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Evaluating Methionine Hydroxyl Manganese and Manganese Sulfate Sources for Dairy Cows During Peak-and Mid-lactation Stage

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ABSTRACT

Thirty Holstein cows (10 cows per treatment) were blocked according to calving date, milk yield and party and randomly assigned to a study to determine the effect of manganese (Mn) sources on lactating cows. Treatments were (1) all Mn (14 ppm) supplied by sulfate (S), (2) Mn sulfate ($MnSO_4$) and methionine hydroxyl manganese (Mn-$(HMTBA)_2$) contributed half of the dietary Mn (SM) or (3) all Mn supplied by (Mn-$(HMTBA)_2$) (M). The average 4% Fat-corrected Milk (FCM) yield and fat yield of cows supplied with M dietary were significantly higher than the cows fed with SM and the S ($p<0.05$). The average lactose rate of SM was significantly lower than S and M ($p<0.05$). Numerically, the apparent digestibility of organic matter, crude protein, crude fat and acid detergent fiber of S were the lowest but no significant difference were found between groups ($p>0.05$). Significant increases was observed for serum High-density lipoprotein cholesterol concentration of M compared with S and SM ($p<0.05$). The average detectable follicle numbers and ovarian score for cows of M was generally lower than cows of SM and S but no significant difference was observed ($p>0.10$). The present study suggested that replacing a portion of dietary Mn sulfate with Mn-$(HMTBA)_2$ during the peak-and mid-lactation periods will improve the lipid metabolism and milk fat secretion; however, the follicular development may be hampered.

Key words: Dairy cow, manganese, organic mineral, lactation performance, fertility, nutrient digestibility

INTRODUCTION

The bioavailability and requirement study of Mn were not much concerned. It was generally believed that the manganese tolerance dose of cows is about 1000 ppm (DM) NRC (2001). The exact minimum requirement for manganese in cow has not been established. The NRC (2001) model uses Absorption Coefficients (AC) to convert dietary Mn into absorbed Mn and a diet with 14 mg of Mn kg^{-1} of DM will meet the Mn requirement for a 600-kg cow producing 30 kg day^{-1} of milk. However, the AC for organic sources of supplemental Mn does not provide and generally assumed equal to inorganic sources.

In recent years, large-scale livestock farms have given rise to environmental concerns, since the excess mineral concentrations in manure lead to mineral depositions that exceed crop requirement (Benke *et al*., 2008; Leeson and Caston, 2008; Wang *et al*., 2008). A growing awareness of environmental impact caused by undigested mineral compounds has led to numerous research

studies examining viable alternatives of more bioavailability sources in animal feed industry. The use of organic trace elements in the poultry nutrition have been suggested, due to their higher bioavailability (Cao *et al*., 2000; Leeson and Caston, 2008; Gheisari *et al*., 2011) and lower manure loading (Acda and Chae, 2002). Researches have proved that the broilers received diet with the reduced doses of trace elements in the organic forms has the growth performance (Nollet *et al*., 2007, 2008).

During the last decades, the diets for cows have been routinely supplemented with manganese (Mn) in the form of inorganic salts to avoid mineral deficiency which is very common in dairy industry (Hall *et al*., 2007). There is little information regarding the effect of the level or source of Mn on lactation performance of cow. Methionine hydroxy Mn, Mn-$(HMTBA)_2$, produced by Novus International Inc., the organic source of Mn used in the current study, is composed of two molecules of 2-hydroxy-4-methythio butanoic acid (HMTBA) chelated with one molecule of Mn. Mn-$(HMTBA)_2$ was reported to have greater bioavailability than inorganic forms of Mn (Yan and Waldroup, 2006). The first objective of this experiment was to determine whether the lactation and reproduction performance and the nutrient digestibility of cows were level influenced by dietary source Mn-$(HMTBA)_2$ (or Mn sulfate) of Mn supplied in the NRC (2001) recommended dose (14 ppm). The second objective was to use data from blood analysis to explain how those influences happen in the hope of being helpful for the future researches of organic Mn on dairy cows and other ruminants.

