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Effects of partial versus complete separation after weaning on plasma serotonin, tryptophan and pituitary-adrenal pattern of Anglo-Arabian foals

G. Bruschetta[a,*], E. Fazio[b], C. Cravana[b], A.M. Ferlazzo[a]

[a] Unit of Veterinary Biochemistry, Department of Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, 98168 Messina, Italy
[b] Unit of Veterinary Physiology, Department of Veterinary Sciences, University of Messina, Polo Universitario dell'Annunziata, 98168 Messina, Italy

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ABSTRACT

The study investigated the effect of partial and complete separation after weaning on circulating serotonin, tryptophan, adrenocorticotropic hormone and cortisol concentrations of foals. Twenty Anglo-Arabian colts, aged 24 weeks, were submitted to partial or complete separation from their mother at the same time. The subjects were divided in two groups on the basis of different separation programs. Foals were randomly assigned to one of two groups based on different treatments: group A was designated as partial separation and included 10 colts; group B was designated as complete separation, and included other 10 colts. The foals were evaluated 21 days before separation (T0), at 10 min before separation (T1), and 30 min (T2), 24 h (T3), 48 h (T4), and 30 days (T5) after separation. One-way analysis of variance (ANOVA) showed a significant timing effect in group B for serotonin ($P=0.002$), tryptophan ($P < 0.0001$), adrenocorticotropic hormone ($P=0.0024$) and cortisol ($P=0.003$) concentrations. Two-way analysis of variance (ANOVA) showed a significant separation effect ($P < 0.0001$) in group B compared to group A, with higher tryptophan concentrations after every post-separation times (T2-T5), and higher cortisol concentrations shortly after separation times (T2-T4). A significant positive correlation between adrenocorticotropic hormone and cortisol values ($r=0.67$; $P < 0.05$) was observed in group B at T2, T3 and T4, respectively. The results support the effects of partial and complete separation in modulating the neuroendocrine response of Anglo-Arabian foals, providing additional knowledge of serotoninergic and pituitary-adrenal involvement. These findings indicate that complete separation already at 30 min causes higher neuroendocrine responses than partial separation during weaning of foals.

1. Introduction

Genetics, environment, facilities and management play significant role in determining individual growth and well-being, including early handling, feeding practices and weaning method (Lansade et al., 2004; Moons et al., 2005; Simpson, 2002). Weaning is defined as the stage at which the mother most sharply reduces the time and effort that she devotes to the offspring (Martin, 1984). However, in farming the weaning is considered as the point in time of man-induced separation of young and mother. Under domestic conditions foals are usually weaned at 4–6 months of age. Depending on the weaning method separation of the mother-offspring is more or less stressful (Rogers et al., 2004; Waran et al., 2008). The most traditional method is the complete, abrupt separation of the mare and foal; moreover, incomplete and gradual separation are also used to wean foals in the horse industry. Each system has its advantages and disadvantages, and there is no one system that is right for everyone. Weaning breaks the emotional, physical and nutritional bounds between the mare and foal that were initiated at birth (Waran et al., 2008). Foals respond immediately to weaning in a different changes of haematological, metabolic and hormonal parameters, according to management on the individual farms (Qureshi et al., 2013). Even if a remarkable amount of studies, carried out in animal models, reported an association of brain serotoninergic system with stressors during critical periods of early life, including maternal care, weaning and puberty, conversely the effects of these physical and mental stressors on blood serotonin (5-HT) are still largely unknown. The complex interplay between 5-HT and its receptors and the modulator role on feeding behaviour have become of great interest in the scientific community (Magalhães et al., 2010). A role of tryptophan (TRP) metabolites, especially 5-HT (5-hydroxytryptamine), in intestinal function and in the pathogenesis of gastrointestinal diseases was reported (Keszthelyi et al., 2009), although mechanisms of action are still poorly understood.

Actual occurrences, the exposure to short separations prior to weaning has been found intensive, and therefore sensitise the stimulus

* Corresponding author.
E-mail addresses: gbruschetta@unime.it (G. Bruschetta), fazio@unime.it (E. Fazio), ccravana@unime.it (C. Cravana), alferl@unime.it (A.M. Ferlazzo).

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as evidenced through the increase in salivary cortisol levels of foals experiencing weaning (Moons et al., 2005). Foals' cortisol concentrations taken 28 h after weaning showed a significant increase, with a subsequent decrease in immune response of foals weaned in pairs as opposed to those weaned singly or those who remained with the dam (Malinowski et al., 1990). Significant higher adrenocorticotropic hormone (ACTH) and cortisol concentrations at 2 and 3 days of weaning than the 1 day were found in Thoroughbred foals (Fazio et al., 2009), thus confirming their metabolically role in response to stress syndrome.

The different neuroendocrine response of foals will play an important role in providing complementary physiological assessment of serotoninergic and pituitary-adrenal involvement according to stress-inducing treatment. Therefore the main objective of the present study was to compare the potential effect of partial versus complete separation methods on circulating 5-HT, TRP, ACTH and cortisol concentrations of healthy Anglo-Arabian foals, evaluating their early and/or later neuroendocrine responses. We could expect that partial separation compared to complete separation reduces neuroendocrine activity in terms of the brain and HPA-axis responses measured in blood.

2. Materials and methods

2.1. Animals

This study was performed in compliance with the guidelines of the Italian law on the care and use of animals (D.L. 4/3/2014n. 26) and EU Directive (Directive 2010/63).

