# Investigation of the effect of *Parapoxvirus ovis*, *Corynebacterium cutis* lysate and vitamin c on immunosuppression caused by long-term transport stress in Morkaraman sheep*

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## SUMMARY

The purpose of the study is to investigate the effectiveness of the inactive *Parapoxvirus ovis* (iPPVO), *Corynebacterium cutis* Lysate (CCL) as well as Vitamin C (Vit-C) on immunosuppression induced by long-term transport stress in Morkaraman ewes. The study consisted of 4 groups: 1 control, 3 trial groups (iPPVO, CCL and Vit-C), and 8 ewes were allocated into each treatment group (n=32). While levels of cortisol and adrenaline significantly ($P<0.05$) increased at post-transport (PT), the lowest increase was obtained from the Vit-C group. Lymphocyte counts in iPPVO group significantly ($P<0.05$) increased at PT. Additionally, ADA activities in the iPPVO, CCL and Vit-C groups significantly ($P<0.05$) increased. However, activities of ADA in the control group decreased ($P<0.05$). A significant ($P<0.05$) positive correlation ($r=0.24$) was also obtained between ADA activities and lymphocyte counts. Hp levels of the control group at 7$^{th}$ days after transport (PT7) approached levels at Pre-transport (PrT). However, the Hp levels at PT, PT1 and PT7 were similar to that of the iPPVO group at PrT. In the CCL group, the Hp level at first day after transport (PT1) attained a similar level at PrT.

In conclusion, immediately before the transport, administration of Vit-C reduced transport stress more than others. Additionally, it could be suggested that the administration of iPPVO before transport was more effective toward immunosuppression occurring after transport, when lymphocyte counts, ADA activities and Hp levels were taken into consideration.

## KEY WORDS

Transport, stress, *Parapoxvirus ovis*, *Corynebacterium cutis* lysate, vitamin C, sheep.

## INTRODUCTION

Animal transport, which is an important element for comprehensive production systems, is very common especially for sheep[1]. Various reasons, for example the use of pasture-based farming systems, breeding of animals, fattening, slaughter, grazing opportunities, or sales lead to the need for transport[2]. Road transport is the most economically important form of transportation in the European Union countries. Twenty-five million cattle, 7 million calves, 171 million pigs, 75 million sheep and lambs and 9 million goats are transported annually, and 90% of the animal transportation is carried out by road[3]. If the journey distance of the animals is shorter than 50 km, the transportation is considered as short distance journey[4]. On the other hand, it was reported that the duration of the transportation was longer than 8 hours (including loading and unloading time) as well as distance of the journey was greater than 400 km in the long route transport of animals[5,6].

In the road transport of ruminants, many factors, for instance, loading and unloading moments, duration of the journey, improper vehicle use, bad road conditions, vibration and vehicle movements, very hot or cold climate, insufficient ventilation, high stocking densities, water and feed insufficiency result in stress, and adversely affect animal welfare, animal health, animal performance as well as the quality of the products[7]. The hypothalamo-pituitary-adrenocortical axis (HPA) and sympatho-adrenomedullary axis activation are held responsible for the stress[8]. Researchers have reported increases in cortisol and catecholamines levels at different stages of the transport due to the HPA activation[9,10]. Increases in the activity of the Adrenal cortex negatively affect animal welfare and may lead to immunosuppression[11].

Behavioral changes, hormonal changes, oxidative stress markers, immunological indicators, genomic and proteomic indicators are used as biomarkers for the determination of stress in animals[12,13]. The immunological responses of ruminants to HPA activation that occurred during transport are leukocytosis and lymphopenia[14]. Results of the studies revealed that especially the T lymphocytes counts decreased during transport. On the other hand, there was no significant change in the B lymphocytes counts[15,16].

ADA is an enzyme widely found in tissues and body fluids. However, the most important biologic activity is associated with lymphoid tissue, since ADA is required for the proliferation and differentiation of T lymphocytes[17]. A positive significant correlation between ADA activity and lymphocyte count was also determined by Rao *et al.*[18] and Abdi *et al.*[19]. It was also reported

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that the decrease in ADA activity was associated with suppression of lymphocyte count, lymphocyte proliferation and differentiation, which led to a lack of immune response[20,22]. Additionally, Lomborg *et al.*[23] suggested the usage of Hp as a physiological indicator of stress in cattle during road transportation. It was also noted that increased Hp levels were responsible for immunosuppression after transport, and the HP fraction (33 k+20 K Dalton) obtained from serums led to dose-dependent suppression in lymphocyte blastogenesis[24].

