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Short Communication

Cytokine levels in colostrum and in foals' serum pre- and post-suckling

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ABSTRACT

The purpose of this study is to investigate the presence of IL-4, IL-8, IL-13 and IFN-γ in equine colostrum and in foals' serum. Samples were obtained from 14 mares and their healthy foals. Soon after parturition, 10 ml of colostrum was collected, filtered, centrifuged and frozen until assayed. Blood samples were obtained from each foal at birth (TO) and again after 24 h (T24), after which they were frozen until assayed.

Serum IgG was measured at 24 h of age with an immunoturbidimetric quantitative method. Cytokine concentration was determined using commercially available ELISA tests. Statistical analyses revealed a significant difference in serum concentration of IL-4 at T0 and at T24 ($p < 0.05$) and a significant correlation between the serum IL-4 at T24 and colostral IL-4. These results suggest the absorption of IL-4 from colostrum. The presence of IL-8 in the pre-suckle foal's serum may be due to an endogenous production. With the exception of two samples, there was no IL-13 detected in the foals' serum at birth and remained undetectable in 8/14 samples after 24 h. This cytokine was also undetectable in four colostrum samples, where its concentration showed a wide range and a high standard deviation. IFN-γ was present in both the colostrum and in the foals serum at birth.

1. Introduction

The epitheliochorial structure of the equine placenta does not provide transfer of immunoglobulins to the fetus whereby the neonate is essentially devoid of circulating antibodies until it absorbs them from the colostrum. For this reason, the immune system of neonatal foals is highly dependent on the ingestion of colostrum in the first hours of life, wherefore the foal is considered "immunodeficient" at birth. This is also because, even with a complete transfer of passive immunity, foals remain more susceptible to some pathogens than adult horses, pathogens which typically infect only neonatal and young foals and many of which rarely occur in adults and whereas they occur only as opportunistic pathogens (Hines et al., 2003; Dawson et al., 2010). Although experimental evidence suggests that the equine fetus is immunologically competent from mid gestation (Battista et al., 2014), it is evident that foals at birth are immunologically naïve to environmental antigens because of the immature adaptive immunity, such as preformed pathogen-specific antibodies, B- and T- cell memory and effector cells (Galan et al., 1986). The adaptive immunity is mainly regulated by cytokines produced by different Th-cell subsets. Cytokine expression in foals during the first year of life differs from the one in adult (Lopez et al., 2003; Breathnach et al., 2006; Wagner et al., 2010). There is some controversy in the current equine literature about Th-cell responses of the neonatal and young foals. Some authors suggest that foals are Interferon-gamma (IFN-γ) deficient (Breathnach et al., 2006) or that the immune system exhibits a Th2-bias (Boyd et al., 2003). More recent studies suggest that the immune response of young foals is Th1-biased and that the Th2 response is barely detectable for an extended time after birth (Wagner et al., 2010; Mariella et al., 2013).

Cytokines in colostrum and milk are mainly studied in human species (Saito et al., 1991; Bocci et al., 1993; Takahata et al., 2003) but also in bovine (Goto et al., 1997; Hagiwara et al., 2000; Hagiwara et al., 2008) and in swine (Nguyen et al., 2007). Little is known about cytokine content of the equine colostrum. Two studies investigated cytokine content of the equine colostrum and their transfer to the neonate. Burton et al. (2009) found that prior to ingestion of colostrum, serum Interleukine (IL)-6 concentrations are undetectable in healthy foals and that equine colostrum, like in other animal species, contains IL-6 (Hagiwara et al., 2000; Nguyen et al., 2007; Zanardo et al., 2007), while IL-10 was generally undetectable. The study of Secor et al. (2012) confirmed the presence of Tumor

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Necrosis Factor (TNF)-α in colostrum and the transfer of TNF-α to foals.

The goal of this study was to analyse the protein expression of key cytokines, representing Th1 (IFN-γ), Th2 (IL-4 and IL-13) response and a chemokine (IL-8) in colostrum and in pre- and post-suckle foals' serum. The authors hypothesized that colostrum may contain the analysed cytokines and that after the ingestion of colostrum there would be a significant increase of these cytokines in post-suckle foals serum. It was also tested the hypothesis that cytokine concentration in post-suckle serum and colostrum is correlated with Immunoglobulin G (IgG) in post-suckle serum.

2. Materials and methods

2.1. Subjects

Fourteen healthy Standardbred mares were hospitalized for attending parturition in the Equine Perinatology Unit (EPU) of the University of Bologna during the 2011 and 2012 foaling season.

Mares were admitted to the EPU at about 310 days of gestation and received a complete physical examination, blood count and biochemical exams. They were housed in separate wide straw-bedded boxes, fed hay ad libitum and concentrates twice a day and were allowed to go to pasture during the day. All foalings were attended indoors and were eutocic. The foals were considered healthy if they had an Apgar score ≥ 9 (Vaala et al., 2002) and, normal clinical, haematological and biochemical exams, if they began to nurse from the mares within two hours after birth, and if they had a complete transfer of passive immunity (IgG > 800 mg/dl at 24 h of life). Both mares and foals remained under observation for at least 7 days post-partum and physical examination was performed two times a day by the clinicians of the EPU.

