.

This study aimed to evaluate changes in serum concentrations of total protein (TP), albumin (Alb), serum amyloid A (SAA), and haptoglobin (Hp) following

experimentally induced inflammation in Caspian horses. A randomized controlled trial was conducted on the healthy, purebred Caspian horses in the Khojir Research Center, Tehran, Iran.

Materials & Methods: Thirty 10-year-old male Caspian horses were randomly assigned to a treatment group (subcutaneous turpentine injection, n = 15) or a control group (saline injection, n = 15). Inflammation was induced by administering 5 mL of turpentine. Blood samples were collected at baseline (1 h before injection) and at multiple time points up to 144 h post-injection. Serum TP and Alb were measured by spectrophotometry using commercial kits, while SAA and Hp were quantified via ELISA. Statistical analysis was performed using t-tests, with significance at $P < 0.05$.

Results: TP concentrations were significantly lower in the treatment group compared to controls at 48 and 72 h ($P < 0.05$). Alb concentrations were significantly reduced in the treatment group from 4 to 72 h ($P < 0.05$). Hp concentrations were significantly elevated in the treatment group at 72 and 144 h ($P < 0.05$), while SAA concentrations were significantly raised from 16 to 144 h ($P < 0.05$). Among the biomarkers, SAA showed the most rapid and pronounced response to inflammation, highlighting its reliability for early detection of the acute phase response in Caspian horses. Hp also increased significantly, though with a delayed onset.

Conclusion: Turpentine-induced inflammation in Caspian horses resulted in significant alterations in acute-phase proteins and serum biochemical parameters. SAA exhibited the earliest and most pronounced increase, highlighting its

sensitivity as a biomarker for the early detection of inflammation in Caspian horses. Hp also increased significantly, although its response was delayed. These findings indicate that acute-phase proteins, particularly SAA, are valuable biomarkers for monitoring inflammatory responses in this breed.

Keywords: Acute phase response, Caspian Horse, Inflammation, SAA, Haptoglobin

1. Introduction

The Caspian horse is among the rarest and oldest horse breeds, with origins tracing back over 3,000-5,000 years in northern Iran, particularly regions surrounding the Caspian Sea. Rediscovered in 1965 after being presumed extinct for centuries, the breed remains critically endangered due to its small population size and restricted geographic range [1]. Although domestic horses (*Equus ferus caballus*) as a species are not classified as endangered by the IUCN, the Caspian breed faces significant conservation challenges.

Various stressors and immunosuppressive factors can subtly impair animal health, often manifesting subclinically without overt clinical signs [2]. Laboratory biomarkers, particularly acute phase proteins (APPs), serve as valuable tools for detecting underlying stress and inflammation, or infection both in subclinical and clinical conditions [3]. In chronic or low-grade stress, APP levels may show mildly but persistent elevations, making them sensitive indicators of physiological perturbations [4].

Acute phase proteins are classified as either positive APPs, which increase in response to inflammation or infection (e.g., C-reactive protein, serum amyloid A (SAA), ceruloplasmin, fibrinogen, alpha-1 antitrypsin, and haptoglobin), or negative APPs, decreasing under similar conditions (e.g., albumin and transferrin) [5]. In equine medicine, commonly measured APPs include SAA, haptoglobin, fibrinogen, and albumin, which are aid in assessing inflammation and infection [6]. Among these, SAA is considered a major APP, characterized by its rapid onset (within 24–48 h) and substantial increase (up to tenfold or more) during inflammatory/infectious events [7].

To date, no published studies have evaluated these biomarkers in Caspian horses. Given the importance of health monitoring in this rare breed, the present study aimed to assess serum concentrations of total protein, albumin, haptoglobin, and SAA in response to experimentally induced inflammation in Caspian horses.

2. Materials And Methods

2.1 Horses

This study was conducted at the Khojir Research Center, Tehran, Iran, from August 2023 to August 2024. All horses were confirmed as purebred Caspian via pedigree records and phenotypic evaluation. Health status of animals were assessed on physical examination. Sample size was determined based on data from previous studies [8, 9]. Thirty clinically healthy, 10-year-old male Caspian horses were included in the study. Horses were randomly allocated to two equal groups (n=15 each): treatment (turpentine-induced inflammation) and control (saline placebo).