Purposes of the immunomodulation in animals are as follows: activating a strong and long-term immune response to diseases caused by microorganisms; accelerating the maturation of specific and non-specific immunity in the neonatal period and young animals; developing local protective immune reactions in sensitive areas such as the mammary gland in dairy cattle or the gastrointestinal tract in neonatal ruminants; reducing the immunosuppressive effects of stress and environmental pollution; increasing immunity time after vaccination[25]. Neuroendocrine hormones, thymus products, inactive *Parapoxvirus ovis*, *Corynebacterium cutis* lysate, Probiotics, Levamisole, Vit-E and Vit-C are some of the immunomodulators[26,29.]

Vit-C has been shown to enhance differentiation and proliferation of B- and T-cells as well as maturation of immature T cells[30,31]. *Parapoxvirus ovis* and *Corynebacterium cutis* lysate are non-selective (innate) immunmodulators which often resulted in the activation and modulation of T lymphocyte function[32]. Therefore, they could have potential positive effect on reducing immunosuppression caused by long route transport stress.

To the best of our knowledge, no single study exists which compares the immunomodulatory efficacy of iPPVO, CCL and Vit-C on the ADA activity and Hp in long-term road transport of sheep. Therefore, it was aimed to compare the effects of some immunostimulants, such as iPPVO, CCL and Vit-C on immunosuppression that occurred as a result of stress-induced by long-term road transportation of Morkaraman ewes in this study.

## MATERIALS AND METHODS

The study has been approved by the ethics committee for animal experiments in Ataturk University (Decision No: 2018/78). A total of 32 Morkaraman ewes at 1-2 years old which were subjected to the same care and feeding conditions were used in the current study. After the feces of the animals were examined regarding diarrhea, blood and constipation, and then healthy ewes were included in the research. Prior to one week from the beginning of the study, all animals were administered 1 ml/50 kg single dose of the antiparasitic drug (Ivomec®, Boehringer Ingelheim, France) containing 10 mg/ml ivermectin as well as 1ml/20 kg single dose of antibiotic (Reptopen®, Ceva, France) containing 200.000 IU/ml Benzilpenicilinprokain and 200 mg/ml Dihidrostreptomicin sülfate.

### Experimental design

The study consisted of 4 groups as 1 control and 3 experimental groups (iPPVO, CCL and Vit-C). A total of 32 ewes was randomly allotted to 4 groups containing 8 ewes in each one. In Group I, 2 ml of a commercial product (Zylexis®, Zoetis, USA) containing a minimum of 230 IFN inactive strain of *Parapoxvirus ovis* (iPPVO) D 1701 in per 1 ml was administered intramuscularly to ewes at two times with 3-days interval[33]. Animals in Group II were injected subcutaneously with 2 ml of Ultra-Corn® (Virbac, France) containing 20 mg of *Corynebacterium cutis* lysate (CCL) per 1 ml, at 2 times with an interval of 8 days[34]. Last injections of ewes in Group I (iPPVO) and Group II (CCL) were performed 24 h before transport. In Group III, another commercial drug (Maxivit-C®, Bavet, Turkey) containing 200 mg of ascorbic acid per 1 ml was administered intramuscularly at a single dose of 200 mg/kg to ewes just before transport[35]. Animals in the control group (Group IV) were received 10 ml of 0.9 % saline intramuscularly (placebo) 24 h before transport.

### Transport procedure

The animals from different treatment groups were mixed and loaded on the same truck by using a loading ramp. The sizes of the trailer of the truck utilized for transportation were 8 m in length and 2.2 m in width. Therefore, stocking density was about 0,4 $m^2$/ewe. The distance traveled was 510 km, and the journey took 8 hours including the time needed for loading and unloading.

### Obtaining clinical findings

Temperature (°C), pulsation (beat/min) and respiration rate (per min), cough, runny nose/tear, diarrhea and pathological lung sounds of animals in all groups were determined and recorded at pre-transport (PrT), post-transport (PT) as well as 1st (PT1) and 7th (PT7) days after transport.

### Blood sampling

Blood samples from *Vena Jugularis* of all ewes were taken in 10 ml serum tubes (Vacutainer tube with clot activator, Becton Dickinson Co. USA) and sterile test tubes with 0.14% anticoagulant (EDTA K3, Pty Ltd., Adelaide, SA, Australia) in the sampling days at PrT, PT, PT1 and PT7. In group III, PrT measurements were taken before administering the treatment. Serum samples were obtained after centrifugation at 3000 g for 10 min, and were put into Eppendorf tubes. They were stored in a freezer at -80 °C until they were analyzed. For hematological analysis, Abacus® Junior Vet 5 brand hemogram device was used. Adrenaline and cortisol levels at PrT and PT as well as ADA activity and Hp levels at PrT, PT, PT1 and PT7 were determined by using EL SA specific test kits for sheep (YL Biont® Sheep Epinephrine/Adrenaline, Cortisol, ADA and Haptoglobin ELISA Kit, Shanghai YL Biotech Co., Ltd.).

