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Research Paper

Dynamics of Serum Amyloid A and Haptoglobin After Experimentally Induced Inflammation in Caspian Horses (*Equus ferus caballus*)

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ABSTRACT

Introduction: The Caspian horse (*Equus ferus caballus*), one of the rarest and oldest horse breeds worldwide, is native to northern Iran. Although it is not listed on the International Union for Conservation of Nature (IUCN) endangered species list, the breed is endangered due to its small population and limited distribution. Health monitoring of this breed is crucial, particularly using biomarkers such as acute phase proteins (APPs) to detect inflammation and subclinical stress. This study aimed to evaluate changes in serum levels 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 healthy, purebred Caspian horses from 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 using 5 mL of turpentine, and 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 level set at $P<0.05$.

Results: TP concentrations were significantly lower in the treatment group compared to control at 48 and 72 h post-injection ($P<0.05$). Alb concentrations were significantly reduced in the treatment group from 4 to 72 h post-injection ($P<0.05$). Hp concentrations were significantly elevated in the treatment group at 72 and 144 h post-injection ($P<0.05$), while SAA concentrations were significantly increased from 16 to 144 h post-injection ($P<0.05$). 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: In contrast, while TP and Alb showed statistically significant changes, their values remained within reference intervals, suggesting limited diagnostic utility for these markers in this breed.

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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 in 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 International Union for Conservation of Nature (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 mild 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 (CRP), serum amyloid A (SAA), ceruloplasmin, fibrinogen, alpha-1 antitrypsin, and haptoglobin [Hp]), or negative APPs, which decrease under similar conditions (e.g. albumin (Alb) and transferrin) [5]. In equine medicine, commonly measured APPs include SAA, Hp, fibrinogen, and Alb, which 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 or 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 (TP), Alb, Hp, 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 and deemed clinically healthy based 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 of 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 vacutainer tubes. Sampling was performed at -60 min, 0, 8, 16, 24, 48, 72, and 144 h following turpentine injection.

Serum was harvested after clotting for 4 h at room temperature, followed by centrifugation for 10 min at 3000 rpm. Samples 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 TP (STP) and Alb were measured by colorimetric spectrophotometry using commercial kits (Zist Shimi Company, Iran). Serum amyloid A and Hp concentrations were quantified using ELISA kits (Tridelta Developments Ltd, Ireland) according to the manufacturer's instructions. Samples, including standards of known SAA content, were added into micro wells along with anti-SAA monoclonal antibody. Any SAA present in the well is both captured on the plate by the immobilized antibody and labelled with the conjugate antibody in a one step procedure. After washing to remove all of the unbound material, TMB substrate solution was added. The intensity of the colour produced is proportional to the concentration of SAA present in the original specimen.

The lowest and highest concentrations of calibrators used were 0 t0 30 mg/L, respectively in SAA-ELISA. CV% was 12.1% according to manufacturers' manual.

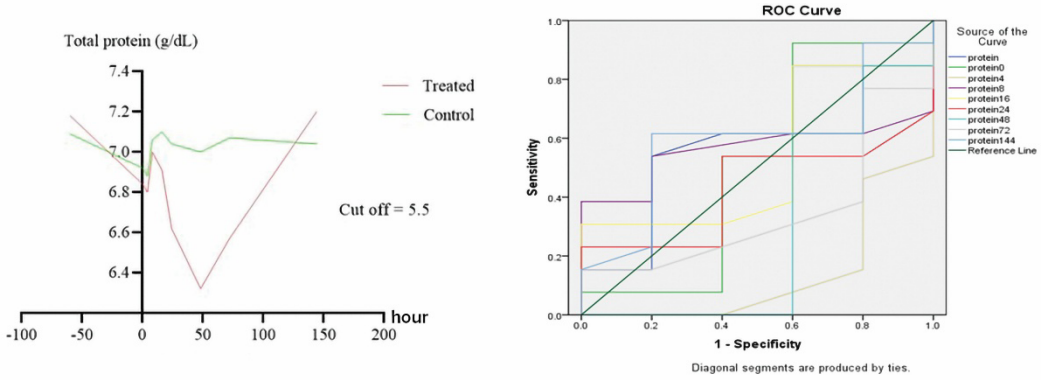

Figure 1. STP–time dynamics (left) and ROC (right) analysis in control and treatment groups after inflammation induction

