Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

Document heading doi: 10.1016/S2305-0500(14)60024-5

Age-related rump fat, fat percent, body fat mass, leptin, androgens and semen parameters of Arab stallions

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ARTICLE INFO

Article history:
Received 15 April 2014
Received in revised form 16 May 2014
Accepted 17 May 2014
Available online 20 September 2014

Keywords:
Stallions
Rump fat
Semen
Leptin
Androgens
Age

ABSTRACT

Objectives: To study the effect of age and body fat on leptin levels and semen parameters of Arab horse. **Methods:** Fifteen fertile Arab stallions of different ages belonging to Police Academy were divided into three equal groups according to their age. Old horses are those of >18 yeas (18-27), Mid-age horses ≥13 to18 years (13-18), Young horses are those of <12 years (7-11). Semen was evaluated three times for each stallion. Blood and seminal plasma were assayed for measuring leptin, testosterone and estradiol. Subcutaneous rump fat thickness was measured using ultrasound for estimating body fat percent and fat mass percent. **Results:** All body fat parameters were significantly high in Young stallions and decreased with increasing age. As age increased, testosterone levels increases but leptin levels decreased. Age was inversely correlated with fat %, fat mass and leptin. All fat parameters had direct correlation with leptin in semen and serum but an inverse one with serum testosterone. Serum leptin directly correlated with sperm cell concentration in Mid- age stallions and inversely correlated with percent of live sperm in Old stallions. Semen leptin correlated directly with both percent of live sperm and percent of abnormal sperm in Old stallions. **Conclusion:** This study proved that aging in stallions is related to a drop in fertility, a decrease in body fat and in turn leptin. Arab stallions of age 7 to 18 years could be used in the breeding efficiently.

1. Introduction

Effects of aging on the stallion are less well documented but include a reduction in sperm output associated with progressive testicular degeneration and potential compromise of libido and mating ability that is often associated with degenerative conditions of the musculskeletal system[1]. Age effect was not observed in adult stallions' endocrine hormones considering testosterone and estradiol[2] and insulin like growth factor-I [3] especially those of 11 to 15 and 16 to 23 years old.

Leptin hormone is mainly secreted by adipocytes and appears to modulate feeding behaviour, and energy expenditure and therefore plays an important role in body weight regulation[4] and reproduction[5]. The net effect of leptin upon male reproductive function may depend on its circulating level[6]. In fact, circulating concentrations of leptin are directly correlated to body mass index and percentage of body fat in horses[7, 8]. Leptin and leptin receptors are present in equine tissues and that peripheral concentrations of leptin reflect a significant influence of fat mass in equine[9]. Studies on association between changes in leptin levels and semen parameters are controversial, leptin may be crucial in ejaculated spermatozoa to manage their energy status and capacitation[10]. Semen quality

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(Volume, sperm concentration, total sperm numbers and sperm abnormalities) was poorest in stallions under 3 year of age and over 11 year[11].

Due to the lack of information on the associations between age, body fat, leptin, testosterone hormone and semen quality in Arab stallions, the following experiment was designed to test the hypothesis that there would be a relationship between advancing age, serum concentrations of those hormones, adiposity, and semen quality in Arab stallion.

2. Materials and methods

2.1. Experimental animals

Fifteen horses (7 to 23) years belonged to Police Academy, Abbasia Horse stud, were used during this study. The stallions were kept under standard nutritional and managerial conditions. All stallions were housed in separate box stalls and were exercised for at least 1 h daily. The stallions were classified into three equal groups. Young horses (7 to 11years), Mid-age horses (13-18 years) and Old horses (>18 years).

2.2. Body composition measurement

All stallions were weighted and assigned to a body condition score (BCS, 1 = extremely emaciated through 9=extremely fat) by two independent, experienced veterinarians; the mean of the two estimates was used[12].

Rump fat thickness was measured by ultrasound frequency of 7.5-MHz using B-mode ultrasonography (NOVEK, Germany) equipped with linear array real time multi-frequency 2.6- 7.5 MHz transducer and used to calculate percent body fat (% fat) as previously published[13]. The site was determined by placing the probe over the rump at approximately 5 cm lateral from the midline at the 13^{th} rib [14]. The region was scanned and same site was measured in all horses. Percent fat was estimated from the equations of Kane *et al.* [15] % fat = 2.47 + 5.47*rump fat/cm.

Fat mass was determined by multiplying % fat and total body mass.

