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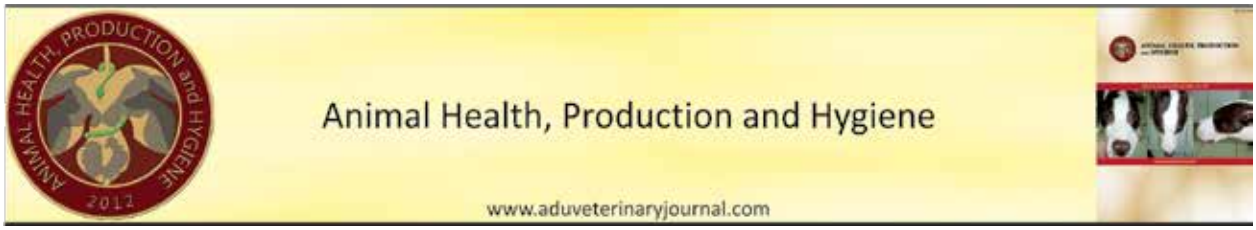

Blood Levels of Leptin and Lymphocyte Subpopulations in Haflinger Mares on Winter and Summer Solstices

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ABSTRACT

Characterization of lymphocyte subpopulations provides important physiological information for understanding specific immune functions. Leptin, produced by the adipose tissue, plays a key role in both metabolic and immune system in many species including horse. The purpose of this study was to characterize blood levels of leptin and lymphocyte subpopulations of Haflinger mares on winter and summer solstices (on June 21st and December 22nd, respectively). Additionally, we also determined possible correlation between leptin and lymphocyte subpopulations. After body weight and body condition score (BCS) were calculated, peripheral blood samples were collected from *v. jugularis* on winter (n=7) and summer (n=10) solstices. Leptin and lymphocyte subpopulations [CD2, CD3, CD4, CD8, CD19, and natural killer (NK) cells] were analyzed by radioimmunoassay (RIA) technique and flow cytometry, respectively. The mean leptin levels of Haflinger mares were 4.80±2.99 ng/mL. The mean percentage of CD2, CD3, CD4, CD8, CD19, NK cells, and CD4/CD8 ratio were determined as 53.8±7.3%, 50.6±7.2%, 33.8±8.2%, 16.8±4.5%, 46.2±7.3%, 3.2±1.4%, and 2.23±1.03 respectively. No statistical significance for leptin, lymphocyte subpopulations and CD4/CD8 ratio was found between winter and summer solstices ($P>0.05$). However, mean percentage of CD2 and NK cells on summer solstice and mean percentage of CD19 on winter solstice were slightly increased ($P=0.055$). No correlation between leptin and lymphocyte subpopulations was found ($P>0.05$). However, there was a significant positive correlation between leptin and BCS (r_s: 0.601, $P=0.011$). Our results suggest that lymphocyte subpopulations could be altered regarding photoperiodic conditions but no effect of leptin was existed on these alterations in Haflinger mares.

Keywords: Haflinger, Horse, Leptin, Lymphocyte subpopulations

Haflinger Kısraklarda Yaz ve Kış Gündönümlerindeki Kan Leptin ve Lenfosit Alt Tipleri Seviyeleri

ÖZET

Lenfosit alt tiplerinin belirlenmesi spesifik immun fonksiyonlar için önemli bilgiler sunmaktadır. Yağ dokusunda üretilen leptinin at türü de dahil pek çok türün metabolik ve immun sisteminde önemli rolü vardır. Bu çalışmanın amacı kış ve yaz gündönümlerinde Haflinger kısrakların kandaki lenfosit alt tipleri ile leptin miktarlarını belirlemektir. Ayrıca leptin ve lenfosit alt tipleri arasındaki muhtemel ilişki de değerlendirilmiştir. Vücut ağırlığı ve vücut kondisyon skorları (VKS) hesaplandıktan sonra kış (n=7) ve yaz (n=10) gündönümü zamanlarında *v. jugularis*'ten periferal kan örnekleri alındı. Leptin ve CD2, CD3, CD4, CD8, CD19 ve doğal öldürücü (NK) hücreleri kapsayan lenfosit alt tipleri sırasıyla radioimmunoassay (RIA) ve akım sitometri tekniği ile analiz edildi. Haflinger kısrakların ortalama leptin seviyeleri 4.80±2.99 ng/mL'di. CD2, CD3, CD4, CD8, CD19 ve NK hücrelerinin yüzde ortalamaları ile CD4/CD8 oranı sırasıyla 53.8±7.3, 50.6±7.2, 33.8±8.2, 16.8±4.5, 46.2±7.3, 3.2±1.4 ve 2.23±1.03 olarak belirlendi. Leptin seviyeleri, lenfosit alt tipleri ve CD4/CD8 oranı için kış ve yaz gündönümleri arasında istatistiksel önem bulunmadı ($P>0.05$). Fakat yaz gündönümünde CD2 ve NK hücre yüzdeleri, kış gündönümünde ise CD19 yüzdeleri hafif artış belirlendi ($P=0.055$). Leptin ile lenfosit alt tipleri arasında ilişki bulunmadı ($P>0.05$). Bununla birlikte leptin ile VKS arasında pozitif bir ilişki vardı (r_s: 0.601, $P=0.011$). Sonuçlar Haflinger kısraklarda lenfosit alt tiplerinin mevsime bağlı değişimler gösterebileceğini fakat leptinin bu değişimlerde etkili olmadığına işaret etmektedir.