Twenty Anglo-Arabian colts, aged 24 weeks, were reared with their mothers on pasture and were allowed to suck freely. A limitation of fillies' number in the current study was determining for its statistical exclusion. Foals were weaned when they were six months old. The subjects were divided in two groups on the basis of different separation from their mother programs. Foals were randomly assigned to one of two groups based on different treatments: group A was designated as partial separation and included 10 colts; group B was designated as complete separation and included other 10 colts. The two groups were managed as one entire group prior to start of the separation program. Group A was submitted to a partial separation of the mare and foal with a common fence line using a V-mesh; hence, the mares and foals were able to see, hear and smell each other while in separate paddocks. Group B was submitted to a complete separation of the mare and foal; hence, the mares and foals were not in contact by sight or sound of either the mare or foal. The foals were placed in separate paddocks in presence of conspecifics, and the mares were turned out to pasture.

The only difference between the two groups was that separation from their mother was partial for group A, whereas separation for group B was complete.

Foals were fed with concentrate (1.5 kg of fodder/foal-day) and pasture (grass hay mixed with other native grasses) in the best conditions of amount and quality. There was a period of adaptation to the concentrate feeding, in order to avoid colics that usually appear with a sudden change in the diet. The amount of commercial feed was gradually increased, starting with small quantities to reach the final amount. Composition (%) of commercial feed was: protein (15.2%), fibre (7.1%), ashes (6.3%), calcium (1.2%), potassium (1.2%) and phosphorous (0.7%). Commercial feed was composed of soya bean flour, wheat bran, corn, alfalfa, sugar cane molasses, soya oil, calcium carbonate, dicalcium phosphate, sodium chloride and powder lactose. This ration was supplemented with the next mineral/vitamin mix: vitamin A (6000 UI/kg), vitamin D3 (600 UI/kg), zinc (150 mg/kg), manganese (70 mg/kg), iron (90 mg/kg), copper (10 mg/kg), cobalt (0.30 mg/kg) and iodine (2 mg/kg) and butyl-hydroxyanisol (0.03 mg/kg), etoxiquine (0.03 mg/kg).

2.2. Blood collection

All samples were taken from the jugular vein using vacutainer tubes (Venoject®, Terumo Europe N.V., Leuven, Belgium), with heparin for HPLC analysis (5-HT and tryptophan), and without anticoagulant (serum) for ACTH and cortisol analysis, between 08:00 a.m. and 09:00 a.m. to minimize the effect of circadian rhythm on neurohormone measurements. Blood samples were taken at 21 days before separation from mother in baseline conditions, at separation day (before 10 min), and 30 min (T2), 24 h (T3), 48 h (T4), and 30 days (T5) after separation. All samples were taken in quiet conditions by the same operator, 10 colts at day for two consecutive days (the first day: group A, the second day: group B).

Foals were accustomed to clinical routines and blood sampling; subjects were individually held in a corner of the paddock, one at a time, and were easily restrained by the same handler assistant who held their halter during the blood samples. All animals were sampled in the same order; thus, blood samples from groups A and B were obtained at the same time of day for each group, to minimize the effect of blood samplings on stress reactions.

2.3. 5-HT and TRP analysis

Blood samples, in heparin, were centrifuged at 4 °C at 4500×*g* for 10 min, to obtain a platelet poor plasma (PPP) fraction, devoid of 98% of platelets (Bruschetta et al., 2014). Equal volumes (100 μL) of internal standard represented by N-methylserotonin (Chromsystems, München, Germany) and protein precipitation reagent (Chromsystems, München, Germany) were added to 100 μL of heparin PPP. The solutions were vortex-mixed for 30 s, incubated at 4 °C for 10 min, and then centrifuged at 4500×*g* for 10 min. The resulting supernatants were stored at −20 °C for the following HPLC analysis. Separation of PPP 5-HT and tryptophan were carried out by an isocratic reverse phase HPLC method and qualitative and quantitative analyses of 5-HT and TRP were performed as detailed elsewhere (Bruschetta et al., 2013). The assay sensitivity of HPLC detector was 0.5 ng/mL for 5-HT and 3 ng/mL for tryptophan. The intra- and inter-assay CVs for 5-HT were 3.8% and 4.9%, respectively, and the intra- and inter-assay CVs for tryptophan were 4.4% and 6.3%, respectively.

2.4. ACTH and cortisol analysis

Immediately after withdrawal, blood samples were refrigerated at 4 °C and were subsequently (within 1 h) centrifuged for 15 min at 1500×*g*. Serum was harvested and stored in polystyrene tubes at −20 °C and assayed for ACTH and cortisol.

Serum ACTH concentrations were analyzed in duplicate using a commercially available RIA kit (ELSA-ACTH, CIS-BioInternational, Gif-sur-Yvette, France). The hormone assay used has a range for the amount of ACTH detected of 0–440 pmol/L. The sensitivity of the assay ACTH was 0.44 pmol/L. The intra- and inter-assay CVs were 6.0% and 15.0%, respectively.

Total serum cortisol concentrations were analyzed in duplicate using a competitive enzyme-linked immunoassay (EIA, RADIM, Rome, Italy) by a commercial test kit and a BRIO automated analyzer (SEAC, Rome, Italy). During the first incubation, the cortisol sample competed with cortisol conjugated to horseradish peroxidase (HRP) for the specific sites of the antiserum coated on the wells. Following incubation, all unbound material was removed by aspiration and washing. The enzyme activity bound to the solid phase was inversely proportional to cortisol concentration in calibrators and samples, and is made evident by incubating the wells with a chromogen solution (tetramethylbenzidine, TMB) in substrate-buffer. Colorimetric reading was carried out using a spectrophotometer at 450, 405 nm wavelength (Sirio S, SEAC, Florence, Italy). Assay sensitivity was 13.80 nmol/L. The intra- and inter-assay CVs were 4.0% and 6.9%, respectively.