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Changes in the cortisol and some biochemical patterns of pregnant and barren jennies (*Equus asinus*)

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ABSTRACT

Changes in cortisol and some biochemical values are likely to occur during pregnancy in different species, although homeostatic mechanisms function to keep substrates in the blood at comparatively constant concentrations. There are no complete data for this important endocrine and metabolic status in jennies. Since metabolic functions are increased during pregnancy to satisfy the demands of the foetus, the placenta and the uterus, the purpose of the present study was to determine which physiological changes occur in plasma cortisol and total protein, creatinine, urea, metabolism in pregnant jennies and hence to compare these findings with those of barren jennies. The study was carried out in 24 healthy Ragusano jennies (10 pregnant and 14 barren). Blood samples were collected monthly from the jugular vein of pregnant and barren jennies in one year. As compared to barren jennies, pregnant jennies showed lower cortisol ($P<0.05$) and urea ($P<0.05$) values and higher creatinine ($P<0.01$) values. Total protein values and AST and ALT activities showed a similar pattern both in pregnant and in barren jennies, and did not differ significantly. The existence of significant differences in cortisol, creatinine and urea concentrations between pregnant and barren jennies showed the relevant involvement of the physiological state on the endocrine and biochemical homeostasis. These findings were confirmed by positive and significant correlations between cortisol and biochemical parameters.

Key words: *Equus asinus*, pregnancy, cortisol, biochemical pattern

Introduction

Adrenocortical activity is involved during physical (FERLAZZO and FAZIO, 1997; FERLAZZO et al., 2007) and psychological (FAZIO et al., 2008a; FAZIO et al., 2008b; FAZIO et al., 2009a) challenges in domestic animals through the release of cortisol. Although many reproductive aspects in jennies are very similar to mares (GINTHER et al., 1987;

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GUILLAUME et al., 2006; CARLUCCIO et al., 2008) they are also some different and specific characteristics (FORHEAD et al. 1994; BURNHAM, 2002; PUGH, 2002).

The role of the adrenocortical function in seasonal reproduction has not been studied extensively in mares (BERGHOLD et al., 2007; FAZIO et al., 2009b) and no data at all are available for jennies.

Cortisol probably regulates seasonal reproductive activity and the oestrous cycle in mares (GILL et al., 1985; FLISIŃSKA-BOJANOWSKA et al., 1991; OUSEY, 2004) and a negative relationship between adrenocortical function and oestrogen changes has been investigated (NETT et al., 1973; NETT et al., 1975). However, few studies have been carried out on the physiology of reproduction in jennies (HENRY et al., 1998; CARLUCCIO et al., 2007; CARLUCCIO et al., 2008; TABERNER et al., 2008) and limited studies have been performed on the biochemical analytes in non pregnant subjects (ZINKL et al., 1990; JORDANA et al., 1998; MORI et al., 2004). Although homeostatic mechanisms function to keep substrates in the blood at comparatively constant concentrations, some changes in adrenocortical and biochemical values are likely to occur in different species, like camel, sheep and goat (ALILA-JOHANSSON et al., 2003; NAZIFI et al., 2003; YILDIZ et al., 2005; SAEED et al., 2009).

Since metabolic functions are increased during pregnancy to satisfy the demands of the foetus- placenta activity, the purpose of the present study was to determine which physiological changes occur in plasma cortisol and biochemical patterns in pregnant jennies and hence compare these findings with those of barren jennies.

Since there are no complete data for cortisol and some biochemical parameters in pregnant and barren jennies, the results obtained may represent a contribution to a better understanding of homeostatic processes in this species, as well as for estimating its physiological status, and for diagnostic purposes.

Material and methods

Animals, diets and feeding. The study was performed on 24 clinically healthy Ragusana jennies belonging to a farm located in Agrigento, Southern Sicily (37° 19' 19" N latitude; 13° 35' 23" E longitude). The breeding season of jennies in Sicily takes place from February to July. The study was conducted from June 2007 to June 2008. The animals were assigned to one of two observation groups based on physiological status (group 1: 10 pregnant jennies; group 2: 14 barren jennies). The animals were kept in paddocks during the day and placed in 20.9 m^2 individual boxes at night, with inter-individual visual contact. They were familiar with their group members. All jennies had free range to pasture during the day and they were also individually fed with 2 kg of a grain supplement, straw, vetch hay twice a day. The composition and quantity of individual

supplement was equal between the two groups, to minimize the effect of different diets on the parameters studied.