Anahtar Kelimeler: At, Haflinger, Lenfosit alt tipleri, Leptin

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Introduction

Evaluation of lymphocyte subpopulations is the one of the main methods to assess specific immune functions and pathophysiological processes of disorders such as infectious and myeloproliferative disease (Gershwin et al., 1995; Tarrant, 2005; Satue et al., 2010). Normal values of these parameters should be established in clinical diagnosis of diseases such as leukemia. However, there is variation in the immunological parameters including lymphocyte subpopulations depending on age (Adams et al., 2008), pregnancy (Agricola et al., 2008) and gender (Satue et al., 2010) in the horse. In addition, season or day-length is the one of the most important factors regarding variations of immune parameters (Gill et al., 1985; Gill et al., 1994). But, effect of season on lymphocyte subpopulations is specifically addressed in only a few studies in the horse (Ferreira-Dias et al., 2005). Thus, assessment of season or day-length related characterization of lymphocyte subpopulations is important in the establishment of reference values in the horse.

Leptin, the 16-kDa protein product of *ob* gene, is a pleiotropic hormone and regulates food intake as well as metabolic and endocrine functions. Leptin also plays a regulatory role in immunity (Lord et al., 1998), inflammation (Cottam et al., 2004), hematopoiesis (Sanchez-Margalet and Martin-Romero, 2001) and reproduction (Hausman et al., 2012). Buff et al. (2002) reported that leptin and leptin receptors are existed in horse tissues and peripheral concentrations of leptin is an indicator of fat mass in the horse (Buff et al., 2002) as well as human (Kolaczynski et al., 1996) and rodent (Trayhurn et al., 1995). Although the lack of leptin and/or leptin receptor expression results impaired immune function in mouse (Lord et al., 1998) and human (Ozata et al., 1999), seasonal alterations of immune functions are not dependent of leptin action in the horse (Ferreira-Dias et al., 2005).

In this research, determination of blood leptin levels and lymphocyte subpopulations on winter and summer solstices in Haflinger mares was aimed. We also determined if there is a link between leptin and lymphocyte subpopulations.

Materials and Methods

The study was approved with the serial number B.30.2.ANK.0.06.00.01/1385 by the Local Ethical Committee.

Animals

Under natural photoperiodic conditions, seventeen adult, non-pregnant Haflinger mares, which are housed at General Directorate of Agricultural Enterprises of Turkey, were used in the study. The mares were fed with the standard adult horse diet and were also maintained on pasture twice a day. All the mares receive clinical examinations periodically and were checked for important viral and bacterial diseases annually. The body weights and body condition scores (BCS) of the mares were recorded according to Henneke et al. (1983). Non-pregnant mares, which demonstrate estrous behavior on summer solstice and also only non-cyclic and non-pregnant mares on winter solstices were used.

Blood collection and leptin analysis

Peripheral blood samples were collected from each horse via jugular venipuncture, before feeding in early morning of the winter and summer solstices. Blood samples were centrifuged at 3000 rpm (1500 *g*) for 10 minutes. The plasmas were decanted and stored at - 70 °C until assayed for leptin. Leptin levels and expressions of lymphocyte subpopulations were evaluated on winter and summer solstices. Plasma leptin levels were measured using radioimmunoassay kit (Linco Research Inc., St. Charles, MO). The intra and inter-assays of coefficient of variations were less than 10% according to manufacturer's knowledge.