### Statistical analysis

According to the results of the normality test, it was found out that data obtained from this study had a normal distribution. Therefore, the data were statistically analyzed by the One-Way ANOVA procedure of the SPSS program (version 22.0)[36]. Different immunomodulators (iPPVO, CCL, Vit-C and control groups) and sampling days (PrT, PT, PT1 and PT7) were considered as main effects in the statistical analysis and the main effects were analyzed separately. The comparisons were first made among immunomodulator groups, and then within the same group among the data obtained at PrT, PT, PT1 and PT7 sampling days.

The Duncan's Multiple Comparison Test was applied for comparison of subclass means when F-test for the main effect was significant. Pearson correlation coefficients were also calculated to analyze the covariation between lymphocyte and ADA variables by the SPSS computer software program[36].

# RESULTS

## Clinical findings

Temperature, pulsation and respiration rate obtained at PrT, PT, PT1 and PT7 are presented in Table 1. At PT, temperature, pulsation and respiration rate were major clinical findings exhibited by the animals. The temperature in all groups except for the Vit-C group increased in PT compared to PrT. On the other hand, the value in PT1 decreased in comparison with PT. However, pulsation and respiration rate significantly increased in all groups immediately after transport (PT) comparing to their baselines (Table 1).

In this study, cough, nasal discharge, tear discharge, diarrhea, and pathological sounds in the lungs were not observed in any animal at PrT, PT and PT7. However, 2 animals in the iPPVO group, 3 animals in the CCL group, 4 animals in Vit-C group as well as 7 animals in the control group had a slight serous and mucopurulent nasal discharge at PT1. Pathological lung sounds were also detected in 2 animals in the iPPVO group and 1 animal in the CCL group at PT1. Additionally, medium pitched vesicular sounds were determined in the lungs of 3 animals in the Vit-C Group in addition to 7 animals in the control group. None of the animals had diarrhea.

## Hematological findings

Hematological results of ewes in iPPVO, CCL, Vit-C and control groups are presented in Table 2. Statistically significant ($P<0.05$) increases concerning leukocyte counts at PT and PT1 comparing to PrT were detected in the iPPVO group and Vit-C groups respectively. Lymphocyte counts in iPPVO group at

**Table 1** - Means and standard errors for clinical findings at PrT, PT, PT1 and PT7 days.

| Parameters | Sampling Days | iPPVO (Group I) $\bar{X}$±SE | CCL (Group II) $\bar{X}$±SE | Vit-C (Group III) $\bar{X}$±SE | Control (Group IV) $\bar{X}$±SE | |
|---|---|---|---|---|---|---|
| Temperature °C | PrT | 39.41±0.07$^{AB}$ | 39.52±0.11$^{A}$ | 39.55±0.12$^{AB}$ | 39.61±0.1$^{A}$ | P>0.05 |
| | PT | 39.96±0.09$^{C}$ | 39.97±0.12$^{B}$ | 39.82±0.16$^{B}$ | 40.11±0.06$^{B}$ | P>0.05 |
| | PT1 | 38.96±0.24$^{A}$ | 39.42±0.15$^{A}$ | 39.33±0.09$^{A}$ | 39.47±0.17$^{A}$ | P>0.05 |
| | PT7 | 39.58±0.15$^{B}$ | 39.50±0.16$^{A}$ | 39.42±0.16$^{AB}$ | 39.73±0.1$^{AB}$ | P>0.05 |
| | | P<0.05 | P<0.05 | P<0.05 | P<0.05 | |
| Pulsation (Beat/min) | PrT | 94.12±3.89$^{Aab}$ | 94.12±3.19$^{Aa}$ | 107.12±4.24$^{Ab}$ | 99.5±4.08$^{Aab}$ | P<0.05 |
| | PT | 119.5±2.44$^{B}$ | 117.50±3.01$^{B}$ | 125.50±4.06$^{B}$ | 117.75±3.0$^{B}$ | P>0.05 |
| | PT1 | 100.5±4.04$^{A}$ | 96±2.59$^{A}$ | 106.25±3.53$^{A}$ | 97.25±4.86$^{A}$ | P>0.05 |
| | PT7 | 99.75±4.41$^{A}$ | 96.75±4.27$^{A}$ | 112.75±7.2$^{AB}$ | 110±7.86$^{AB}$ | P>0.05 |
| | | P<0.05 | P<0.05 | P<0.05 | P<0.05 | |
| Respiration (Rate/min) | PrT | 44.12±2.42$^{A}$ | 51.62±2.92$^{AB}$ | 44.5±2.57$^{A}$ | 45±3.27$^{A}$ | P>0.05 |
| | PT | 65.50±3.14$^{B}$ | 65.50±5.44$^{C}$ | 55.5±3.81$^{B}$ | 57.75±3.53$^{B}$ | P>0.05 |
| | PT1 | 48.75±2.78$^{AB}$ | 48.25±1.75$^{A}$ | 51.75±3.57$^{AB}$ | 52.25±4.58$^{AB}$ | P>0.05 |
| | PT7 | 53±3.29$^{B}$ | 60±2.26$^{BC}$ | 53.75±2.65$^{AB}$ | 59.25±4.86$^{B}$ | P>0.05 |
| | | P<0.05 | P<0.05 | P<0.05 | P<0.05 | |

$^{A, B, C}$ The means shown in different capital letters within the group (in the column) are statistically significant.
$^{a, b}$ The means shown in different lowercase letters between the groups (on the line) are statistically significant.