Group 1 jennies became pregnant at the same time of the year (April-June) and the pregnancy was nearly of the same duration in all animals studied. The stage of pregnancy at the time of blood sampling was different in all jennies studied and the data were defined on the basis of a pregnancy test carried out 40-days post-breeding and then in relation to medical history, confirmed successively by the timing of parturition that occurred 5 to 10 days after the final blood sampling. Consequently, at beginning of the first month of sampling (June) the pregnant jennies were not necessarily in the same month of pregnancy. They showed normal pregnancy and foaled spontaneously, eutocic delivery, after 372.3 ± 5.48 days of pregnancy. All foals were healthy and viable.

Group 2 barren jennies were selected as the control group, by random selection.

Sample collection and analysis. Blood samples were taken from the jugular vein once monthly over the 12 months. All samples were collected between 7.00 and 9.00 a.m. to minimize the effect of the circadian rhythm on cortisol measurements, and by the same operator. Blood samples were collected in evacuated tubes (Venoject, Terumo®; Belgium) and then were immediately refrigerated at 4 °C after withdrawal. Blood samples were centrifuged at 1,500 g for 10 minutes; the serum was separated and stored in polystyrene tubes at -20 °C until used for analyses. Serum cortisol concentrations were analysed in duplicate using a commercial EIA Kits supplied by Radim (Pomezia, Roma, Italy). Intra- and interassay coefficients of variation were 4.6 % and 6.9 % respectively. Serum biochemical parameters were measured by a biochemistry auto analyzer (SLIM, SEAC, Calenzano, Florence, Italy) using SEAC/Radim kits (Pomezia, Roma, Italy). The parameters analysed were total protein, creatinine, urea, aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

Statistical analysis. Data are presented as means ± standard deviation (SD). Significant differences between the previous month and between pregnant and barren jennies were established using the Student's unpaired *t*-test. The level of significance was set at $P<0.05$. All calculations were performed using the PRISM package (GraphPad Software Inc., San Diego, CA). The relation between cortisol values and biochemical changes was evaluated by linear correlation analysis (Pearson's method).

Results

Pregnant jennies (Table 1) had higher cortisol concentrations in the 3rd ($P<0.001$) and the 11th ($P<0.05$) month, and lower in the 5th ($P<0.01$) and 8th ($P<0.05$) months than the previous month.

Table 1. Circulating endocrine and biochemical values (Mean ± SD) in pregnant jennies from the 1st to the 12th month of pregnancy

Pregnant jennies						
Months	Cortisol, nmol/L	Total protein, g/L	Creatinine, mg/dL	Urea, mg/dL	AST, UI/L	ALT, UI/L
1st	217.82 ± 130.87	6.40 ± 1.05	1.18 ± 0.21	33.00 ± 6.26	186.00 ± 53.38	6.00 ± 2.85
2nd	249.38 ± 14.57	8.60 ± 0.35***	1.49 ± 0.12	45.33 ± 7.09**	330.00 ± 110.91	10.67 ± 2.08
3rd	609.33 ± 51.38***	8.43 ± 1.62	1.17 ± 0.03	30.67 ± 7.09	295.00 ± 123.19	10.00 ± 1.73
4th	620.82 ± 151.01	9.50 ± 0.62	1.12 ± 0.01	38.00 ± 10.82	197.00 ± 77.49	12.33 ± 3.06
5th	304.03 ± 79.46**	9.33 ± 0.97	1.43 ± 0.29	22.25 ± 3.59*	170.50 ± 16.74	11.50 ± 4.04
6th	411.63 ± 143.61	9.45 ± 0.78	1.79 ± 0.10	36.00 ± 7.07***	104.00 ± 11.41**	12.50 ± 6.36
7th	437.36 ± 124.26	8.78 ± 1.05	1.32 ± 0.41	17.50 ± 3.11***	203.50 ± 35.80*	9.75 ± 4.35
8th	243.37 ± 99.27*	7.77 ± 0.76	0.91 ± 0.47*	29.00 ± 3.00***	236.33 ± 74.41	8.83 ± 1.72
9th	413.65 ± 189.06	8.78 ± 0.76***	1.35 ± 0.14*	27.83 ± 11.87	240.86 ± 46.52	10.33 ± 5.86
10th	305.21 ± 116.45	7.75 ± 0.58***	1.36 ± 0.18	32.29 ± 4.79	209.40 ± 71.13	9.00 ± 1.26
11th	564.49 ± 46.67*	8.10 ± 0.14	1.36 ± 0.21	30.00 ± 5.66	236.00 ± 21.21	9.33 ± 0.58
12th	310.59 ± 149.58	7.84 ± 1.67	1.33 ± 0.21	36.00 ± 9.17	251.67 ± 31.33	10.20 ± 1.79