MATERIALS AND METHODS

Animals, diets and experimental design: The study was conducted from July 20, 2008 to April 12, 2009 at Hutubi dairy farm in Xinjiang (northwest of China). The cows selected were cared for in accordance with the practices outlined in the Guide for the Care and Use of Agriculture Animals in Agriculture Research and Teaching (FASS, 2010). Ten days before the start of the experiment, 30 clinically healthy Holstein dairy cows (DIM (days in milk) = 45±26; milk yield = 32.97±2.00 kg; parities = 1-6 and BW = 597.62±64.19 kg) were allocated to 10 blocks of three cows and assigned to one of three treatments using the randomized block design according to milk yield, lactation days, parities and body weights to ensure no statistically significant differences between groups. Treatments were: (1) Mn sulfate only (S): 14 ppm dietary Mn provided by $MnSO_4$; (2)Mn sulfate plus methionine hydroxyl manganese (SM): 7 ppm dietary Mn provided by $MnSO_4$ and 7 ppm by Mn-$(HMTBA)_2$; (3) methionine hydroxyl manganese only (M): 14 ppm dietary Mn provided by Mn-$(HMTBA)_2$. The level of diet Mn was determined according to the NRC (2001) requirement. The trial lasted for 120 days, including 20 days for adaptation. Milk components were determined before the start of formal experimental period. No significant differences were found between treatments and no adjustment was conducted. Foot and mouth disease vaccine (3 mL per cow; concentrated, Chinese Academy of Agricultural Sciences, Lan Zhou Veterinary Research Institute) was given at day 107 (DIM) during the trail.

Cows in the experiment were component fed, and the Cu supplement was included in the compound premixes (1% of concentrate). Six feeders (5 cows per feeder) were responsible for the premix daily feeding. The compound premix was pre-weighted for each cow three times a day, immediately after the basic concentrate delivered to cow, the premix was added on top and general stirred into concentrate every time. During the adaptation period, the premixes were prepared using control premix (premix of S) and treatment premix (premix of S, SM or M), by replacing control premix with corresponding treatment, and the replacing rate was increased by 10% per day

Table 1: Ingredients and composition of the experimental diets (% DM basis)

	Treatment[1]		
Item	S	SM	M
Feed ingredients ([2]%)			
Corn silage	24.60	24.60	24.60
brewers grain	4.90	4.90	4.90
Alfalfa hay	17.70	17.70	17.70
Concentrate without premix[3]	52.80	52.80	52.80
Total	100.00	100.00	100.00
Composition			
Crude protein	15.90	15.90	15.90
Neutral detergent fiber	40.20	40.20	40.20
Acid detergent fiber	23.60	23.60	23.60
Organic matter	91.60	91.60	91.60
Ether extract	6.23	6.23	6.23
Calcium	0.79	0.79	0.79
Magnesium	0.45	0.45	0.45
Phosphorns	0.44	0.44	0.44
Potassium	1.20	1.20	1.20
Sodium	0.37	0.37	0.37
Chlorine	0.41	0.41	0.41
Manganese from $MnSO_4 \cdot 4H_2O$ (mg kg^{-1})	14.00	7.00	-
Manganese from Mn-$(HMTBA)_2$[4] (mg kg^{-1})	-	7.00	14.00
Basal Mn levels ([5]mg kg^{-1})	29.11	29.11	29.11
Iron (mg kg^{-1})	214.44	214.44	214.44
Zinc (mg kg^{-1})	64.41	64.41	64.41
Copper (mg kg^{-1})	21.78	21.78	21.78
Molybdenum (mg kg^{-1})	2.38	2.38	2.38
NE_L[6] (MJ kg^{-1})	6.78	6.78	6.78