Lymphocyte subpopulation analysis

Lymphocyte subpopulations were evaluated by flow cytometry using monoclonal antibodies, including rat anti-equine CD2 (Clone Mac288), mouse anti-equine CD4 (Clone CVS4), and mouse anti-equine CD8 (Clone CV8) (Serotec, Oxford, United Kingdom) as primary antibodies and Star 9 FITC (F(ab')2 Rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate, Serotec, Oxford, United Kingdom) and Star 17B FITC (F(ab')2 Rabbit anti-rat IgG conjugated to fluorescein isothiocyanate, Serotec, Oxford, United Kingdom) as secondary antibodies (Roberta da Costa et al., 2003; Ferreira-Dias et al., 2005; Uner et al., 2013).

Heparinized blood (150 µL) was added into three tubes (Becton Dickinson, Rutherford, NJ). Primary antibodies, including anti-CD4, anti-CD8, and anti-CD2 were added into tubes, respectively and vortexed. After incubation of 30 minutes and washing with phosphate buffered saline (2 mL, PBS), solutions were centrifuged at 1090 rpm (300 *g*) for 5 minutes and supernatants were removed. Secondary antibodies were added and solutions were incubated 30 minutes protecting from light. Lysing solution (2 mL) (Becton Dickinson, Rutherford, NJ) was added and vortexed. After incubation of 10 minutes, the solutions were centrifuged and supernatants were removed and the pellets were washed again with PBS (2 mL). The

Table 1. Blood leptin levels and lymphocyte subpopulations in winter and summer solstices.
Tablo 1. Kış ve yaz gündönümünde kan leptin ve lenfosit alt tiplerinin seviyeleri.

Parameters	Summer solstice (n=10)	Winter solstice (n=7)	Overall (min-max)	*P**
Leptin (ng/mL)	4.20±2.13	5.65±3.95	4.80±2.99 (1.08-10.64)	0.536
CD2 (%)	56.48±6.35	49.86±7.16	53.75±7.30 (39.30-65.50)	0.055
CD3 (%)	53.00±6.40	47.23±7.40	50.62±7.20 (37.20-62.50)	0.161
CD4 (%)	34.84±6.35	32.31±10.6	33.80±8.20 (15.10-47.70)	0.813
CD8 (%)	18.16±4.21	14.91±4.51	16.82±4.50 (8.00-25.10)	0.109
CD19 (%)	43.50±6.35	50.10±7.16	46.25±7.30 (34.50-60.70)	0.055
NK (%)	3.54±1.15	2.63±1.70	3.17±1.40 (1.40-6.40)	0.055
CD4/CD8 ratio	2.03±0.69	2.51±1.40	2.23±1.03 (0.68-4.80)	0.475

* Expresses *P* values obtained from Mann-Whitney U test. The results show mean ± standard deviation. NK: Natural killer cells killer cells.
* Mann-Whitney U testinden elde edilen *P* değerlerini ifade etmektedir. Sonuçlar ortalama ± standart sapmayı göstermektedir. NK: Do ğal öldürücü hücreler.

solutions were further centrifuged and PBS (500 µL) was added to pellets and mixed gently. Obtained cells were measured using a flow cytometry machine (Cytomic FC500, Beckman Coulter, Miami, FL) (Roberta da Costa et al., 2003; Ferreira-Dias et al., 2005; Uner et al., 2013) and analyzed with a software program (Cell Quest, Becton Dickinson, Rutherford, NJ).

CD4+ cells were estimated by using CD8 plot because anti-CD4 monoclonal antibody did not work. Hence, firstly, NK cells, which express lower fluorescence intensity than those of CD8 cells, were determined on the CD8 plot (Figure 1) (Campbell et al., 2008; Uner et al., 2013). CD3+, CD4+, CD19+ cells and CD4/CD8 ratio were calculated mathematically according to previous studies (CD3% = CD2% – NK%. CD4% = CD3% – CD8%. CD19% = 100 – CD2%) (Roberta da Costa et al., 2003; Ferreira-Dias et al., 2005; Uner et al., 2013). CD2+ cells were defined as total T-lymphocytes and NK cells. CD3+, CD4+, CD8+, and CD19+ cells were defined as total T-lymphocytes, T-helper cells, T-cytotoxic cells, and total B-lymphocytes, respectively (Roberta da Costa et al., 2003; Ferreira-Dias et al., 2005; Uner et al., 2013).