[a] Asterisks indicate significant (*P<0.05; **P<0.01; ***P<0.001) differences in average parameters *vs* previous month.

Cortisol values (Table 2) of pregnant jennies ranged between 235.54 and 556.37 nmol/L, and barren jennies ranged between 329.01 and 654.98 nmol/L. As compared to barren jennies cortisol concentrations were lower ($P<0.05$) in pregnant jennies.

Pregnant jennies had total protein values (Table 1) higher in the 2nd ($P<0.001$) and the 9th ($P<0.001$) month, and lower in the 10th ($P<0.001$) than the previous month.

Total protein values (Table 2) of pregnant jennies ranged between 7.25 and 9.65 g/L, and of barren jennies ranged between 6.77 and 9.86 g/L. No differences were observed between pregnant and barren jennies.

In pregnant jennies creatinine values (Table 1) had lower levels in the 8th ($P<0.05$) month, and higher levels in the 9th ($P<0.05$) than the previous month.

Creatinine values (Table 2) of pregnant jennies ranged between 1.04 and 1.55 mg/dL, and of barren jennies ranged between 0.99 and 1.42 mg/dL. Circulating creatinine levels were higher in pregnant jennies than in barren jennies ($P<0.01$).

In pregnant jennies urea values (Table 1) were at higher levels in the 2nd ($P<0.01$), 6th ($P<0.001$) and 8th ($P<0.001$) months, and lower levels in the 5th ($P<0.05$) and 7th ($P<0.001$) month than the previous month.

Urea values (Table 2) of pregnant jennies ranged between 18.40 and 44.00 mg/dL, and of barren jennies ranged between 23.45 and 48.75 mg/dL. Circulating urea values were significantly lower in pregnant ($P<0.05$) than in barren jennies.

Table 2. Circulating endocrine and biochemical values (Mean ± SD) in pregnant and barren jennies during 12 months (from June to June)

Parameters	Pregnant	Range	Barren	Range	Significance
Cortisol, nmol/L	361.22 ± 136.18*	235.54 - 556.37	443.49 ± 151.92	329.01 - 654.98	P<0.05
Total protein, g/L	8.35 ± 0.84	7.25 - 9.65	7.99 ± 1.14	6.77 - 9.86	N.S.
Creatinine, mg/dL	1.31 ± 0.16**	1.04 - 1.55	1.23 ± 0.18	0.99 - 1.42	P<0.01
Urea, mg/dL	30.89 ± 5.33*	18.40 - 44.00	32.98 ± 5.77	23.45 - 48.75	P<0.05
AST, UI/L	231.03 ± 63.17	156.00 - 324.00	251.89 ± 84.89	197.50 - 322.80	N.S.
ALT, UI/L	10.34 ± 2.65	8.67 - 14.00	10.83 ± 2.93	9.11 - 14.29	N.S.

[a] Asterisks indicate significant (*P<0.05; **P<0.01) differences in average parameters *vs* barren jennies

In pregnant jennies AST changes (Table 1) had lower activities in the 6th ($P<0.01$) month, and higher in the 7th ($P<0.05$) month than the previous month.

AST changes (Table 2) in pregnant jennies ranged between 156.00 and 324.00, and of barren jennies ranged between 197.50 and 322.80 UI/L. No significant differences were observed between pregnant and barren jennies.

In pregnant jennies ALT changes (Table 1) did not shown significant differences as compared to the previous month.

ALT changes (Table 2) of pregnant jennies ranged between 8.67 and 14.00, and of barren jennies ranged between 9.11 and 14.29 UI/L. No significant differences were observed between barren and pregnant jennies.