2.2 Induction of inflammation

Inflammation was induced by subcutaneous injection of 5 ml turpentine (*Sigma-Aldrich, CAS 8006-64-2*) into the anterior shoulder region. Controls received an equivalent volume of 0.9% sodium chloride. Turpentine injection is a standard well-established model for sterile acute inflammation in veterinary experimental studies [8]. All procedures were performed under veterinary supervision with strict adherence to animal welfare protocols.

2.3 Sample collection and laboratory analyses

Blood samples were collected from the jugular vein into plain tubes (without anticoagulant) and EDTA venoject tubes. Sampling was performed at -60 min, 0, 8, 16, 24, 48, 72, and 144h following turpentine injection.

Serum samples were separated by using centrifugation for 10 minutes at 3000 rpm and were immediately transported, under cold conditions to the Biochemistry Laboratory, Danesh Animal Hospital, Islamic Azad University, Tehran, Iran, and stored at -20˚ C until analysis.

Serum total protein and albumin were measured by colorimetric spectrophotometry using commercial kits (*Zist Shimi Company, Iran*). Serum amyloid A and haptoglobin concentrations were quantified using commercial ELISA kits (*Tridelta Developments Ltd, Ireland*) according to the manufacturer's instructions.

All assays followed kit protocols precisely.

2.4 Statistical analyses

Data normal distribution was evaluated using the Shapiro–Wilk test. Inter-group comparisons were performed with independent t-tests in SPSS version 22 (IBM

Corp., Armonk, NY, USA). Differences were considered significant when P values were <0.05. Receiver operating characteristic (ROC) analysis determined optimal cutoff values, sensitivity (Se), specificity (Sp), and area under the curve (AUC) for diagnostic performance.

3. Results

3.1 Serum total protein (STP)

No significant differences in TP were observed between groups at −60 min, 0, 4, 8, 16, 24, and 144 h ($P > 0.05$;Table 1, Figure 1). However, at 48 and 72 h, the mean STP concentration in the treatment group was significantly lower than that in the control group ($P < 0.05$).

TP was significantly lower in the treatment group at 48 and 72 h ($P < 0.05$). At 4 h post-induction, its diagnostic performance was poor (cutoff 6.7 g/dL; Se 46.2%, Sp 20%, AUC 0.131; Figure 1). Throughout the study period, TP values remained within the normal equine reference interval (5.5–7.5 g/dL).

3.2 Serum Albumin (Alb)

No significant differences were found at −60 min, 0, or 144 h ($P > 0.05$; Table 1, Figure 2). Alb was significantly lower in the treatment group from 4 to 72 h ($P < 0.05$). Despite statistical significance at 4 h ($P < 0.01$), diagnostic utility was limited (cutoff 3.45 g/dL; Se 15.4%, Sp 20%, AUC 0.092; Figure 2). Concentrations stayed within normal ranges (2.6–3.7 g/dL).

3.3 Serum Haptoglobin (SHp)

No differences occurred at −60 min, 0, 4, 8, 16, 24, or 48 h ($P > 0.05$; Table 1, Figure 3). Hp was significantly higher in the treatment group at 72 and 144 h ($P <$

138 *0.05*). Hp showed reliable diagnostic performance at 4, 48, 72, and 144 h (*P* < *0.01*),
139 with cutoffs of 1.35 g/L (4 h), 3.10 g/L (48 h), 1.40 g/L (72 h), and 0.43 g/L (144
140 h). Se/Sp reached 86.7%/80% at 4 h and 93.3%/100% thereafter; AUC was 0.853
141 at 4 h and 1.00 at later time points (Figure 3).

142 **3.4 Serum amyloid A (SAA)**

143 No differences at −60 min, 0, 4, or 8 h (*P* > *0.05*; Table 1, Figure 4). SAA was
144 significantly elevated in the treatment group from 16 to 144 h (*P* < *0.05*). SAA
145 proved highly reliable at 24, 48, 72, and 144 h (*P* < *0.01*), with cutoffs of 479.48
146 mg/L (24 h), 162.81 mg/L (48 h), 151.31 mg/L (72 h), and 52.8 mg/L (144 h); Se/Sp
147 91.7%/100% and AUC 1.00 at these points (Figure 4).

148 Table 2 summarizes ROC-derived diagnostic performances across biomarkers.

149 **4. Discussion**

150 The present study is the first research which aimed to characterize changes in serum
151 total protein (TP), albumin, haptoglobin (Hp), and serum amyloid A (SAA)
152 concentrations in Caspian horses following experimental induction of
153 inflammation.