**Table 2** - Means and standard errors for hematological findings at PrT, PT, PT1 and PT7 days.

| Parameters | Sampling Days | iPPVO (Group I) $\bar{X}$±SE | CCL (Group II) $\bar{X}$±SE | Vit-C (Group III) $\bar{X}$±SE | Control (Group IV) $\bar{X}$±SE | |
|---|---|---|---|---|---|---|
| Leukocyte (x10$^3$/µL) | PrT | 9.27±0.39$^{Aa}$ | 10.09±0.70$^{ab}$ | 10.11±0.37$^{Ab}$ | 11.82±0.81$^{b}$ | P<0.05 |
| | PT | 11.23±0.52$^{B}$ | 12.48±2.49 | 11.31±0.86$^{AB}$ | 12.08±0.77 | P>0.05 |
| | PT1 | 10.48±0.24$^{AB}$ | 10.60±1.88 | 12.34±0.59$^{B}$ | 12.27±0.96 | P>0.05 |
| | PT7 | 10.41±0.41$^{AB}$ | 11.25±2.82 | 10.84±0.61$^{AB}$ | 10.86±0.54 | P>0.05 |
| | | P<0.05 | P>0.05 | P<0.05 | P>0.05 | |
| Lymphocyte (x10$^3$/µL) | PrT | 6.24±0.19$^{Aa}$ | 6.26±0.52$^{a}$ | 6.59±0.38$^{a}$ | 8.79±0.50$^{b}$ | P<0.05 |
| | PT | 7.46±0.29$^{B}$ | 7.31±0.54 | 6.93±0.49 | 7.61±0.48 | P>0.05 |
| | PT1 | 6.96±0.18$^{ABa}$ | 6.70±0.46$^{a}$ | 7.79±0.59$^{ab}$ | 8.61±0.62$^{b}$ | P<0.05 |
| | PT7 | 7.48±0.94$^{B}$ | 7.63±0.79 | 7.55±0.56 | 8.49±0.38 | P>0.05 |
| | | P<0.05 | P>0.05 | P>0.05 | P>0.05 | |
| Neutrophil (x10$^3$/µL) | PrT | 2.98±0.25$^{AB}$ | 3.78±0.28$^{A}$ | 3.46±0.47 | 3.32±0.59$^{AB}$ | P>0.05 |
| | PT | 3.71±0.27$^{Ba}$ | 5.10±0.52$^{Bb}$ | 4.33±0.73$^{ab}$ | 4.12±0.57$^{Bab}$ | P<0.05 |
| | PT1 | 3.47±0.19$^{AB}$ | 3.84±0.32$^{A}$ | 4.48±0.44 | 3.81±0.62$^{AB}$ | P>0.05 |
| | PT7 | 2.88±0.30$^{Aab}$ | 3.56±0.28$^{Ab}$ | 3.22±0.50$^{ab}$ | 2.32±0.27$^{Aa}$ | P<0.05 |
| | | P<0.05 | P<0.05 | P>0.05 | P<0.05 | |

$^{A, B}$ The means shown in different capital letters within the group (in the column) are statistically significant.
$^{a, b}$ The means shown in different lowercase letters between the groups (on the line) are statistically significant.

PT and PT1 sampling days were significantly higher (P<0.05) than those at PrT. iPPVO and CCL groups at PT1 had significantly (P<0.05) lower lymphocyte counts than Vit-C and control groups.

## Biochemical findings

Means for adrenaline, cortisol, ADA activity and Hp levels at PrT and AT sampling days are presented in Table 3. Although adrenaline and cortisol levels in all groups increased significantly (P<0.05) after transport, the values in the Vit-C group were lower (P<0.05) than those in iPPVO, CCL and control groups at PT. ADA activity in the control group decreased significantly (P<0.05) at PT compared to PrT. However, activities of ADA in iPPVO, CCL and Vit-C groups increased significantly (P<0.05) on the contrary of the control group (Table 3). ADA activity in the control group at PT1 approached the ADA level at PrT (Figure 1). On the other hand, ADA activity in other groups at PT1 remained significantly higher (P<0.05) than ADA levels at PrT.

