Correlations of whole blood heavy metals with serum immunologic and oxidative markers on the early dry period and transition period of dairy cattle

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SUPPLEMENTARY FILE

Materials and Methods

Animals and Setting

The current research was confirmed by the Animal Welfare Committee of the Ferdowsi University of Mashhad with the code of 3/44869 under the institutional, national, and international guidelines. This cross-sectional study was performed in a commercial dairy herd consisting of 3000 Holstein cows and 970 lactating animals in Neyshabur, Iran. The average milk production of the herd was recorded as 40 L. Animals were fed twice a day and had free access to water throughout the study period. The ingredients and nutritional composition of diets for far-off, close-up, and fresh cows are presented in Table 1.

Thirty-five pregnant cows on the second and higher pregnancy, which were at the beginning of the dry period (6 weeks before the expected calving) and were healthy in clinical examinations were enrolled in the study. Data of 30 subjects, which had normal easy calving and had no

clinical abnormalities, such as retained placenta, mastitis, metritis, laminitis, milk fever, or displaced abomasum during the study were finally analyzed. The parity of all animals was obtained from herd records and animals were divided into three groups of two, three, and more than three parturitions.

Sample Collection

Blood sampling was carried out on the early dry period (-6w), one week prior to the expected calving (-1w), and one week postpartum (+1w). The specimens were collected through coccygeal venipuncture into commercial evacuated tubes (Novin-pyrex, Iran) that had clot activators and tubes containing lithium heparin. Next, the blood samples in the tubes with clot activator were left steady at the room temperature for 30 min to clot and then, were centrifuged at 3000 g for 15 min and the sera were harvested instantly. All the samples were chilled and delivered to the laboratory on ice packs. Serum samples were aliquoted in Eppendorf tubes to avoid repeated freeze and thaw. The sera and whole blood specimens were stored at -80°C until further analyses. All the serum and blood samples were thawed at room temperature before the tests.

Heavy Metals

Whole blood samples were analyzed for Pb, As, and Cd using inductively coupled plasma optical emission spectrometry (ICP-OES) (SPECTRO ARCOS, Germany) with the blank of ultrapure water (18.2 MΩ cm^{-1} water) added to the digest mixture (3 ml water, 3 ml nitric acid, and 2 ml hydrogen peroxide). Following melting, each sample was pipetted thoroughly to avoid settling and 3 ml of whole blood was transmitted into acid-washed tubes. Next, 3 ml of nitric acid 65% (HNO_3, Merck, Darmstadt, Germany) was added to each tube and after 30 min in the clean hood as the pre-reaction time, 2 ml of hydrogen peroxide 30% (H_2O_2, Merck, Darmstadt, Germany)

was added to all specimens. Afterwards, the tubes were sealed and placed in a water bath at 80°C for approximately 45 min until the complete digestion of samples and were then centrifuged at 3000×g for 15 min. Supernatants were harvested immediately and 18.2 MΩ cm^{-1} ultrapure water was added to provide the final volume of 10 ml. The detection limit for all measured trace elements was l μg/L and the average accuracy was 95%.

Oxidative Markers

The level of malondialdehyde (MDA), as one of the products of polyunsaturated fatty acids peroxidation, in the serum samples was assessed by reaction with thiobarbituric acid (TBA) at 95°C to generate an MDA-TBA adduct. The pink-color adduct was measured spectrophotometrically at 530-540 nm (Nalondi™ assay kit, Navand Salamat, Orumieh, Iran). In addition, the total antioxidant capacity (TAC) of the serum samples was measured through the colorimetric evaluation of ferric iron (Fe^{3+}) reduction to ferrous iron (Fe^{2+}) by the serum antioxidants at 593 nm (Naxifer™ assay kit, Navand Salamat, Orumieh, Iran).

Immunologic and Inflammatory Markers

The immunologic markers, including Immunoglobulin G (IgG), interleukin 4 *(*IL-4), interleukin 10 *(*IL-10), interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), and haptoglobin (Hp) were measured in the serum samples by enzyme-linked immunosorbent assay (ELISA) commercial bovine kits (Bioassay Technology Laboratory, Shanghai, China) according to the manufacturer manual. The ELISA automatic washer (BioTek, ELx-50, Winooski, USA) and reader (BioTek, ELx-800, Winooski, USA) were utilized.

Statistical Analysis

All the data were statistically analyzed using SPSS software version 22 (IBM, USA). The normality of data distribution was assessed by the Shapiro-Wilk test along with skewness and kurtosis showing that none of the factors had a normal distribution. Therefore, all the parameters were modified by the logarithmic transformation.

The repeated measures analysis was performed using a 3 (time) × 3 (parity) mixed analysis of variance (ANOVA) to evaluate the effects of time, parity, and interactions between these factors on all the measured variables. The Tukey post hoc test was run for parity to assess the differences between different parity groups as the between-subject factor. Pairwise comparisons for time and factor interactions were completed utilizing Bonferroni adjustment to investigate the differences between the repeated measures. Moreover, the correlations between all the transformed variables were investigated separately for the three measured times using the Pearson correlation coefficient. The significance level was considered as $P < 0.05$ for all tests.