154 Total protein, combined concentration of albumin and globulins, is considered a
155 useful but nonspecific biomarker of inflammation [10]. Normal TP concentrations
156 in horses range from 5.5 to 7.5 g/dL, and deviations may reflect dehydration,
157 chronic inflammation, or protein loss associated with various pathological
158 conditions [11, 12]. In the present study, although TP concentrations decreased
159 significantly at 48 and 72 h after inflammation induction, values remained within
160 the reference interval. This finding suggests that TP alone has limited diagnostic

value for assessing acute inflammation in Caspian horses. Similar observations have been reported previously, indicating that TP fluctuations during acute inflammatory responses are often inconsistent and should be interpreted in conjunction with other acute phase proteins [13, 14].

Atyabi (1999) investigated serum biochemical parameters in Caspian ponies and Arabian horses and reported that TP and globulin concentrations increased with age in both breeds, with a significant difference between the 0–36-month and 37–72-month age groups. In contrast, no significant age-related differences were observed in the present study, likely due to the broad age range (0–72 months) included within the first age group [15]. Jahn et al. (1994) demonstrated that TP concentrations increase in healthy riding horses following exercise. Additionally, in a study conducted in 2008 on foals, the authors reported a gradual postnatal increase in serum protein concentration, primarily attributable to elevated globulin levels resulting from colostrum absorption. In the present study, however, TP concentrations did not exhibit a sustained increase, which may be explained by the absence of dehydration or prolonged physiological stress [16].

Serum albumin is a major plasma protein involved in maintaining oncotic pressure, molecular transport, and antioxidant defense. During the acute phase response, albumin concentrations typically decrease due to reduced hepatic synthesis, increased catabolism, and redistribution into extravascular compartments [17, 18]. In the present study, albumin levels declined significantly from 4 to 72 h post-injection but remained within the established reference range (2.6–3.7 g/dL). Similar to TP, these findings suggest that albumin concentration alone has limited

184 diagnostic value for assessing acute inflammation in Caspian horses. Richard et al.
185 (1994) observed no significant breed-related differences in TP or albumin
186 concentrations in Thoroughbred and Saddlebred horses, highlighting that albumin
187 changes may be subtle and not clinically significant in certain breeds or
188 physiological conditions [19].
189 Haptoglobin, a liver-synthesized glycoprotein that binds free hemoglobin and limits
190 oxidative damage, showed a significant increase at 72 and 144 h following
191 inflammation induction. The elevation of Hp concentrations beyond the reference
192 range (0–0.3 mg/mL), with a peak observed at 144 h (0.34 mg/L), highlights Hp as
193 a sensitive marker of inflammatory status in Caspian horses, although with a
194 delayed response compared with SAA. This delayed kinetic profile should be
195 considered when interpreting Hp levels in both clinical and experimental settings.
196 In horses, Hp is classified as a moderate acute phase protein and is typically
197 expected to increase 2–5 days after inflammatory stimulation [20]. The results of
198 the present study are consistent with those reported by Canisso et al. (2014), who
199 reported similar temporal patterns of Hp elevation in equine inflammatory
200 conditions [20].
201 Serum amyloid A, a major acute phase protein rapidly synthesized by the liver in
202 response to inflammatory stimuli, exhibited a marked and early increase beginning
203 at 16 h post-induction, peaking at 48 h, and remaining elevated throughout the study
204 period. These findings are in agreement with previous studies that have identified
205 SAA as a highly sensitive biomarker of inflammation in horses [21, 22]. The rapid
206 kinetics and pronounced elevation of SAA support its utility in routine health

monitoring and early diagnosis of inflammatory conditions in this threatened equine breed [23].

ROC analysis demonstrated clear differences in the diagnostic performance of inflammatory indices in Caspian horses, reflecting their biological behavior during the acute phase response. SAA showed the highest AUC, Se, and Sp, confirming its role as a major and rapidly responsive acute phase protein in horses. Haptoglobin, a moderate positive acute phase protein, demonstrated good discriminatory power at later time points, with increased AUC values once inflammation was established. This delayed but reliable ROC performance aligns with reports showing slower kinetics and lower amplitude increases of Hp compared with SAA in horses subjected to sterile or infectious inflammatory stimuli [9, 24]. In contrast, total protein and albumin, classified as minor or negative acute phase proteins, showed poor sensitivity, specificity, and low AUC values. These results are in agreement with earlier studies indicating that TP and albumin decrease modestly and inconsistently during inflammation, limiting their utility as standalone diagnostic markers in ROC-based evaluations [25, 26]. Overall, the ROC analysis supports the combined use of SAA for early detection and Hp for confirmation of ongoing inflammation, while TP and albumin remain indicators of systemic or chronic alterations rather than acute inflammatory status.