[1]S: 14 ppm Mn in concentrate supplied by $MnSO_4$; SM: 7 ppm Mn supplied by $MnSO_4$ and another 7 ppm by Mn-$(HMTBA)_2$; M: 14 ppm Mn supplied by Mn-$(HMTBA)_2$. Mineral-vitamin mixtures (1% of concentrate) of S, SM and M were offered to cows according to treatments allocation.[2]Defined based on the consecutive-three-days intake of all cows determined in the adaptation period. [3]Contained per kilogram of concentrate without mix: 48% Corn grain, 16% Wheat bran, 12% Cottonseed meal, 6% Cottonseed protein, 4% Beet meal, 1% Sunflower mea l (exp.), 3.4% Sunflower meal (sol.), 3% Grape seed meal, 2% Plastered Starch Urea, 1.5% limestone, 1.2% Calcium phosphate, 1% Premix, 0.1% Detoxifier, 0.8% Salt. [4]Mintrex Mn, (Mn-$(HMTBA)_2$, Novns International Inc.) is composed of two molecules of 2-hydroxy-4-Methythio Butanoic Acid (HMTBA) chelated with one molecule of Mn. [5]From corn silage, brewers dried grain, alfalfa hay and concentrate. [6]NRC (2001) was used to calculate nutrient composition

as a transition, gradually increased to the model of experimental design at d 10. All ingredients including corn silage, alfalfa, brewers (a by-product from beer industry) and concentrate were weighted basing on the daily dry matter intake requirement of the experimental cows, and component fed three times a day (0400, 1100 and 1700), in the order of corn silage and part of alfalfa, brewers and concentrate, at last alfalfa was finally offered to guarantee the forage intake of cows. For example, in this experiment, 8 kg corn silage and 0.6 kg alfalfa, 2 kg brewers and 4 kg concentrate, and then 1.5 kg alfalfa were delivered to every cow in the morning, and 6 kg corn silage and 0.2 kg alfalfa, 1.5 kg brewers and 3 kg concentrate, and then 1 kg alfalfa were supplied to every cow in the noon and afternoon. Cows were access to water and open lot without bricks. Mn-(HMTBA)2 was supplied by the Novus International Trade Company. Ingredients and nutrient levels of diets were shown in Table 1.

Data and samples collection

Temperature and humidity index calculation (THI): Environmental temperature and humidity were monitored by thermometers hanging 1.5 m above the ground in the stadium and the average THI was calculated using the dry and wet bulb temperatures recorded at 07:00, 13:00 and 20:00 daily according to the following equation:

$$THI = 0.72 (Td+Tw)+40.06$$

where, Td is dry bulb temperature and Tw is wet bulb temperature.

Milk yield monitoring and milk sample collection: Individual milk yields (three milkings per day) were recorded every ten days apart using milk-sampling devices (Waikato Milking Systems NZ Ltd., Waikato, Hamilton, New Zealand). Milk samples (two battles in total of 100 mL) from all cows were collected, fresh raw milk samples for milk composition analysis and other tests were stored at -20°C for later analysis.

Reproduction performance: Calving records were reviewed and cows with uterine infection or retention were excluded from selection. Uterus recovery in each cow was confirmed during the adaptation stage. Cows were visually observed for estrus twice daily (06:00 and 16:00). B-ultrasonic (50 s Tringa Vet, PIE MEDICAL, Netherlands) examination of all cows were conducted at the end of the trial, including examination of the ovaries and determination of the numbers and diameters of folhcles and luteums. The health status of the cows was recorded by an experienced veterinarian.

Dry Matter Intake (DMI) estimate and digestibility testing: Feed (alfalfa hay) was offered to ensure approximately 10% orts. To determine DMI (distinguish DMI from the aforementioned DIM), diets offered to and refused by individual cows were weighed monthly for three consecutive days. Dietary samples were collected weekly. Five cows per treatment with similar feed intakes and milk yields were selected for five consecutive days of digestibility testing (from day 155 to 160) and the operation process referencing to Cao *et al.* (2008, 2009).

Serum preparation and analysis: Ten milliliters of jugular blood were taken from all cows before fasting (before 04:00) and h 1 (06:00), 2 (07:00) and 4 (09:00) after first feeding per 30 days with blood collection tubes (Vacuum Tube, Becton-Dickinson, USA). Before sampling, the skin was washed with Milli-Q-water (Milli-Q, Milli Corp., Bedford, MA., USA). The tubes were centrifuged at 3,500×g for 15 min to obtain the serum which was stored at -80°C in an ultra low temperature freezer (DW-86L286, Haier, China) in several fractions until further analysis of serum biochemical parameters.