Hp levels increased (P<0.05) in CCL, Vit-C and control groups at PT, PT1 and PT7 days, while those in the iPPVO group did not significantly change during the period between PrT and PT (Table 3). In the control group, Hp level (P<0.05) returned to the initial level at PT7, while the Hp level in the CCL group

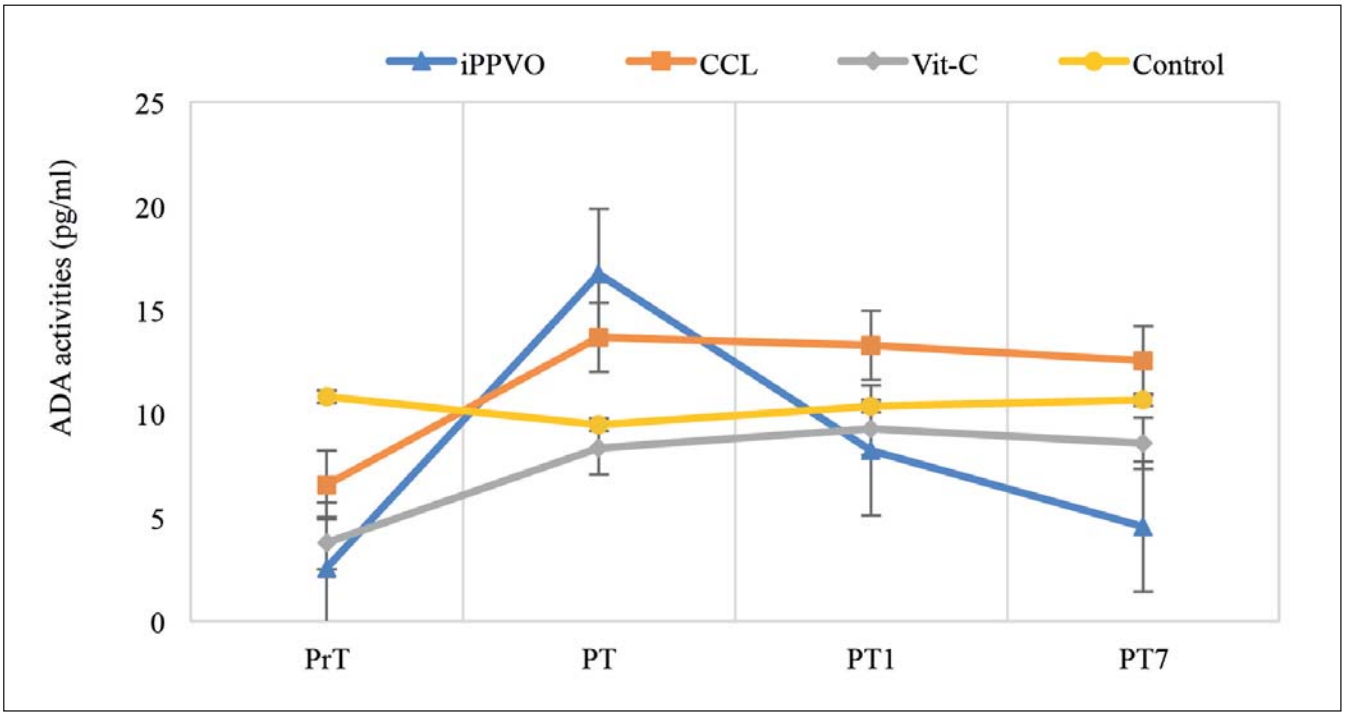


**Figure 1** - ADA activities at PrT, PT, PT1 and PT7 sampling days.

**Table 3** - Means and standard errors for biochemical Findings at PrT and PT as well as ADA and Hp levels at PrT, PT, PT1 and PT7 days.