5. Conclusion

Turpentine-induced inflammation in Caspian horses produced significant alterations in acute-phase proteins and selected serum biochemical parameters, confirming the activation of a systemic inflammatory response in this breed.

Among the evaluated biomarkers, serum amyloid A (SAA) demonstrated the earliest, most pronounced, and most consistent increase, indicating its high sensitivity as an early indicator of inflammation. The rapid rise of SAA shortly after induction of inflammation highlights its strong diagnostic value for the early detection of inflammatory conditions in Caspian horses.

Haptoglobin (Hp) also increased significantly; however, its response was comparatively delayed and became more evident during the later stages of the inflammatory process. This temporal pattern suggests that Hp may serve as a complementary biomarker, particularly for assessing the progression and persistence of inflammation. The differing response dynamics between SAA and Hp emphasize the benefit of evaluating multiple acute-phase proteins to obtain a more comprehensive assessment of inflammatory status.

Although certain serum biochemical parameters, including total protein (TP) and albumin (Alb), showed statistically significant changes, their values remained largely within established reference ranges, suggesting limited clinical relevance for the detection of acute inflammation. In contrast, acute-phase proteins demonstrated greater sensitivity and diagnostic utility for monitoring inflammatory responses.

Overall, these findings highlight the clinical value of acute-phase proteins "particularly SAA" as sensitive and reliable biomarkers for detecting and monitoring inflammation in Caspian horses. The results provide new insights into the dynamics of inflammatory responses in this breed and may contribute to improved diagnostic and monitoring approaches in equine veterinary medicine.

Data availability

The data supporting the findings of this study are available.

Ethical approval

All procedures complied with the ethical guidelines of the Islamic Azad University and were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran.

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Conflict of interest

The Authors declare no conflict of interest.

Acknowledgments

The authors gratefully acknowledge Dr. Hossein Khalesi, Dr. Shahram Dardari, Dr. Hormoz Hamidieh, Dr. Ali Parsa Khaneghah, Engineer Kiarash Mohebbi, Engineer Dehghani, Ms. Andisheh, and all staff of the Khojir Research Center for their valuable support and cooperation throughout this study.

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Figure 1: STP–time dynamics (left) and ROC (Right) analysis in control and treatment groups after inflammation induction

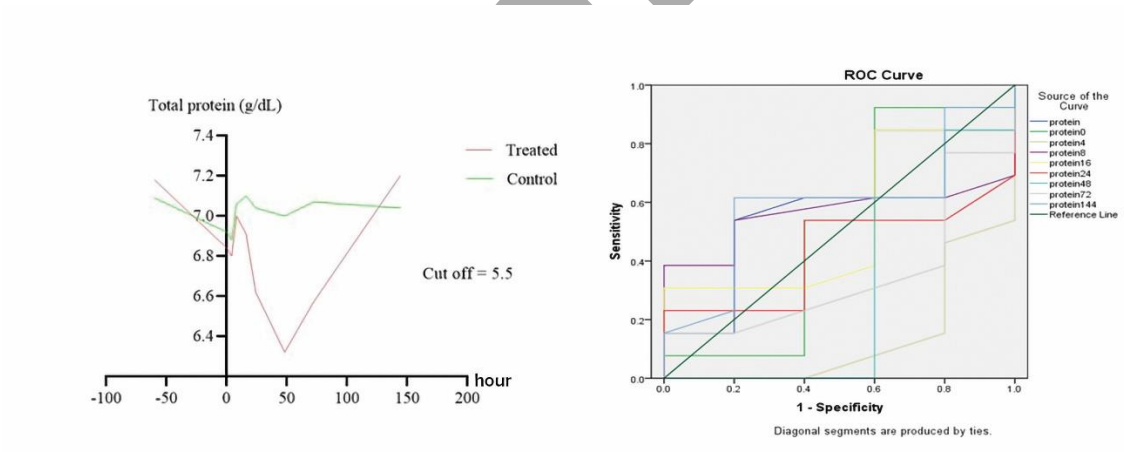

Figure 2: SA–time dynamics (left) and ROC (Right) analysis in control and treatment groups after inflammation induction