2.3. Semen collection and evaluation

Breeding mares starts from October to May every year[16]. Semen was collected from all stallions during the breeding season at weekly intervals from December till the next May. Semen ejaculates were collected using estrus mare and Colorado artificial vagina (Lane manufacturing co., Denver, Colorado) supplied with its in-line filter to remove the gel portion. After semen collection, the gel-fraction was removed. The remaining volume was measured in a graduated cylinder.

Semen was subjected to conventional semen evaluation including volume, pH, motility, concentration, total sperm concentration, live sperm % and total sperm abnormalities [17]. Volume was measured in graduated cylinder after removal of gel-fraction. Hydrogen ion concentration (pH) of freshly ejaculated semen was measured using pH paper graduated from 6 to 7.8 with 0.3-unit sensitivity (Merck, Darm-stadt, Germany). Sperm cell concentration was determined using the Thomarulling of the Neubaur haemocytometer in samples diluted 100 times with 2% eosin-colored sodium hydroxide. Concentration was expressed as the number of spermatozoa in million per ml of neat semen. Total number of sperm per ejaculate was calculated by multiplying the volume and the concentration of each ejaculate. Mass and individual motility were examined using light microscope equipped with hot plate[11]. For determining live sperm percentage, a thin drop of freshly collected semen was mixed with a large drop of eosin-nigrosin stain. A thin smear was then made, rapidly dried in air and examined microscopically. The percentage of unstained (presumably alive) sperm cells was calculated. Random examination of at least 200 spermatozoa in each smear was done[18]. The stained semen smears used for estimation of live sperm percent were also used to determine the percentage of total sperm abnormalities. Not less than 200 spermatozoa were examined in each smear [18]. After semen evaluation semen immediately centrifuged at 1 000 g for 10 minute and the seminal plasma samples were collected and kept at - 20 ℃ until hormones assay.

2.4. Blood sampling

Blood samples were collected via jugular vein puncture after each stallion dismount in the morning (8 to 10 a.m.) and before the next meal. Sera were harvested and stored at -20 ℃ until hormone analysis.

2.5. Leptin , estradiol and testosterone assays

Plasma leptin concentration was determined from a commercial kit (Multi-species Leptin RIA Kit, Linco Research Inc., St. Charles, MO, USA),) that had been validated for measuring leptin in equine serum and plasma and previously used for the horse[8,19]. The kit utilized 125I-

labeled recombinant human leptin with a specific activity of 135 lCi/lg, a guinea pig multispecies leptin primary antibody, and a goat anti-guinea pig IgG serum for the precipitating reagent. Purified recombinant human leptin was used for the kit standards and quality controls. Samples were run in duplicate and counted for one minute in a gamma counter (Packard Instrument Company). In the absence of purified equine leptin, results were expressed as human equivalents of immunoreactive leptin (ir-leptin HE). Serial dilution of standards and equine plasma demonstrated both parallelism and linearity with a sensitivity of 0.5 μg/L HE. Intra- and inter-assay coefficients of variation were 2.8% and 8%.

Testosterone was determined by using a total testosterone commercial kit (Biosource, Testo ELISA, Belgium). The sensitivity of the assay was 0.05 μg/L and intra-assay coefficients of variation were 6.3 and 8.3. Estradiol was assayed using RIA DSL-43100 commercial kit of diagnostic laboratories. Intra- and inter-assay coefficients of variation were 5.9 and 10.1%. Sensitivity of the assay was 2.0 pg/mL.

2.6. Statistical analysis

Results were presented as means ± standard error of the mean (SEM) using SPSS[20]. The effect of age on different parameters was studied using simple one way ANOVA. Duncan Multiple Range Test was used to separate between significant means. Pearson correlation coefficients were also performed.

3. Results

Means (±SEM) of body weight, BCS, rump fat, fat% and fat mass are presented in Table 1. Although mean age of horses was significantly (P<0.01) different but both body weight (P=0.8) and BCS (P=0.2) are not significantly different. Young and mid-age horses had significantly high rump fat thickness (P<0.01), fat % (P<0.01) and fat mass (P<0.01) compared to old ones.

Table 1
Average age, weight, body condition scores (BCS), and fat mass of Arab stallions with different ages.