| Parameters | Sampling Days | iPPVO (Group I) $\bar{X}$±SE | CCL (Group II) $\bar{X}$±SE | Vit-C (Group III) $\bar{X}$±SE | Control (Group IV) $\bar{X}$±SE | |
|---|---|---|---|---|---|---|
| Cortisol (pg/ml) | PrT<br>PT | 1.58±0.08$^{A}$<br>2.84±0.18$^{Bb}$<br>P<0.05 | 1.61±0.13$^{A}$<br>2.69±0.12$^{Bb}$<br>P<0.05 | 1.65±0.05$^{A}$<br>1.96±0.04$^{Ba}$<br>P<0.05 | 1.67±0.03$^{A}$<br>3.00±0.10$^{Bb}$<br>P<0.05 | P>0.05<br>P<0.05 |
| Adrenaline (pg/ml) | PrT<br>PT | 6.09±0.32$^{A}$<br>12.37±0.79$^{Bb}$<br>P<0.05 | 6.43±0.29$^{A}$<br>11.45±0.36$^{Bb}$<br>P<0.05 | 6.63±0.24$^{A}$<br>8.44±0.24$^{Ba}$<br>P<0.05 | 6.30±0.17$^{A}$<br>12.23±0.40$^{Bb}$<br>P<0.05 | P>0.05<br>P<0.05 |
| ADA Activities (pg/ml) | PrT<br>PT<br>PT1<br>PT7 | 2.55±1.12$^{Aa}$<br>16.72±1.94$^{Cb}$<br>8.2±1.03$^{Ba}$<br>4.54±1.27$^{ABa}$<br>P<0.05 | 6.55±1.10$^{Ab}$<br>13.65±0.78$^{Bb}$<br>13.28±0.23$^{Bc}$<br>12.53±0.40$^{Bc}$<br>P<0.05 | 3.75±0.97$^{Aa}$<br>8.31±0.56$^{Ba}$<br>9.25±0.61$^{Bab}$<br>8.56±0.70$^{Bb}$<br>P<0.05 | 10.80±0.37$^{Bc}$<br>9.45±0.28$^{Aa}$<br>10.35±0.60$^{ABb}$<br>10.66±0.26$^{ABbc}$<br>P<0.05 | P<0.05<br>P<0.05<br>P<0.05<br>P<0.05 |
| Hp (pg/ml) | PrT<br>PT<br>PT1<br>PT7 | 24.13±1.03$^{ABb}$<br>22.28±1.22$^{Aa}$<br>26.89±1.22$^{Ba}$<br>26.63±1.40$^{Bb}$<br>P<0.05 | 21.31±0.84$^{Aab}$<br>28.66±1.21$^{Bb}$<br>22.75±0.80$^{Ab}$<br>21.94±0.38$^{Aa}$<br>P<0.05 | 20.69±1.22$^{Aa}$<br>28.92±1.26$^{Cb}$<br>26.30±0.68$^{BCa}$<br>24.94±0.73$^{Bb}$<br>P<0.05 | 22.26±0.80$^{Aab}$<br>31.01±1.14$^{Bb}$<br>27.10±0.65$^{Ba}$<br>18.27±2.68$^{Aa}$<br>P<0.05 | P<0.05<br>P<0.05<br>P<0.05<br>P<0.05 |

$^{A, B, C}$ The means shown in different capital letters within the group (in the column) are statistically significant.
$^{a, b, c}$ The means shown in different lowercase letters between the groups (on the line) are statistically significant.

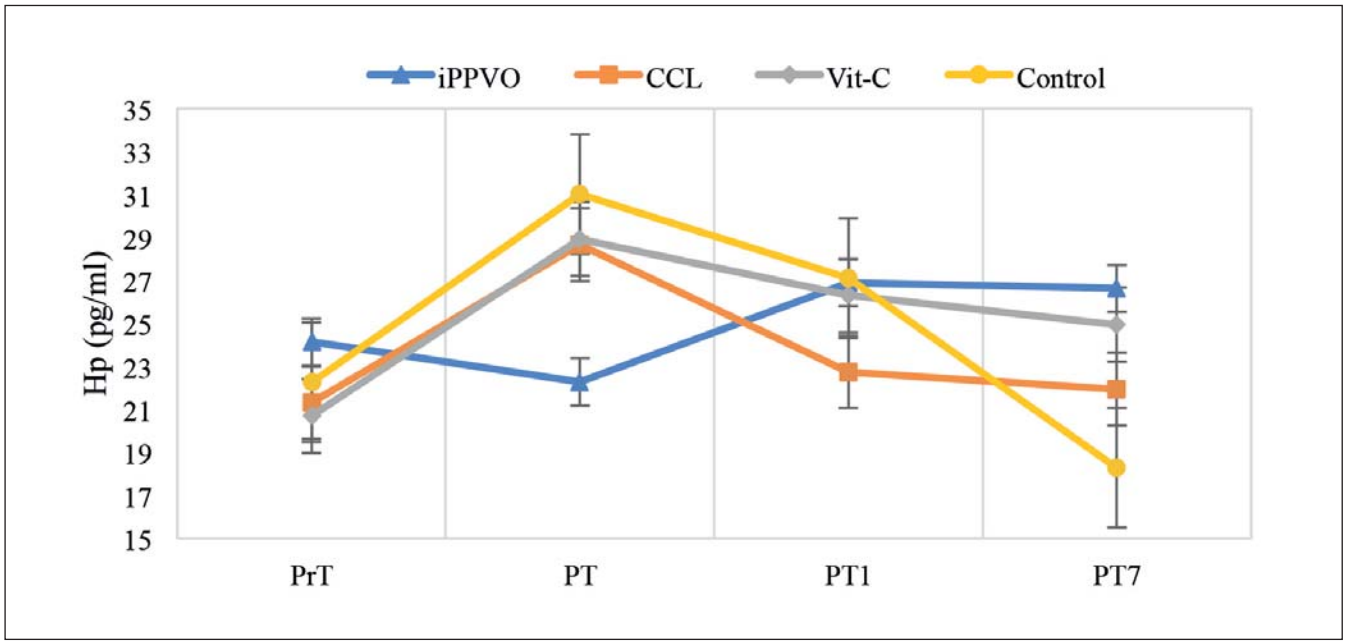


**Figure 2** - Hp levels at PrT, PT, PT1 and PT7 sampling days.

was measured at a level similar to PrT in PT1 (Figure 2). On the other hand, the Hp levels of the Vit-C group in PT, PT1 and PT7 were statistically higher ($P<0.05$) than these at PrT (Table 3).