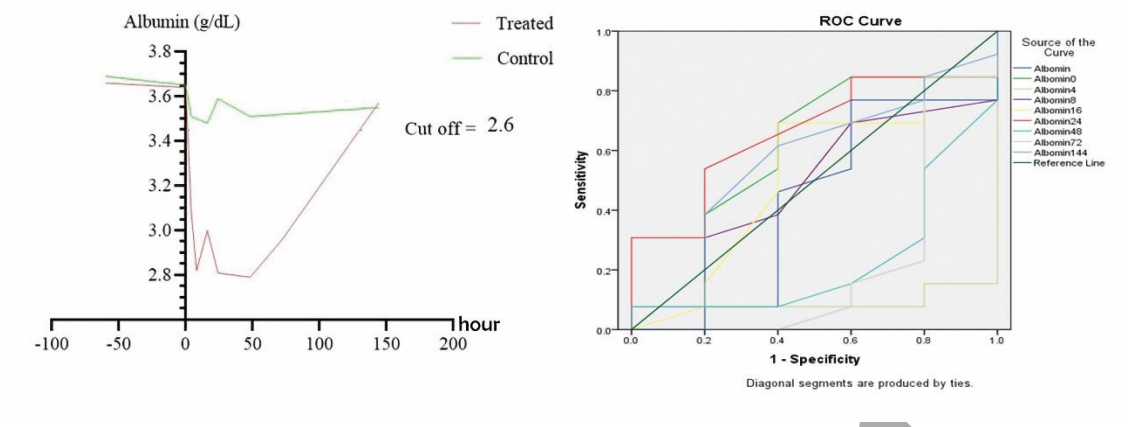

Figure 3: SHp–time dynamics (left) and ROC (Right) analysis in control and treatment groups after inflammation induction

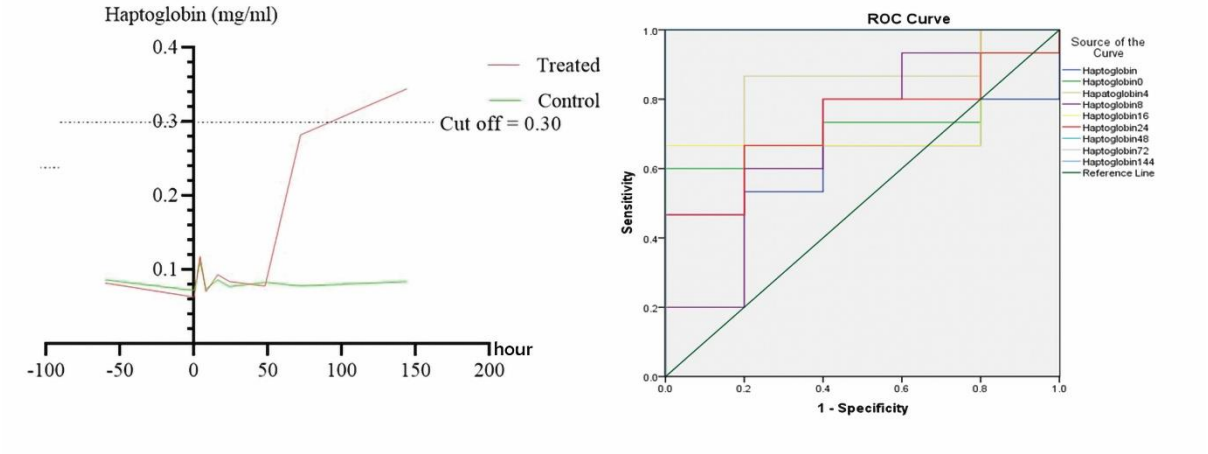

Figure 4: Serum SAA–time dynamics (left) and protein ROC (Right) analysis in control and treatment groups after inflammation induction