Table 1. Ingredients and nutritional composition (% DM unless noted) of diets fed to cows during dry and lactation periods

Item	Far-off	Close-up	Fresh cow
Ingredients			
Alfalfa hay	8.55	5.7	8.25
Corn silage	76.7	58.54	42.38
Sugar beet pulp	-	-	6.64
Wheat straw	3.54	2.22	0.92
Alfalfa silage	-	-	2.29
Sugar beet molasses	-	-	9.16
Water	-	9.49	-
Barley meal	0.88	5.56	11.14
Cornmeal	3.17	8.18	6.03
Soybean meal	0.98	3.89	4.97

Cottonseed	-	0.63	2.96
Wheat grain	1.88	-	-
Meat meal	3.36	-	-
Fish meal	-	2.22	3.3
Full fat soybean	-	1.68	-
Urea	0.21	-	-
Salt	0.06	-	0.18
Calcium carbonate	0.26	0.49	0.23
Sodium bicarbonate	-	-	0.73
Dicalcium phosphate	-	-	0.09
Magnesium oxide	0.06	0.03	0.23
Anionic salts	-	0.95	-
Mineral-vitamin supplement[1, 2]	0.21	0.42	0.26
Toxin binder	0.14	-	0.14

Energy and nutrients

Net energy for lactation (Mcal/kg)	1.32	1.5	1.61
Neutral detergent fiber	44.7	28.6	29.1
Non-fiber carbohydrates	30.2	38.5	37.5
Ether extract	2.1	3.6	4.3
Crude protein	12.1	14.7	16.7
Calcium	0.75	0.97	0.86
Phosphorus	0.35	0.37	0.48

[1] Anionic pre-fresh supplement: 250000 IU/Kg vit A, 40000 IU/Kg vit D_3, 40000 IU/Kg vit E, 40 mg/Kg biotin, 12 g/Kg niacin, 168 g/Kg Ca, 65 g/Kg Mg, 1300 mg/Kg Mn, 2210 mg/Kg Zn, 600 mg/Kg Cu, 10 mg/Kg Co, 8 mg/Kg Se, 12 mg/Kg I, 52 g/Kg S, 120 g/Kg Cl

[2] Fresh cow supplement: 550000 IU/Kg vit A, 120000 IU/Kg vit D_3, 5000 IU/Kg vit E, 100 mg/Kg biotin, 100 mg/Kg niacin, 160 g/Kg Ca, 30 g/Kg P, 40 g/Kg Mg, 16000 mg/Kg Mn, 22500 mg/Kg Zn, 5750 mg/Kg Cu, 200 mg/Kg Co, 125 mg/Kg Se, 200 mg/Kg I

Table S2. Correlations of heavy metals with immunological and oxidative markers at six weeks before expected parturition

		Pb	As	Cd	MDA	TAC	IgG	IL4	IL10	IFN-γ	TNF-α	Hp
Pb	***r***	1	-0.249	0.208	-0.6	0.004	-0.009	0.01	-0.125	0.109	-0.154	0.002
	P		0.184	0.271	0.754	0.985	0.964	0.96	0.512	0.568	0.418	0.992
As	***r***	-0.249	1	0.224	0.417	-0.366	-0.092	-0.031	-0.074	-0.075	-0.151	0.046
	P	0.184		0.234	0.022*	0.046*	0.629	0.871	0.696	0.692	0.425	0.81
Cd	***r***	0.208	0.224	1	0.083	-0.027	-0.118	-0.127	-0.06	-0.09	-0.107	0.051
	P	0.271	0.234		0.663	0.886	0.535	0.503	0.752	0.637	0.572	0.79

one week expected parturition

		Pb	As	Cd	MDA	TAC	IgG	IL4	IL10	IFN-γ	TNF-α	Hp
Pb	***r***	1	0.169	-0.098	-0.04	-0.127	-0.088	-0.009	0.235	-0.593	0.045	0.069
	P		0.371	0.608	0.835	0.504	0.644	0.962	0.211	0.001*	0.815	0.721
As	***r***	0.169	1	0.114	0.033	0.335	-0.157	-0.377	-0.087	0.168	-0.127	0.221
	P	0.371		0.548	0.864	0.071	0.408	0.04*	0.648	0.375	0.505	0.25
Cd	***r***	-0.098	0.114	1	-0.214	0.234	-0.048	-0.18	-0.124	-0.281	-0.372	-0.152
	P	0.608	0.548		0.256	0.213	0.802	0.342	0.516	0.133	0.043*	0.43

and one week after expected parturition

		Pb	As	Cd	MDA	TAC	IgG	IL4	IL10	IFN-γ	TNF-α	Hp
Pb	***r***	1	-0.062	-0.39	0.06	-0.538	0.28	0.289	0.153	0.049	0.122	0.275
	P		0.745	0.033*	0.755	0.022*	0.134	0.121	0.42	0.799	0.519	0.142
As	***r***	-0.062	1	0.451	-0.103	0.277	0.348	0.238	0.156	-0.103	0.157	0.153
	P	0.745		0.012*	0.588	0.139	0.06	0.206	0.411	0.587	0.409	0.42
Cd	***r***	-0.39	0.451	1	0.193	0.312	0.088	0.037	0.04	-0.09	0.101	-0.097
	P	0.033*	0.012*		0.306	0.093	0.645	0.845	0.833	0.636	0.594	0.612