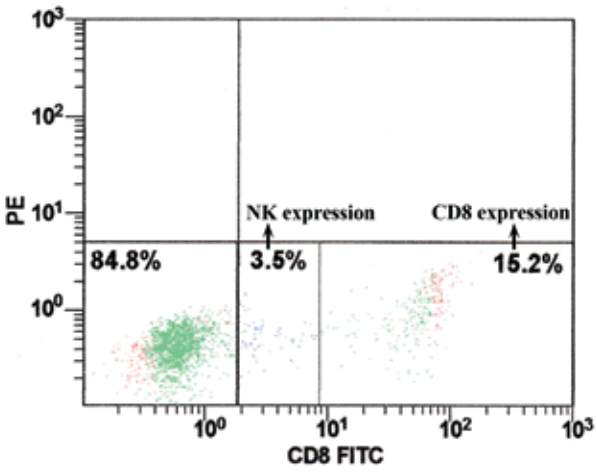

Figure 1. A representative example in calculation of NK expression using by CD8 plot. NK: Natural killer cell.

Şekil 1. Örnek bir CD8 plotu üzerinden NK ekspresyonunun hesaplanması. NK: Doğal öldürücü hücreler.

Statistical analysis

The data were not normally distributed. Because of this, non-parametric Mann-Whitney U test was used to compare the mean differences of leptin and lymphocyte subpopulations between winter and summer solstices. Spearman correlation analysis was performed to investigate relationship between leptin, BCS, and lymphocyte subpopulations. *P* values ≤ 0.05 were considered to be significant. The results were presented as the mean ± standard deviation of means.

Results

The mean leptin levels and lymphocyte subpopulations were depicted in Table 1. No statistically significance (*P*>0.05) between winter and summer solstices was found for plasma leptin levels, percentage of lymphocyte subpopulations and CD4/CD8 ratio. However, mean percentage of CD2 and NK cells was tended (*P*=0.055) to increase on summer solstice compared to winter solstice. Similarly, mean percentage of CD19 were slightly increased on winter solstice compared to summer solstice (*P*=0.055). Correlation analysis did not confirm any correlation between leptin and lymphocyte subpopulations (*P*>0.05) (Table 2). However, it was found a significant positive correlation between leptin and BCS (r_s: 0.601, *P*=0.011) (Table 2).

Discussion

Day length, which influences reproductive and immune activity, is the one of the most important environmental factors in seasonal breeders (e.g. horse and hamster). In long-day breeders such as hamster, change in day length influences some endocrine factors including melatonin and therefore, it modulates immune and reproductive system. In addition of this, several studies (Fitzgerald and McManus, 2000; McManus and Fitzgerald, 2003) suggest that day length dependent changes in melatonin levels do not modulate entirely overall immune and reproductive activity in the seasonal breeders. Therefore, it has interested in other factors that influence these activities. Leptin is the most interesting hormone in regulation of immune and reproductive activity in the horse. But, according to the authors' knowledge, very little is known about blood leptin levels and its relationship with BCS in Haflinger horse. Several studies (Deichsel et al., 2005; Deichsel et al., 2006) focused on reproductive cyclicity and its related hormones (e.g. progesterone and luteinizing hormone) and metabolic factors such as insulin and insulin-like growth factor-1. In a study (Salimei et al., 2002), leptin levels in colostrum and milk have been reported in five Haflinger mares.

Leptin, a hormone-cytokine produced by the adipose tissue, is an important endocrine regulator in metabolic, reproductive, and immune processes in many species. In the present study, the mean leptin levels of Haflinger mares are slightly higher when compared to other studies (Fitzgerald and McManus, 2000; Buff et al., 2002; Gentry et al., 2002; Ferreira-Dias et al., 2005). However, results on Thoroughbred mares reported by Piccione et al. (2004) are consistent with our results. Additionally, in our previous study (Uner et al., 2013), we detected that the mean plasma leptin levels of Arabian and Thoroughbred mares were 2.05±0.10 ng/ml, which was lower than those of Haflinger mares, suggesting Haflinger mares have higher adipose tissue mass than those of Arabian and Thoroughbred mares. But this statement was not consistent with Piccione et al.'s (2004) statement. Nevertheless, many factors including diet and blood collection time are considered important influencing the plasma leptin levels.

In the horse as in other species, it is well determined correlation between leptin and body fat mass (Buff et al., 2002). Our results confirm this relationship (Table 2) and it seems that BCS is highly correlated with plasma leptin level and body fat in Haflinger mare as well as other breeds of horses (Fitzgerald and McManus, 2000; Buff et al., 2002; Gentry et al., 2002). However, although season dependent variations in leptin levels have been reported (Fitzgerald and McManus, 2000; Buff et al., 2007), in the present study, no significant difference in plasma leptin levels was found between winter and summer solstices. We consider that the reason of this inconsistent result is our blood collection protocol. Blood collection was performed twice a year in this study and therefore, the overall seasonal changes in leptin levels may not be determined.