Table 3. Correlation and linear regression between cortisol concentrations and biochemical parameters in pregnant and barren jennies

Parameters	Pregnant		Barren	
Cortisol : total protein	0.9957	.01	0.9996	.001
Cortisol : creatinine	0.9713	.05	0.9220	N.S.
Cortisol : urea	0.9909	.01	0.9898	.05
Cortisol : AST	0.9971	.01	0.9754	.05
Cortisol : ALT	0.9944	.01	0.9966	.01
Creatinina : urea	0.9909	.05	0.9220.	N.S
AST : ALT	0.9971	.01	0.9966	.05

In pregnant jennies (Table 3) positive and significant correlations were observed between cortisol and total protein ($P<0.01$), creatinine ($P<0.05$), urea ($P<0.01$), AST ($P<0.01$), ALT ($P<0.01$) changes, and between creatinine and urea ($P<0.05$), AST and ALT ($P<0.01$) changes. In barren jennies (Table 3) positive and significant correlations were observed between cortisol and total protein, urea, AST, ALT changes, and between AST and ALT changes.

Discussion

Circulating cortisol concentrations obtained in jennies are higher than the physiological range reported in horses (KANEKO, 1989). However, the comparison of results obtained in this study with published data reported for mares revealed a similar trend of cortisol levels, with lower concentrations in pregnant than in barren subjects (GILL et al., 1985). Our data confirm the progressive decrease of circulating cortisol concentrations from the 3rd to the 10th month of pregnancy, as reported by FLISIŃSKA-BOJANOWSKA et al. (1991); these results could be related to the negative correlation reported for Equine species (ASA et al., 1983) between plasma cortisol and oestrogen levels, which increase from the 3rd to the 7th-

8th months in pregnant mares (NETT et al., 1975; NODEN et al., 1978). The higher cortisol levels in the 11th month of pregnancy could be related to equine foetus production at the end of the pregnancy, according to the decrease of maternal progesterone concentrations. However, foetal adrenocortical glands are rich in 17α-hydroxylase, which modifies pregnenolone in cortisol only after the 310th day of pregnancy (CHAVATTE et al., 1995; OUSEY, 2004; OUSEY et al., 2005).

Generally, the highest cortisol levels observed both in pregnant and barren jennies could be due to their half-wild state. Although the blood sampling was carried out on the animals in pasture, this state was probably more stressful than sampling carried out in jennies stabled in single boxes. However, it is well-known that stabling (KOELKEBECK and CAIN, 1994) and management (IRVINE and ALEXANDER, 1994) may induce the activation of the hypothalamus-hypophysis-adrenocortical axis (HPA), with an increase in cortisol levels.

The different adrenocortical response between pregnant and barren jennies may be interpreted in terms of the different metabolism of cortisol. Hence, maternal and foetal cortisol synthesis could be considered as hormone concentrations that may substantially enhance circulating cortisol levels. These changes may subsequently result in the apparent decrease in adrenocortical activity of pregnant jennies, with lower cortisol levels compared to barren jennies. These data could be due to the existence of negative feedback on maternal cortisol levels induced by foetal cortisol synthesis.

The results obtained do not exclude the fact that certain stressful conditions, like stabling or management (HOFFMAN et al., 1995; MOONS et al., 2005) and diet (MESSER et al., 1995; STICKER et al., 1995; POWELL et al., 2000), may have led to an increase in cortisol levels; it cannot be excluded that stress was caused by blood drawing, which was always performed in jennies put out to pasture. However, the data obtained exclude the possible influence of cortisol rhythms because blood sampling was always performed at the same time of day.

Pregnancy has a major influence on the intensity of metabolism and on circulating metabolic parameters. The overall mean total protein values were found to be close to those reported for donkey (ZINKL et al., 1990; MORI et al., 2004; AL-BUSADAH and HOMEIDA, 2005) and confirm the gradual increase of total protein concentrations obtained in the plasma of mares during pregnancy. The significant increase in total protein concentrations in the 2nd and 9th months of pregnancy, with a general slight increase in the second and third period of pregnancy, compared to the beginning of pregnancy, could be a result of hormonal changes in the organism, which in turn intensifies metabolic events (CUNNINGHAM, 1997).