Analytical procedures

Nutritional evaluation: Feed and feces samples were determined for DM at 65°C for 48 h in a forced air oven (Model, 2000; Experimental Mill, Beijing, China) and Organic Matter (OM) (method 942.05 (AOAC, 2000). Crude Protein (CP) was determined using automated nitrogen analyzer (Rapid Nlll, Elementar, DE). Neutral Detergent Fibre (NDF) was mearsured by means of Van Soest *et al.* (1991) using heat-stable α-amylase (#A-3306; Sigma Chemical Co., St. Louis, MO) and sodium sulfite and corrected ash concentration adapted for the Ankom 200 fiber analyzer

(Ankom Technology, Fairport, NY). The feed samples were also analyzed for Acid Detergent Fibre (ADF) (method 973.18 c) (AOAC, 2002), Ether Extract (EE) (method 920.39) Calcium (AOAC, 2000), (Ca) and Phosphorus (P) by atomic absorption spectrometry (method 945.46) (AOAC, 2000).

Milk composition: Milk composition (protein, fat, lactose and SNF) in fresh milk of all cows was determined with near infrared milk ingredients analyzer (MIRIS DMA, Sweden). A subsample of the milk was deproteinized (Ekinci and Broderick, 1997) and analyzed for MUN by colorimetric assay.

Blood biomarkers: Blood metabolites and parameters, such as Total Protein (TP), Albumin (ALB), Serum Urea Nitrogen (SUN), Total Cholesterol (TC), High-density Lipoprotein cholesterol (HDL), Low-density Lipoprotein cholesterol (LDL), Triglyceride (TG) and potassium (K) of serum were analyzed at 37°C by using a chinical auto-analyzer (c8000, Abbott Laboratories, USA). The contents or activities of Malondialdehyde (MDA), Total Superoxide Dismutase (T-SOD), Cu-Zn-superoxide dismutase (Cu-Zn SOD), Mn-superoxide dismutase (Mn-SOD) and glutathione peroxidase (GSH-Px) in serum were detected by use of standard procedures and commercial kits (Nanking Jiancheng Biology Research Institute).

Serum Mn concentrations: Graphite furnace atomic absorption spectrometry (AA-6300, Shimadzu) were used in determination of serum manganese with wavelength 297.5 nm, spectral passband 0.2 nm, wide hollow cathode lamp current 3.0 mA, narrow hollow cathode lamp current 3.9 mA and pure argon gas as protection. Drying current (temperature)/time = 40 A (100°C)/25 s, ash current (temperature)/time = 80A (200°C)/10 s, atomization current (temperature)/time = 410A (2400°C)/2 s, Clear current (temperature)/time = 450 A (2600°C)/3 s, injection volume = 10 μL. The linear relationship is 0.998, the linear range 0.1-40 μg L^{-1}, detection limit 1.58×10^{-11} g, recovery 93.7-101.5% and RSD 0.41%.

Statistical analysis: Data were subjected to covariate analysis using the SAS Institute (2004). Pretreatment measurements were included in the model as a covariate. When measurements were taken over time, repeated measurement data were analyzed using the MIXED procedure of SAS. The model used to analyze the data was:

$$Y_{ijk} = \mu+trt_i+cow_j\ (trt_i)+period_k+trt_i\times period_k+E_{ijk}$$

where, Y_{ijk} is dependent variable; μ is overall mean; trt_i is fixed effect of the ith treatment, i = 1, 2, 3 and cow_j (trt_i) is random effect of the jth cow within the ith treatment, j =1,... 10; $period_k$ = fixed effect of sample collection/measurement period, k = 1,... 12 for milk yield measurements, k = 1,... 4 for milk composition and blood analysis. $trt_i\times period_k$ = fixed effect of the interaction between the ith treatment and the kth period and E_{ijk} is random residual ~$N(0,\ \delta_e^2)$.