All procedures on the animals were carried out with the approval of the Ethical Committee of the University of Bologna, in accordance with national legislation (legislative decree 116/92), communicated to the Ministry of Health. The owners gave oral informed consent.

2.2. Sample collection

Ten milliliters of mare colostrum was collected into tubes with no additives, soon after parturition and before the first suckling. First, to estimate colostral IgG concentration, a Brix refractometer was used. Then, the samples were filtered with a gauze and centrifuged (3000 × *g*, 30 min, room temperature) to separate fat and impurity. The hydrophilic phase was aliquoted and stored at −20 °C until assayed.

Foals blood samples were collected by jugular venipuncture into tubes with gel clotting activator (S-Monovette; Sarstedt, Verona, Italy) at birth (T0), before colostrum uptake, and post-suckle at 24 h of life (T24). Blood samples were centrifuged (3500 × *g*, 10 min, room temperature) and serum was stored at −20 °C until assayed. Serum IgG concentration was measured at 24 h of life with a hand-held quantitative colorimetric immunoassay (DVM Rapid Test™, Immunosystem Inc, USA). This method is demonstrated as an accurate quantitative measurement comparing to the standard radial immunodiffusion (Davis and Giguére, 2005).

2.3. Enzyme-linked immunosorbent assay (ELISA) for equine cytokines

Serum samples were diluted 1:2 and then cytokines were measured in different enzyme linked immunosorbent assay (ELISA) tests. The concentration of IL-4, IL-8 and IL-13 was evaluated using three different commercial kits produced by USCN Life Science Inc. (Milano, Italy), and the concentration of IFN-γ was evaluated using Bethyl Laboratories Inc. (Bologna, Italy) commercial kit. The ELISA tests were performed according to manufacturer's instructions.

2.4. Statistical analysis

Data were tested for Gaussian distribution using the Kolmogorov-Smirnov test. Since they were found to be non-Gaussian, further data analysis were performed with non-parametric tests. The Wilcoxon signed-rank test was used to evaluate serum cytokine concentration over time. The Spearman's rank correlation was tested to characterize the association between: 1) cytokine concentration in post-suckle serum and colostrum; 2) post-suckle serum cytokine concentration and IgG serum concentration; 3) serum ΔT cytokine (T0–T24) and colostral cytokine concentration. Spearman's rank correlation coefficient was indicated as ρ (rho). A P value < 0.05 was considered statistically significant. All analyses were carried out using commercial software (Analyse-it, version 2.03; Analyse-it Software Ltd., Leeds, West Yorkshire, England).

3. Results and discussion

This study investigated for the first time the colostral content of Th1 (IFN-γ), Th2 (IL-4, IL-13) cytokines and chemokine (IL-8) and their possible transfer to neonatal foals via colostrum. The analysed cytokines were detected in all the colostrum samples, except for IL-13, which was not present in 4/14 samples. With the exception of IL-13, all the analysed cytokines were constantly found in foals' serum. The IL-13 was found in only 1/14 serum samples at T0 and in 6/14 at T24; in only one sample it was present both at birth as well as after 24 h. The results of the cytokine concentration in foals' serum and colostrum were summarized in Table 1.

One of the most relevant findings, is that the serum IL-4 concentration is significantly higher in post-suckle samples than at

Table 1
Concentration of the analysed cytokines in foals' serum at birth (T0), after 24 h (T24), their difference (ΔT24-T0) and their concentration in colostrum. Data are expressed as mean ±SD, median, minimum and maximum value.

	IL-4 (pg/mL)	IL-8 (pg/mL)	IL-13 (pg/mL)	IFN-γ (pg/mL)
Serum T_0	43.8 ± 13.9	244.9 ± 119.5	13.8 ± 48.2	3709.1 ± 234.5
	39.8	196.4	0	3626.7
	(30.7–86.1)	(147.6–519.1)	(0–187.7)	(3600.5–4542.6)
Serum T_{24}	80.2 ± 60.5	286.1 ± 147.1	40.8 ± 82.4	3710.2 ± 222
	65.3	236.2	0	3671.1
	(31.3–256.4)	(172.5–758.7)	(0–315.8)	(3497.5–4542.6)
ΔT_{24}-T_0	36.3 ± 62	41.2 ± 88.1	27 ± 82.4	3.7 ± 18.5
	17.3	48.9	0	8.9
	(−17–219.3)	(−69.6–269.3)	(0–315.8)	(−63.2–8.9)
Colostrum	1141.9 ± 2104.3	843.1 ± 421.4	333.1 ± 566.3	5050.7 ± 5283.8
	45.6	706.4	78.2	3515
	(21.8–5349.8)	(383.5–1851.7)	(0–2028.3)	(3139.4–24017.3)