Traits	Young	Mid	Old	Total	P-value
Average age (year)	7.88±0.58[a]	15.17±0.60[b]	20.7±1.37[c]	15.07±1.05	0.0001
Range age	7-11	13-18	18-27	7-27	-
Weight (Kg)	375.00±10.21	389.29±7.43	393.75±6.25	386.70±4.80	0.86
BCS	6.75±0.14	6.64±0.18	6.75±0.14	6.70±0.09	0.21
Rump fat (mm)	6.25±0.29[b]	5.09±0.47[b]	3.15±0.26[a]	4.88±0.38	0.002
Fat %	13.79±0.29[b]	11.51±0.89[b]	7.75±0.54[a]	11.12±0.74	0.002
Fat mass	137.93±2.87[b]	115.08±8.98[b]	77.45±5.39[a]	111.14±7.35	0.001

Means with different superscripts a, b, c are significantly different at P<0.05

Leptin levels (Table 2) in both serum (P=0.05) and seminal plasma (P<0.01) are significantly high in young and mid-age horses compared to old horses. In contrast testosterone levels were increasing with the increase in age but the difference was not significant (P>0.05). Estradiol is not significantly different between all horses groups. Estratiol to testosterone ratio increased from young to mid age horses then decreased in old horses.

Regarding semen parameters (Table 3), old horses had significant lower mass motility, lower live sperm % and higher abnormal sperm% than young and mid-age stallions (P all <0.05). There was no significant difference in ejaculate volume, semen pH, motile sperm % semen concentration among young, mid-aged and old stallion.

Age is inversely related to rump fat (r= - 0.52; P<0.05), fat % (r=-0.53; P<0.05), fat mass (r=-0.60; P<0.01) and seminal plasma leptin (r=-0.45; P=0.09). Although age is directly related to testosterone level (r=0.42), but this correlation is not significant. Both body weight and BCS inversely correlated with all fat parameters (Table 4) but all of them are weak and insignificant. BCS has inverse correlation with serum (r=-0.35), and seminal plasma leptin (r=-0.48; P=0.06). Contrary to the inverse significant correlation that exists between testosterone and all fat parameters, a direct correlations exists between fat parameters and both serum leptin or seminal plasma leptin (Table 4). Testosterone inversely correlated with serum (r=-0.43), and semen leptin (r=-0.46; P=0.08) but estradiol has a non significant direct correlation with serum leptin (r=0.36; P<0.05). Serum (r=0.73) and seminal plasma (r=0.62) leptin directly correlated with sperm cell concentration in mid-age stallions and inversely correlated with % of live sperm in old stallions (r=-0.85; -0.89) respectively. Seminal plasma leptin in young stallions correlated directly (Table 5) with % of abnormal sperm (r=0.84) and inversely with % of live sperm(r=-0.70). Serum (r=0.79) and seminal plasma (r=0.76) leptin in old stallions correlated directly with % of abnormal sperm.

Table 2
Leptin, Testosterone and Estradiol concentrations of Arabians stallion with different ages.

Hormones	Young	Mid	Old	Total	*P*-value
Serum leptin (μg/L)	2.61±0.13[b]	2.58±0.33[b]	1.86 ±0.03[a]	2.35±0.15	0.05
Semen leptin (μg/L)	1.92±0.09[b]	1.82±0.18[b]	1.25 ±0.06[a]	1.66±0.09	0.004
Testosterone (μg/L)	6.87±0.52[a]	7.75±.89[ab]	9.77 ±0.46[b]	8.05±0.52	0.10
Estradiol (pg/mL)	30.69±6.99	39.42±4.21	31.95±0.99	35.09±2.75	0.36
E2:testo	4.39±0.88	5.50±0.88	3.28±0.11	4.61±0.51	0.20

Means with different superscripts a, b, c are significantly different at $P<0.05$.

Table 3
Semen characteristics of Arab stallion with different ages.

Semen traits	Young	Mid	Old	Total	*P*-value
Ejaculate volume/mL	41.88±5.50	48.00±5.59	45.83±5.90	45.5±3.26	0.78
pH	7.03±0.08	6.99±0.05	6.94±0.09	6.98±0.04	0.79
Mass motility	3.38±0.0.18[b]	3.40±0.16[b]	2.83±0.17[a]	3.17±0.11	0.03
Motile sperm%	75.63±2.40	76.50±2.69	67.92±3.23	72.83±1.80	0.07
Concentration $\times 10^6$/mL	372.63±20.66	359.9±17.41	315.25±18.96	345.43±11.63	0.09
Total sperm count $\times 10^9$	17.03±3.00	17.04±2.51	14.61±2.15	16.04±1.40	0.71
% live sperm	77.88±2.33[ab]	80.50±1.79[b]	72.25±2.55[a]	76.50±1.46	0.04
% abnormal sperm	15.00±1.91[ab]	11.80±1.30[a]	17.58±2.02[b]	14.30±1.15	0.03

Means with different superscripts a, b, c are significantly different at $P<0.05$.

Table 4
Correlations between age, body weigh (BW), body condition score (BCS), fat %., fat mass , serum leptin (leptin), seminal plasma leptin (semen leptin), testosterone(T) and estradiol (E_2) in Arab stallion.