Creatinine concentrations came within the physiological range reported for donkeys (GUPTA et al., 1994; MORI et al., 2004; AL-BUSADAH and HOMEIDA, 2005; CALDIN et

al., 2005). Higher creatinine concentrations obtained in pregnant than in barren jennies could be related to changes that occurred over the course of the pregnancy and to foetus-placental activity. Since a correlation between plasma creatinine and body size has been described (SWAMINATHAN et al., 1986) the heavier body weight of pregnant than barren jennies may have had an influence on creatinine changes.

Urea concentrations of jennies were in agreement with the range reported for donkeys (AL-BUSADAH and HOMEIDA, 2005; CALDIN et al., 2005). In addition, changes in plasma creatinine and urea concentrations might be explained if muscle mass increased secondary to decreased muscle catabolism during pregnancy. The highest creatinine and the lowest urea values of pregnant jennies might also reflect a difference in protein metabolism associated with the presence of the foetus.

AST and ALT ranges were in agreement with the findings reported for horses and for Ragusana donkeys (KANEKO, 1989; CALDIN et al., 2005), but not for other donkey breeds (GUPTA et al., 1994; JORDANA et al., 1998; MORI et al., 2004). However, a large individual variability was recorded. Since there are no data about pregnant Ragusana jennies, and as it is known that AST activity varies according to the breed (MILINKOVIĆ-TUR et al., 2005), we are unable to state with certainty that the values obtained are not necessarily outside the physiological range. In addition, our data confirm the absence of significant differences in ALT changes during pregnancy as reported in pregnant mares (MILINKOVIĆ-TUR et al., 2005). These data could be explained by the higher turnover of transaminase changes due to changes in maternal metabolism and functional differentiation of tissues (RAVISHANKAR and MEHTA, 1991), such as the reproductive system. In addition, the seasonal transaminase changes confirm previous data for mares reported by FLISIŃSKA-BOJANOWSKA et al. (1991).

In conclusion, the existence of significant differences of cortisol, creatinine and urea concentrations between pregnant and barren jennies showed the significant influence of the physiological state on the endocrine and biochemical homeostasis. These findings were confirmed by the positive and significant correlations between cortisol and biochemical parameters.

In addition, the significant effect of season on the cortisol, total protein, creatinine and urea changes in both pregnant and barren jennies showed that seasonal cyclicity is an intrinsic peculiarity of these parameters.

This information will certainly help practitioners to identify any metabolic changes due to pregnancy; this will allow veterinarians to establish an appropriate interpretation of laboratory data that can be used as reference values in the clinical evaluation of pregnant jennies.

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SAŽETAK

Promjene u koncentraciji kortizola i određenih biokemijskih pokazatelja često se javljaju tijekom bređosti u različitih vrsta, usprkos činjenici da homeostatski mehanizmi održavaju sastojke krvi u konstantnim i međusobno uravnoteženim koncentracijama. Potpunih podataka o endokrinom i metaboličkom statusu u magarica za vrijeme bređosti nema. Budući da se metaboličke funkcije povećavaju tijekom bređosti da bi zadovoljile zahtjeve ploda, posteljice i maternice, svrha ovog istraživanja bila je odrediti koje se fiziološke promjene javljaju u koncentraciji kortizola u plazmi te u koncentraciji ukupnih bjelančevina, kreatinina, ureje i općenito u metabolizmu bređih magarica te usporediti nalaze s onima u jalovih. Istraživanje je bilo provedeno na 24 zdrave Ragusano magarice (10 bređih i 14 jalovih). Uzorci krvi bili su magaricama uzimani iz jugularne vene jedanput mjesečno tijekom jedne godine. U usporedbi s jalovim magaricama, u bređih magarica bile su ustanovljene niže vrijednosti kortizola ($P<0,05$) i ureje ($P<0,05$), a više vrijednosti kreatinina ($P<0,01$). Vrijednosti ukupnih bjelančevina te aktivnosti AST i ALT bile su slične u bređih i jalovih magarica. Značajne razlike u koncentraciji kortizola, kreatinina i ureje između bređih i jalovih magarica govore o odgovarajućem upletanju fizioloških mehanizama na endokrinu i biokemijsku homeostazu. Ovi su nalazi bili potvrđeni pozitivnim i značajnim korelacijama između koncentracije kortizola i biokemijskih pokazatelja.

Ključne riječi: *Equus asinus*, bređost, kortizol, biokemijski pokazatelji