2.4. Statistical analyses

Data normal distribution was evaluated using the Shapiro–Wilk test. Inter-group comparisons were performed using independent t-tests in SPSS software, version 22 (IBM Corp., Armonk, NY, USA). Differences were considered significant when P<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. STP

No significant differences in TP were observed between groups at −60 min, 0, 4, 8, 16, 24, and 144 h post-injection (P>0.05;Table 1, Figure 1). However, at 48 and 72 h post-injection, 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 post-injection (P<0.05). At 4 h post-injectio, diagnostic performance was poor (cutoff 6.7 g/dL; Se 46.2%, Sp 20%, AUC 0.131; Figure 1). Values remained within normal equine reference intervals (5.5–7.5 g/dL) throughout.

3.2. Serum Alb (SAlb)

No significant differences were found at −60 min, 0, or 144 h post-injection (P>0.05; Table 1, Figure 2). Alb was significantly lower in the treatment group from 4 to 72 h post-injection (P<0.05). Despite statistical significance at 4 h post-injection (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 Hp (SHp)

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

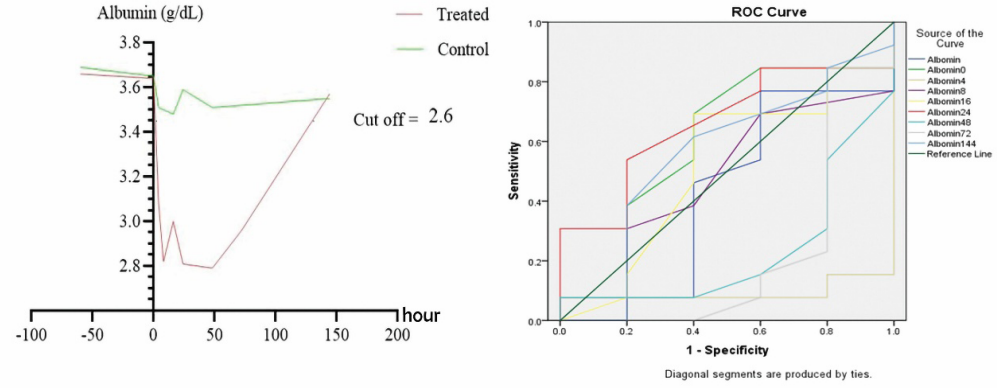

Figure 2. SA–time dynamics (left) and ROC (right) analysis in control and treatment groups after inflammation induction

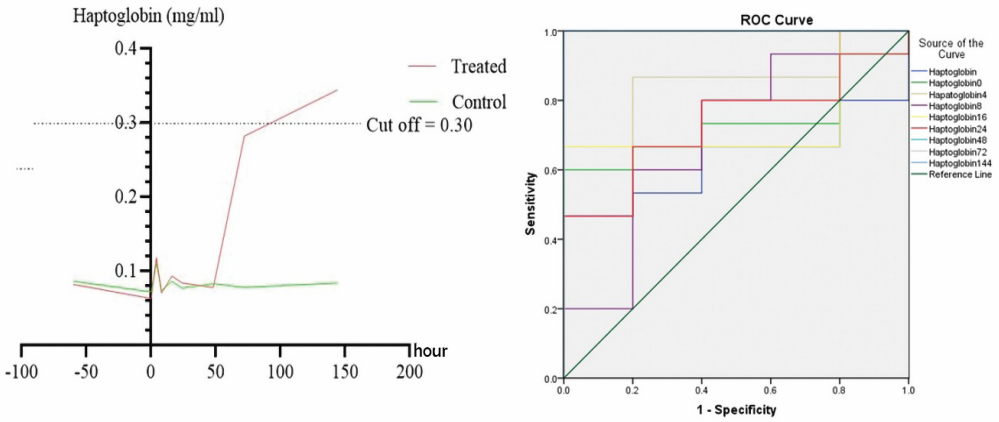

Figure 3. SHp–time dynamics (left) and ROC (right) analysis in control and treatment groups after inflammation induction