## DISCUSSION

Cortisol is an important marker for determining of the stress in animals[37]. Aktas *et al.*[38] found out that there was an increase in adrenaline level in all dairy cattle groups after 22 hours of transport. Similarly, studies carried out on sheep, goats, pigs and horses have reported increases in adrenaline and cortisol levels at post-transport[39,42]. The results were in accordance with findings of the current study which revealed significantly higher levels of adrenaline and cortisol in all groups at PT in comparison with PrT. These findings also indicated that 8 hours (including loading and unloading) and approximately 510 km of road transportation resulted in stress on Morkaraman ewes in iPPVO, CCL, Vit-C and control groups.

In the present study, the least increases of the adrenaline and cortisol levels were obtained from ewes in the Vit-C group, although all of the animals were exposed to the same loading-transport-unloading processes. Vit-C is known as an anti-stress agent[43]. Kassab and Mohammed[44] found out that the increase in the cortisol level of the Vit-C group was lower than that of the control group following 225 km road journey of sheep. They also pointed out that the result was due to the cortisol inhibitory role of Vit-C during the transport. Similarly, studies on different animal species also revealed that Vit-C can alleviate the negative effects of transport[45,47]. For this reason, in the current study, the lower adrenaline and cortisol levels of the Vit-C group at PT compared to the PrT could be attributed to the stress preventing/reducing effect of the Vit-C as stated in the literature.

The increasing body temperature during the transport reflects the animal's physical reaction to maintain balance as the vehicle moves[48]. In other studies, the temperature increased in animals at the beginning of transport, but decreased in the following hours[49,50]. It has been reported that the temperature of cattle after 14 hours of transport started to decrease only at rest[51]. In the current study, the temperature in the iPPVO, CCL and control groups increased significantly ($P<0.05$) at PT compared to PrT. The result could be due to the adrenal cortex response during long-term road transport.

Increases in pulsation take place from the beginning of transport, and this condition continues as long as the transportation[52,53]. It was stated that the pulsation in cattle during loading and unloading was higher than these during the transport[54]. Respiration rate is one of the most important physiological parameters for evaluating the health status of animals and the body against stress[47]. Kassab and Mohammed[44] noted that the respiration rate of ewes significantly higher at the end of journey (225 km) compared to prior to transport. The result was attributed to the road transport stress. Similar to the findings of the other studies, in the current study, respiration rate and pulsation significantly increased in PT comparing to PrT in all groups. The increased pulsation and respiration rate could be ascribed to the release of cortisol and catecholamines into the bloodstream during stress as reported by Getabalew *et al.*[55] Additionally, in the current study, increases of the cortisol and adrenaline levels at PT could also support their suggestion.

Stress causes a decrease in the number of lymphocytes and an increase in the number of neutrophils[56]. In a study conducted on sheep, while the percentage of leukocytes, neutrophils and monocytes increased after transport, a decrease was observed in the percentage of lymphocytes[57]. In another study carried out on sheep, the percentage of leukocytes, neutrophils and monocytes increased, while the percentage of lymphocytes decreased after transport compared to the control group[44]. In the present study, an increase in lymphocyte and ADA activity was observed in the iPPVO group after transport, as a result of the immunomodulatory effect of iPPVO on Morkaraman ewes. There are not limited number of studies concerning the effect of iPPVO on immunosuppression induced by long-term transport in livestock. In a study, Winnicka *et al.*[58] reported that the CD4+/CD8+ showing the ratio of helper cytotoxic T lym-