Parameters	BCS	Rump fat	Fat %	Fat mass%	T	E_2	Serum leptin	Semen leptin
Age	0.15	−0.52*	−0.53*	−0.60**	0.42	0.09	−0.19	−0.45
BW	−0.10	−0.43	−0.44	−0.29	0.30	0.24	−0.04	−0.09
BCS	1	−0.21	−0.21	−0.30	0.08	−0.01	−0.35	−0.48
Rump fat		1	1 .0**	0.98**	−0.74**	−0.14	0.52*	0.71**
Fat%			1	0.98**	−0.74**	−0.14	0.52*	0.71**
Fat mass				1	−0.72**	−0.11−	0.54*	0.73**
T					1	−0.04	−0.43	−0.46
E_2						1	0.36	0.004
Leptin							1	0.79**

Correlation is significant at $P<0.05$(*), $P<0.01$(**).

Table 5
Simple correlations of blood and seminal plasma leptin and semen parameters.

Leptin	Age	Volume	Motility		Sperm cell concentration	Total sperm count	%live sperm	Abnormal sperm%
			Mass	Individual				
Serum	Young	0.20	0.39	0.44	−0.29	0.02	0.18	−0.34
	Mid	−0.11	0.46	0.26	0.73	−0.18	0.34	−0.46
	Aged	−0.67	−0.68	−0.79	−0.54	−0.67	−0.85	0.79
Semen	Young	−0.67	−0.50	−0.50	−0.26	0.06	−0.70	0.84*
	Mid	0.58	0.49	0.39	0.62	0.77*	0.47	−0.33
	Aged	−0.18	−0.97**	−0.98	−0.67	−0.1	−0.89**	0.76*

*$P<0.05$, **$P<0.01$.

4. Discussion

Although BCS is a valuable estimate of apparent adiposity [12], BCS could not be used for measuring obesity in horses of this study because horses in all age groups had nearly similar BCS and body weight so leptin was not correlated with BCS though other studies assumed a relation between them[9]. In mares, BCS and subcutaneous fat measurements are correlated and the amount of fat in areas such as the 13th rib should have an accurate indication of the condition and nutritional health[21]. But in stallions of this study these correlations were weak.

Leptin is secreted into the periphery in proportion to body

fat percentage in horses[7] and also responds to changes in energy balance in Thoroughbred mares[22]. The foregoing results indicated that fat mass and fat% was significantly correlated with serum leptin level in Arab horses. Moreover, circulating leptin levels increased significantly with obesity in rats[23]. Foremost, because leptin is mainly secreted from adipose tissue, the positive correlation found between fat percent and leptin is logical and in agreement with several studies conducted in horses[7,24]. The decrease in leptin in Old Arab horses could be referred to the decrease in fat mass and fat percent compared to Young horses. In agreement with our results, plasma leptin concentrations were positively correlated to percent body fat and fat mass in Standardbred horses[13] both horses and ponies[25].

Leptin levels are high in Arabian horses of this study of >7 to 18 years but declined in horses > 18 years. Similarly, leptin increased in horses from 2 to >12 years[9]. As in horses, adult humans of different body weights showed a gradual decline of serum leptin levels[26]. In contrast to horses, the significant positive correlation observed between serum leptin with patients' age in men[27] was due to the increase in body fat mass and/or decrease serum total testosterone with increasing age[28].

The increase in testosterone concentrations with increasing age in Arab horses and its relation to age and body weight is in agreement with results similar to those of Khalil *et al.* [29] where plasma testosterone increased with age in young horses from 1 to 9 years old. Also both plasma and intratesticular concentrations of testosterone and estradiol increased with age light horse stallions ranging in age from 2 month to 25 years[30]. Moreover, intratesticular testosterone increases with age in horses from 1-20 years old[31]. The age related increase in testosterone levels[32] may refer to the increase Leydig cell volume and numbers with age in horses from 2 to 20 years to more than three times their number at reaching maturity[32,33] Contrary, Thoroughbred stallions had no significant difference in plasma testosterone levels whatever their age or season[2].