For non-repeated measures of reproduction, cow was used as the experimental unit and the model was as follows:

$$Y_{ij} = \mu+trt_i+cow_j+trt_i\times cow_j+E_{ij}$$

where, Y_{ijk} is dependent variable; μ is overall mean; trt_i is fixed effect of the ith treatment, i = 1, 2, 3 and cow_j is random effect of the jth cow, j = 1,... 10; $trt_i\times cow_j$ = fixed effect of the interaction between the ith treatment and the jth cow and E_{ijk} is random residual ~ $N(0,\ \delta_e^2)$.

For both repeated and non-repeated measures, least squares means were calculated and differences between treatments were detected with Tukey's adjustment. Significant differences were declared at $p<0.05$ and trends effected were noted at $0.05<p\leq0.10$, when $p\leq0.10$ treatment differences were explored.

Survival analysis was conducted on "time to event" reproductive parameters, such as days to first estrus and days to first service using survival analysis. The χ^2 log-rank test was used to test treatment effects.

RESULTS

Feed intake and lactation performance: Neither treatment nor period significantly affected DMI ($p>0.05$). The average milk production of all diets gained a decreasing trend in M-type (Fig. 1). The milk yield tended to increase during day 45 to 75 and day 135 to 145 but decreased during day 85 to 125 and day 145 to 165, with the S most greatly decreased. The average milk yield of all thirty cows during the experiment was 30.37 kg day^{-1}. The average milk yield of S (28.79 kg) was lower than the M (31.10 kg day^{-1}) and the SM (31.21 kg day^{-1}). The average milk yield of SM and M increased by 2.24 and 2.31 kg, respectively, compared with S but the effect of treatment was not significant ($p>0.10$). The average 4% FCM yield and fat yield of cows supplied with M dietary (28.01, 1.12 kg day^{-1}) were higher than the cows fed with SM (26.96, 1.08 kg day^{-1}) and the S diet (26.08 kg, 1.04 kg day^{-1}) ($p<0.05$) and the average lactose rate of SM (4.32%) was significantly lower than M (4.46%) and S (4.48%) while there was no effect of treatment on average fat and protein rates and protein yields, content of MUN and non-fat solids and lactose yields ($p>0.05$) (Table 2).

Reproduction and ovarian function: Reproductive performance was shown in Table 3. There was no significant effect of treatment on day to first estrus and service and the estrous cycle length. First service conception rate was not significantly different among treatments ($p>0.10$). The average detectable follicle numbers and ovarian score for cows of M was generally lower than those cows of SM and S but no significant difference was observed ($p>0.10$).

Blood analysis: Except for serum GLB, A/G, HDL-C and Mn concentration, most serum parameters were not influenced by treatments. The levels of serum MAD and K in group S were numerically highest among groups, while the concentration of serum ALB, SUM, GLU, TC, HDL-C,

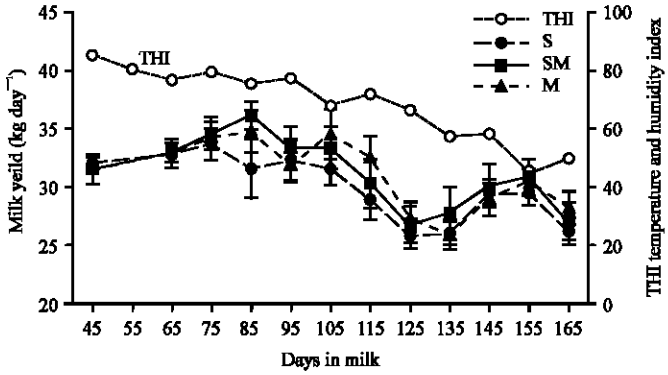

Fig. 1: The trends of average milk yield during the trial period. S (●; n = 10) = 14 ppm Mn in concentrate supplied by $MnSO_4$; SM (■; n = 10) = 7 ppm Mn supplied by $MnSO_4$ and another 7 ppm by Mn-$(HMTBA)_2$; M (▲; n = 10) = 14 ppm Mn supplied by Mn-$(HMTBA)_2$