tic performance at 4, 48, 72, and 144 h post-injection (P<0.01), 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 h). Se/Sp reached 86.7%/80% at 4 h post-injection and 93.3%/100% thereafter; AUC was 0.853 at 4 h post-injection and 1.00 at later time points (Figure 3).

3.4. SAA

No differences at −60 min, 0, 4, or 8 h post-injection was observed (P>0.05; Table 1, Figure 4). SAA was significantly elevated in the treatment group from 16 to 144 h post-injection (P<0.05). SAA proved highly reliable at 24, 48, 72, and 144 h post-injection (P<0.01), with cutoffs of 479.48 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 91.7%/100% and AUC 1.00 at these points (Figure 4).

Table 2 summarizes ROC-derived diagnostic performances across biomarkers.

4. Discussion

The present study is the first to characterize changes in STP, Alb, Hp, and SAA concentrations in Caspian horses following experimental induction of inflammation.

TP, representing the combined concentration of Alb and globulins, is considered a useful but nonspecific biomarker of inflammation [10]. Normal TP concentrations in horses range from 5.5 to 7.5 g/dL, and deviations may reflect dehydration, chronic inflammation, or protein loss associated with various pathological conditions [11, 12]. In the present study, although TP concentrations decreased significantly at 48 and 72 h after inflammation induction, values remained within 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 APPs [13, 14].

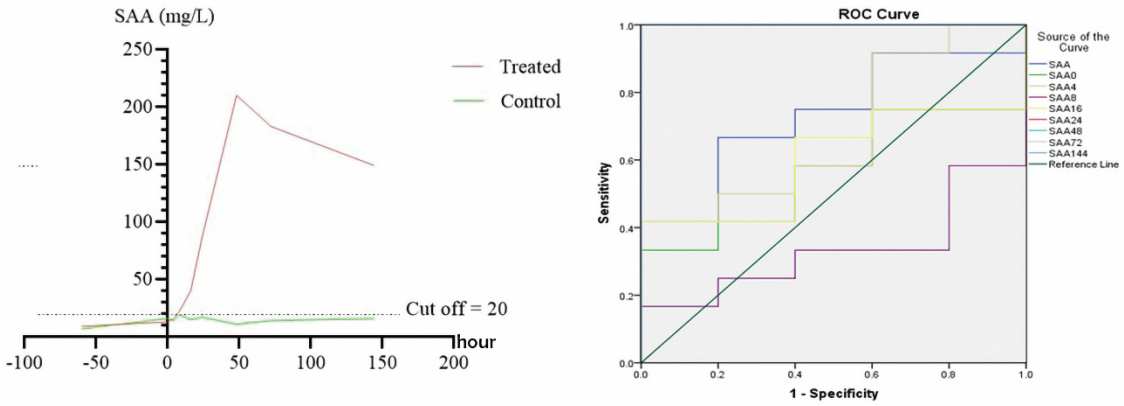

Figure 4. Serum SAA–time dynamics (left) and protein ROC (right) analysis in control and treatment groups after inflammation induction

Table 1. STP, SA, SHp, SAA concentrations (g/dL) in the control and treatment groups

Index	Mean±SD (g/dL)																	
	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±0.5[a]	7.06±0.31[a]	7.1±0.64[a]	7.04±0.44[a]	7±0.32[a]	7.07±0.47[a]	7.04±0.43[a]	7.18±0.52[a]	6.84±0.39[a]	6.8±0.61[a]	7±0.26[a]	6.91±0.38[a]	6.62±0.53[a]	6.32±0.57[b]	6.57±0.54[b]	7.2±0.62[a]
SA	3.69±0.21[a]	3.65±0.47[a]	3.51±0.34[a]	3.5±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±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]	0.07±0.016[a]	0.093±0.02[a]	0.084±0.023[a]	0.078±0.014[a]	0.282±0.02[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±2.4[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]

[a] and [b] Different superscript letters, indicate significant difference between groups (P<0.05).