In agreement with our results, leptin levels whatever in blood or semen was inversely related to testosterone and the only direct correlation existed was between blood serum leptin and estradiol but all these correlations were not significant. Similarly, only semen leptin concentrations inversely correlated with serum testosterone levels in men, but directly correlated with serum leptin concentrations [34]. In Tom cats, leptin also increased significantly in concomitant with a decrease in testosterone concentration after castration and testosterone administration resulted in decreased leptin levels[35]. Moreover, pharmacologic castration of young men led to an increase in serum leptin level[36]. This relation explained why treatment with testosterone reduced leptin levels[37]. The significant inverse correlation between the serum leptin and total testosterone matched the lower serum leptin in males than in females due to the higher serum testosterone in males[37,38]. This inverse correlation was explained by binding of testosterone to androgen binding receptors on the adipocytes with subsequent increase in lipolysis or direct suppressive effect on ob gene expression [39,40]. Also, leptin possibly has a direct inhibitory effect on testosterone production by binding to Leydig cells[41,42] and it appears to act as a direct inhibitory signal for testicular steroidogenesis[43]. It has been observed in obese men that the peripheral leptin receptors in the testis are directly exposed to high-leptin concentrations with possible negative effects on gonadal functions[44,45]. Contrary to our results, testosterone and leptin correlated positively in stallions[46]. Other studies reported no significant relation between leptin and gonadal hormone values[28,47,48] since leptin may have local effects on the function of testis and spermatogenesis[48].

Soyupek *et al.* [49] suggested that the effect of leptin on reproductive functions originates from a systemic effect related to central neuroendocrine system, androgen levels or spermatogenic existence rather than its direct effect on testicular tissue. It has been reported that, testosterone suppresses leptin secretion and estradiol stimulates release of leptin independently of body fat[50,51] which explains the relation between estradiol and blood leptin in Arab horses of this study. Recently, Ishikawa *et al.* [42] showed that the dysfunction of spermatogenesis is associated with an increase in leptin and leptin receptor expression in the testis. Thus infertile oligozoospermic men had higher serum leptin than fertile normozoospermic men[28]. Moreover, leptin in seminal plasma was significantly lower in the 'normal' semen than in the 'pathological' one[52].

This study demonstrated that semen leptin concentrations were inversely correlated with serum testosterone levels and directly with serum leptin concentrations. These results are in accordance with those reported in men[34] and castrated rats[53] Furthermore, semen leptin concentrations display only a fraction of serum leptin levels and the dysfunction of testicular epithelia that found in hypergonadotrophic hypogonadism and high-grade oligozoospermia with

decreased testosterone levels might be caused due to elevated spermal leptin concentrations[34]. However, the correlation of semen with serum leptin concentrations indicates that leptin is not actively transported but rather leaks through the blood-testis barrier. Leptin levels in seminal plasma of healthy men was similar to that of vasectomized men, suggesting that testicular tissues were not the source of seminal leptin and the most likely source being either seminal vesicles or prostate tissue[54]. But recent findings demonstrated that leptin was expressed in and secreted by ejaculated spermatozoa[10].

In contrast to the direct relation between leptin levels in either serum or semen and sperm cell concentration in Mid-age stallions and the inverse relation to %of live sperm in Old horses, no correlation between leptin levels in serum and all semen parameters, though seminal plasma leptin level was inversely related to sperm concentration and motility[48]. Contrary to stallion results, no correlation was recorded between leptin and sperm concentration, motility, vitality and morphology in men[54,55] The negative correlation observed between leptin with percentage of motile spermatozoa and with the velocity straight line[52] was recorded in this study between serum leptin in Old stallions and between seminal plasma leptin in Young and Old stallions. Similar to Old Arab stallions, serum leptin demonstrated significant positive correlation with abnormal sperm morphology and significant negative correlation with sperm concentration and sperm motility[27].

In spite that, semen volume and total sperm count increased in Noriker draught horse stallions with increasing age from 2 to > 9 years but the percentage of stallions with semen abnormalities and lowered sperm motility increased with age[56]. High semen volume observed in Arab stallions obtained from the Mid-age group where age was ranging from 13-18years. Also highest total and gel-free volumes were obtained from stallions from 5-13 years old with a general tendency for the volume of the gel to increase with age until 9 or 10 yr of age and then to[11]. As well as Young Arab stallions of 7-11 years had high sperm cell concentration with a tendency to decrease with age, stallions in the 12- and 13 yr-old groups had the highest sperm concentrations[11]. Moreover, the highest total numbers of spermatozoa were obtained from the 5 and 13-yr-old stallions[11], but in comparison to Arab stallions, it was obtained in Young and Mid- age stallions of age 7-18 compared to Old ones.

In conclusion, these data provide hints for the role of leptin in the Arab stallion reproduction, possibly, by an interaction between leptin, adiposity, semen characteristics and some reproductive hormones and age.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors wish to thank all the officers, doctors in Abbasia Horse Stud for their cooperation during blood sampling, semen collection and ultrasound examination. Chemicals and hormones were supplied and analyzed by authors in National Research Centre and Equine artificial vagina was supplied by Animal Reproduction Research Institute, Agriculture Research Center.

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