Table 2: Effects of $(HMTBA)_2$-Mn on the milk composition of cows

	Treaments[1]				
Item	S	SM	M	SEM	p-value
Dry matter intake (kg day^{-1})	19.24	20.29	19.92	0.12	0.19
Milk yield (kg day^{-1})	28.79	31.21	31.10	0.89	0.31
Fat (%)	3.81	3.77	3.80	0.04	0.68
Protein (%)	3.34	3.30	3.33	0.03	0.68
Lactose (%)	4.48[b]	4.32[a]	4.46[b]	0.03	0.05
Non-fat solids (%)	8.63	8.69	8.69	0.05	0.40
Fat yield (kg day^{-1})	1.04[a]	1.08[a]	1.12[b]	0.02	0.06
Protein yield (kg day^{-1})	0.92	0.97	0.98	0.03	0.26
Lactose yield (kg day^{-1})	1.23	1.26	1.31	0.04	0.31
Urea nitrogen (mg dL^{-1})	18.39	18.02	16.66	0.72	0.22
4% fat corrected milk (kg day^{-1})	26.08[a]	26.96[a]	28.01[b]	1.01	0.05

Means within the same row with different superscripts differ ($p<0.05$) by Tukey-Kramer test. [1]S: 14 ppm Mn in concentrate supplied by $MnSO_4$; SM: 7 ppm Mn supplied by $MnSO_4$ and another 7 ppm by $(HMTBA)_2$-Mn; M: 14 ppm Mn supplied by $(HMTBA)_2$-Mn

Table 3: Effect of $(HMTBA)_2$-Mn on reprodnction and the ovary condition

	Treatment[1]				
Item	S	SM	M	SEM	p-value
Reproductiou					
First estrus (day)	56.00	57.00	62.00	1.10	0.23
First service (day)	64.00	69.00	69.00	1.20	0.14
Estrous cycle length (day^2)	24.60	24.40	25.40	1.16	0.29
First service conception rate (%)	40.00	30.00	20.00	0.96	0.15
Number of follicles					
Small (3-5 mm)	3.70	2.10	2.80	0.78	0.67
Medium (6-9 mm)	2.10	2.20	1.60	0.78	0.64
Large (>9 mm)	1.40	1.20	1.10	0.45	0.78
DDF (^{3}mm)	18.20	18.70	16.90	1.12	0.62
OCS[4]	3.90	4.09	2.10	0.51	0.61

Means within a row with different superscripts differ ($p\leq0.05$). [1]S: 14 ppm Mn in concentrate supplied by $MnSO_4$; SM: 7 ppm Mn supplied by $MnSO_4$ and another 7 ppm by $(HMTBA)_2$-Mn; M: 14 ppm Mn supplied by $(HMTBA)_2$-Mn. [2]Base on all estrous during the experimental period. [3]DDF: Diameter of dominant follicles, DCL: Diameter of the corpus luteum, OCS: Ovarian condition score. [4]Using a scale of 0: Both ovaries static, 1: Ovarian cyst or unilateral ovarian static durable luteinizing; 2: No luteal and dominant follicles; 3: No ovarian follicular development normal; 4: The fetus is not visible; 5: The fetus can be seen

GSH-PX, T-SOD, Mn-SOD, GPT, AKP, LDH and Ca and the A/G and GOT/GPT of cows for group S were the lowest. Significant increases was observed for serum HDL-C concentration of M compared with S and SM ($p<0.05$). The A/G of SM and M were significantly higher than that of S ($p<0.05$). The contents of serum GLB and P for cows of SM were significantly higher than those of cows in S and M ($p<0.05$). According to the comprehensive analysis of serum at fasting and h 1, 2 and 4 after first feeding, the serum manganese concentration for cows of M was significantly higher than those of S and SM ($p = 0.05$) (Table 4).

Nutrient digestibility: Replacing 50 and 100% manganese sulfate with Mn-$(HMTBA)_2$ had no influence on the digestibility of dietary OM, CP, EE, NDF and ADF of cow ($p>0.05$). Numerically, the apparent digestion rates of OM, CP, EE, NDF and ADF of cow fed S diet were the lowest (Table 5).