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	46.2	20	0.131
Albumin	4	3.45	15.4	20	0.092
SHp	4	1.35	86.7	80	0.853
SHp	48	1.43	93.3	100	1
SHp	72	2.14	93.3	100	1
SHp	144	3.10	93.3	100	1
SAA	24	52.8	91.7	100	1
SAA	48	1517.31	91.7	100	1
SAA	72	162.81	91.7	100	1
SAA	144	479.48	91.7	100	1

Atiyabi (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].

SA is a major plasma protein involved in maintaining oncotic pressure, molecular transport, and antioxidant defense. During the acute phase response, Alb concentrations typically decrease due to reduced hepatic synthesis, increased catabolism, and redistribution into extravascular compartments [17, 18]. In the present study, Alb 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 Alb concentration alone has limited diagnostic value for assessing acute inflammation in Caspian horses. Richard et al. (1994) observed no significant breed-related differences in TP or Alb concentrations in Thoroughbred and Saddlebred horses, highlighting that Alb changes may be subtle and not clinically significant in certain breeds or physiological conditions [19].

Hp, a liver-synthesized glycoprotein that binds free hemoglobin and limits oxidative damage, showed a significant increase at 72 and 144 h following inflammation induction. The elevation of Hp concentrations beyond the reference range (0–0.3 mg/mL), with a peak observed at 144 h post-injection (0.34 mg/mL), highlights Hp as a sensitive marker of inflammatory status in Caspian horses, although with a delayed response compared with SAA. This delayed kinetic profile should be considered when interpreting Hp levels in both clinical and experimental settings. In horses, Hp is classified as a moderate APPs and is typically expected to increase 2–5 days after inflammatory stimulation [20]. The results of the present study are consistent with those reported by Canisso et al. (2014), who reported similar temporal patterns of Hp elevation in equine inflammatory conditions [20].

Serum amyloid A, a major APPs rapidly synthesized by the liver in response to inflammatory stimuli, exhibited a marked and early increase beginning at 16 h post-induction, peaking at 48 h, and remaining elevated throughout the study period. These findings are in agreement with previous studies that have identified SAA as a highly sensitive biomarker of inflammation in horses [21, 22]. The rapid 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 APPs in horses. Haptoglobin, a moderate positive APPs, 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 Alb, classified as minor or negative APPss, showed poor sensitivity, specificity, and low AUC values. These results are in agreement with earlier studies indicating that TP and Alb 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 Alb remain indicators of systemic or chronic alterations rather than acute inflammatory status.

5. Conclusion

The early and robust increase in SAA underscores its role as a rapid and reliable biomarker, whereas the delayed elevation of Hp suggests its usefulness as a complementary indicator in the later stages of inflammation. These findings enhance the understanding of inflammatory biomarkers in Caspian horses and support the clinical application of APPss in equine veterinary diagnostics.

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Compliance with ethical guidelines

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.

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Authors' contributions

Conceptualization and study design: Shahabeddin Safi and Nader Vojdanifar; Experiments, data interpretation, and statistical analysis: Abbas Rahimi Forushani; Data acquisition and writing the original draft: Nader Vojdanifar; Project administration, technical, and material support, review and editing: Shahabeddin Safi.

Conflict of interest

The authors declared no conflict of interest.

Data availability

The data supporting the findings of this study are available from corresponding author upon reasonable request.

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