phocytes in goats injected with iPPVO against the immunosuppression resulting from glucocorticoid administration was higher than the reference value. In the present study, statistically insignificant an increase in lymphocyte levels at PT1 comparing to PRT was determined in the iPPVO group. There was also a slight level of serous runny nose and a slightly noticeable increase in pathological lung sounds during clinical examination in sheep at PT1. However, lymphocyte counts of ewes in the iPPVO group at PT and PT7 were significantly higher ($P<0.05$) than those at PrT, and no clinical abnormalities such as cough, nasal discharge, tear discharge, diarrhea and pathological sounds in the lungs were observed. Ziebell *et al.*[59] investigated the clinical findings of the horses for 2 weeks after transport. They reported that the clinical score of the iPPVO group was better than the control group and that iPPVO administration was a successful tool to prevent serious clinical consequences caused by road stress. In the current study, it was suggested that the increase in lymphocyte levels in the iPPVO group at PT, PT1 and PT7 might be related to the administration of iPPVO. The possible cause of this result could be the increase in the number of T lymphocytes as already reported by Winnicka *et al.*[58]. In the current study, higher ADA activities of the iPPVO group after transport may also support this thought. Similarly, an increase in ADA activity was observed in CCL as well as in Vit-C groups after transport and the elevation continued until AT7 days. In the literature, there is no study investigating the effects of CCL application on hematological parameters in acute or chronic stress situations. Dik *et al.*[60] reported that the IL-12 and IL-6 levels were higher in sheep after 1 day of CCL administration with PPR vaccine compared to the group in which PPR vaccine was administered alone. Trinchieri[61] indicated that IL-12 stimulates cytotoxic T lymphocytes and plays a specific role in defense against viruses, and also shows synergistic effects with IFN, IL-4 and IL-6 in the early cellular immune response. In the present study, the increase in ADA activity after transport in the CCL group could be ascribed to the hypothesis that CCL enhances IL-12 level and IL-12 stimulates the production of T lymphocytes, consequently, an increase in lymphocyte count has been formed.

In addition to cortisol inhibition, Vit-C has been reported to be a chain-breaking antioxidant, which plays a role in preventing and restricting free radical chain formation. Thus, it protects blood cells including neutrophils and lymphocytes from oxidative damage[34]. On the other hand, in vitro studies demonstrate that Vit-C is required for the development of T lymphocytes. Additionally, Vit-C increases the proliferation of T lymphocytes and may affect their function. There are limited and controversial results regarding the effects of Vit-C on B lymphocytes that mediate humoral immunity[62]. However, Vit-C also increases the proliferation of NK cells, a group of cytotoxic natural lymphocytes. In summary, Vit-C positively affects lymphocyte development and function. In the present study, it was suggested that the increase in ADA activity in the Vit-C group could be due to the positive enhancement of lymphocyte development and function as well as the anti-stress properties of Vit-C.

Hp levels were determined in various animal species that were transported, and different results were obtained. Kołacz *et al.*[63] noted that the increased Hp level of pigs after transport did not return to a normal level with rest. On the contrary, Hp concentration was 86.2% higher than that measured at the 22nd hours after transport. Fazio *et al.*[64] reported that the Hp level in sheep and cattle reached a peak at the 48th hour after transport. Pascual Alonso *et al.*[57] stated that the Hp level, which increased after 4 hours of transport, continued for 24 hours in sheep. A significant positive correlation between dose-dependent Hp concentration and serum lymphocyte suppression was also determined by Murata and Miyamoto[24]. In the present study, Hp levels in PT and PT1 in the control group were found to be higher than PrT. The induction of the acute phase protein (APP) response in animals under stress is based on the hypothesis that "non-inflammatory and psychological stresses activate the sympatho-adrenal and HPA axis via the afferent nervous system"[65,66]. The sympatho-adrenal axis releases catecholamines, which stimulate immune-related cells and cause them to produce pro-inflammatory cytokines in response to stress[65]. HPA pathway controls glucocorticoid release from the adrenal cortex[66]. Both glucocorticoids and proinflammatory cytokines such as IL-1, IL-6, and TNF- are indicated as potential direct stimuli of the acute phase response[67]. In the current study, it is highly possible that the results obtained from high Hp levels in the control group emerged as the effect of catecholamines and glucocorticoids as already stated by Johnson *et al.*[65] and Leonard[66]. Additionally, high adrenaline and cortisol levels obtained from the control group also support this information. Therefore, transport as a stress factor is seen to induce two main stress pathways (acute and chronic) and stimulates the production of APPs such as Hp. It could be suggested that the high level of Hp resulted in negative effects on the immune system and the result was in accordance with clinical findings of the study.

Hp level increased after transport in the CCL and Vit-C groups. Hp level in the CCL group at PT1 was similar to PrT. It was suggested that pre-transport CCL administration partially stopped the increase of Hp, one of the immune response markers of the body due to stress, and may have a positive effect on the ewes. Clinical findings of the CCL group partially coincide with this result. On the other hand, Vit-C administration at PrT did not have a reducing effect on serum Hp concentration.

## CONCLUSION

In conclusion, it was demonstrated that iPPVO administration could be more effective against immunosuppression induced by long-term road transport of Morkaraman ewes, taking into consideration lymphocyte count, ADA activity and Hp levels. A positive correlation between ADA activity and lymphocyte count was also determined in this study. Additionally, since Vit-C administration before transportation reduced road transport stress more efficiently than others, it could be suggested as a stress inhibitor for ewes.

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## Conflicts of interest

The authors declare no conflicts of interest with respect to their authorship and/or the publication of this manuscript.

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