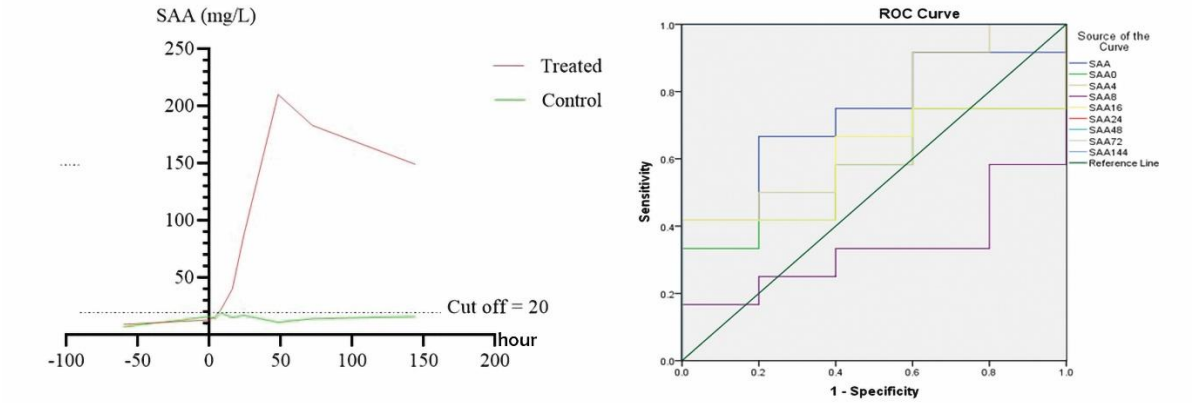

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Table 1: STP, SA, SHp, SAA concentrations (g/dL) in the control and treatment groups (mean ± SD)

Index	Control									Treatment								
	-1	0	+4	+8	+16	+24	+48	+72	+144	-1	0	+4	+8	+16	+24	+48	+72	+144
STP	7.09 ± 0.33^{a}	6.92 ± 0.37^{a}	6.88 ± 0.50^{a}	7.06 ± 0.31^{a}	7.10 ± 0.64^{a}	7.04 ± 0.44^{a}	7.00 ± 0.32^{a}	7.07 ± 0.47^{a}	7.04 ± 0.43^{a}	7.18 ± 0.52^{a}	6.84 ± 0.39^{a}	6.80 ± 0.61^{a}	7.00 ± 0.26^{a}	6.91 ± 0.38^{a}	6.62 ± 0.53^{a}	6.32 ± 0.57^{b}	6.57 ± 0.54^{b}	7.20 ± 0.62^{a}
SA	3.69 ± 0.21^{a}	3.65 ± 0.47^{a}	3.51 ± 0.34^{a}	3.50 ± 0.27^{a}	3.48 ± 0.25^{a}	3.59 ± 0.41^{a}	3.51 ± 0.34^{a}	3.52 ± 0.24^{a}	3.55 ± 0.60^{a}	3.66 ± 0.31^{a}	3.64 ± 0.22^{a}	3.08 ± 0.53^{b}	2.82 ± 0.46^{b}	3.00 ± 0.68^{b}	2.81 ± 0.53^{b}	2.79 ± 0.47^{b}	2.96 ± 0.39^{b}	3.57 ± 0.52^{a}

SHp	0.086 ± 0.029[a]	0.072 ± 0.031[a]	0.111 ± 0.022[a]	0.074 ± 0.019[a]	0.086 ± 0.015[a]	0.077 ± 0.017[a]	0.083 ± 0.021[a]	0.078 ± 0.019[a]	0.084 ± 0.023[a]	0.082 ± 0.027[a]	0.063 ± 0.018[a]	0.118 ± 0.014[a]	0.070 ± 0.016[a]	0.093 ± 0.020[a]	0.084 ± 0.023[a]	0.078 ± 0.014[a]	0.282 ± 0.020[b]	0.344 ± 0.019[b]
SAA	7.31 ± 2.14[a]	16.27 ± 3.26[a]	14.09 ± 2.35[a]	19.38 ± 2.72[a]	15.58 ± 2.54[a]	17.93 ± 2.39[a]	11.34 ± 2.01[a]	14.18 ± 2.49[a]	16.33 ± 2.18[a]	9.59 ± 2.40[a]	13.19 ± 2.93[a]	16.48 ± 3.53[a]	22.84 ± 3.81[a]	40.28 ± 4.11[b]	87.37 ± 6.23[b]	210.63 ± 10.17[b]	183.48 ± 7.44[b]	149.51 ± 9.21[b]

Different superscript levels (a and b) indicate significant difference between groups ($P < 0.05$)

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Table 2: Cut-off points, sensitivity, specificity, and ROC in Caspian horses after inflammation induction.

Index	Time (h)	Cut-off	Sensitivity%	Specificity %	AUC
TP	4	6.7	46.20	20	0.131
Albumin	4	3.45	15.40	20	0.092
SHp	4	1.35	86.70	80	0.853
SHp	48	1.43	93.30	100	1
SHp	72	2.14	93.30	100	1
SHp	144	3.10	93.30	100	1
SAA	24	52.80	91.70	100	1

SAA	48	1517.31	91.70	100	1
SAA	72	162.81	91.70	100	1
SAA	144	479.48	91.70	100	1

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