The mean percentage of CD2, CD3, CD4, and NK cells in this study were lower whereas CD19 was higher than those of reported studies, which carried out in different horse breeds including Andalusian (Satue et al., 2010), Lusitano (Roberta da Costa et al., 2003; Ferreira-Dias et al., 2005; Agricola et al., 2008), Arabian (Uner et al., 2013), and Thoroughbred (Uner et al., 2013). The mean percentage of CD8+ cells and CD4/CD8 ratio is similar to other reports (Roberta da Costa et al., 2003; Ferreira-Dias et al., 2005; Agricola et al., 2008; Satue

Table 2. Correlation coefficients (r_s) among leptin (ng/mL), lymphocyte subpopulations (%) and BCS[#].
Tablo 2. Leptin (ng/mL), lenfosit alt tipleri (%) ve VKS[#] arasındaki korelasyon katsayıları.

Parameters	Leptin	CD2	CD3	CD4	CD8	CD19	NK	BCS
Leptin	1	– 0.025	0.044	0.145	– 0.150	0.025	– 0.082	0.601*
CD2		1	0.971*	0.686*	0.105	– 1.000*	– 0.036	0.276
CD3			1	0.804*	– 0.054	– 0.971*	– 0.199	– 0.322
CD4				1	– 0.574*	– 0.686*	– 0.395	0.430
CD8					1	– 0.105	0.258	– 0.315
CD19						1	0.036	– 0.276
NK							1	– 0.009
BCS								1

* $P<0.05$, n=17. NK: Natural killer cell. BCS: Body condition score.
# BCS was calculated as arbitrary unit (1 = thin, 9 = fat) according to Henneke et al (1983).
* $P<0.05$, n=17. NK: Doğal öldürücü hücreler. VKS: Vücut kondisyon skoru.
# VKS'ler Henneke ve ark.(1983)'a göre ilgili metoda özgü birimler kullanılarak hesaplanmıştır (1 = zayıf, 9 = şişman).

et al., 2010; Uner et al., 2013). There are many physiological and technical factors such as age, gender (Satue et al., 2010), pregnancy (Agricola et al., 2008), and optimization methods for lymphocyte subtyping, to affect these variables. In contrast to human (Chng et al., 2004) and dog (Faldyna et al., 2001) studies and Satue et al.'s (2010) statement, our previous study (Uner et al., 2013) showed that breed in horse was not an important factor affecting lymphocyte subpopulations.

The data obtained from previous studies (Gill et al., 1985; Gill et al., 1994; Ferreira-Dias et al., 2005) on the effect of photoperiod on blood lymphocyte subpopulations and other immune cells have shown conflicting results in the horse because insufficient number of studies conducted. In our previous study (Uner et al., 2013), we determined day-length effect on CD4/CD8 [T-helper/T-cytotoxic] ratio and NK cells and also determined day-length-by-gender interaction for CD2+ cells (total T cells and natural killer cells), mature T cells (CD3+ cells), T-cytotoxic cells (CD8+ cells), and B cells (CD19+ cells) in Arabian and Thoroughbred horses. But, in that study (Uner et al., 2013), it was found that only stallions were responsive to day-length regarding CD2+, CD3+, CD8+, and CD19+ cells. In the present study, Haflinger mares were likely responsive to day-length but our sample size is potential drawback to clarify this. However, it is clear that there is tendency to increase for CD2+ cells and NK cells whereas decrease for CD19+ cells on summer solstice compared to winter solstice.

In order to evaluate the possible role of leptin on lymphocyte subpopulations, we conducted correlation analysis and we did not find the relationship between leptin and lymphocyte subpopulations suggested that circulating leptin levels might not influence circulating lymphocyte subpopulations in Haflinger mares as in other horse breeds (Ferreira-Dias et al., 2005; Uner et al., 2013).

In the present study, the effects of the estrous cycle including luteal and follicular phases of the mares on lymphocyte subpopulations were not evaluated. Therefore, we considered that this obtained data from mares, which were not determined to be in estrous, was a limitation of the present study. However, other reports (Roberta da Costa et al., 2003; Ferreira-Dias et al., 2005) suggest that the estrous cycle of the mare does not affect the immune function such as lymphocyte subpopulation count and neutrophil functions.

In conclusion, blood leptin levels reflect body fat mass in Haflinger mares as in other horse breeds. Our results also suggest that blood lymphocyte subpopulations may change according to seasonal conditions but it seems like that leptin does not modify circulating lymphocyte subpopulations in Haflinger mares.

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