Table 4: Effects of $(HMTBA)_2$-Mn on serum enzyme activities and metabolites

	Treaments[1]				
Item	S	SM	M	SEM	p-value
TP (g L^{-1})	49.68	58.03	47.07	1.41	0.43
ALB (g L^{-1})	22.50	27.99	23.69	0.56	0.35
GLB (g L^{-1})	27.17^{b}	30.04^{c}	23.42^{a}	0.92	0.09
A/G	0.89^{a}	1.02^{b}	1.05^{b}	0.03	0.06
SUN (mmol L^{-1})	5.93	6.83	6.45	6.44	0.10
GLU (mmol L^{-1})	0.69	0.72	0.76	0.06	0.69
TC (mmol L^{-1})	4.14	4.46	4.45	0.12	0.33
HDL-C (mmol L^{-1})	1.94^{a}	2.06^{a}	2.22^{b}	0.06	0.06
LDL-C (mmol L^{-1})	2.16	2.25	2.35	0.08	0.40
TG (mmol L^{-1})	0.07	0.08	0.07	0.00	0.35
GSH-PX (U L^{-1})	310.43	333.33	331.97	4.32	0.67
T-SOD (U L^{-1})	141.52	169.11	171.18	7.62	0.73
Cu-Zn SOD (U L^{-1})	101.21	113.29	101.55	2.11	0.69
Mn-SOD (U L^{-1})	37.42	46.29	56.21	4.99	0.18
GPT (U L^{-1})	22.27	28.23	23.75	0.84	0.46
GOT (U L^{-1})	44.06	50.70	41.85	1.32	0.48
GOT/GPT	2.38	1.86	1.79	0.09	0.77
AKP (U L^{-1})	26.73	36.70	29.63	0.99	0.20
LDH (U L^{-1})	606.79	696.28	604.18	15.89	0.14
MAD (U L^{-1})	8.67	7.99	7. 23	1.11	0.19
Potassium (mmol L^{-1})	5.34	4.88	4.67	0.18	0.16
Sodium (mmol L^{-1})	114.21	128.14	113.75	1.81	0.91
Calcium (mmol L^{-1})	1.52	1.99	1.68	0.06	0.12
Manganese (μg L^{-1})	17.82^{a}	19.36^{b}	20.75^{c}	0.33	0.08
Phosphorus (mmol L^{-1})	1.50^{a}	1.77^{b}	1.61^{a}	0.04	0.09

Means within a row with different superscripts differ ($p \leq 0.05$). [1]S: 14 ppm Mn in concentrate supplied by $MnSO_4$; SM: 7 ppm Zn in concentrate supplied by $MnSO_4$ and another 7 ppm by Mn-$(HMTBA)_2$, M: 14 ppm Mn supplied by Mn-$(HMTBA)_2$. [2]MDA: Malondialdehyde, GSH-PX: Glutathione-Peroxidase, T-SOD: Total superoxide dismutase, Cu-Zn SOD: Cu-Zn-superoxide dismutase, GPT: Glutamic-pyruvic transaminase, GOT: Glutamic-oxaloacetic transaminase, AKP: Alkaline phosphatase, LDH: Lactate dehydrogenase, TP: Total protein, ALB: Albumin, SUN: Serum urea nitrogen, TC: Total cholesterol, HDL: High-density lipoprotein cholesterol, LDL: Low-density lipoprotein cholesterol, TG: Triglyceride, Mn-SoD: Mn-Superoxide dismutase

Table 5: Effects of $(HMTBA)_2$-Mn on apparent nutrient digestibility

	Treatment[1]				
Item,% DM	S	SM	M	SEM	p-value
OM	62.20	64.31	64.10	1.45	0.79
CP	63.73	63.15	62.56	0.62	0.91
EE	50.40	52.09	52.78	0.88	0.52
NDF	49.13	50.43	51.11	1.97	0.83
ADF	44.01	46.99	46.47	1.04	0.52

[1]S = 14 ppm Mn in concentrate supplied by $MnSO_4$; SM = 7 ppm Zn supplied by $MnSO_4$ and another 7 ppm by $(HMTBA)_2$-Mn; M = 14 ppm Mn supplied by $(HMTBA)_2$-Mn. OM: Organic matters, CP: Crude protein, EE: Ether extract, NDF: Neutral detergent fiber, ADF: Acid detergent fiber

DISCUSSION

Experimental design: Many previous studies on trace elements involved more than two elements combination (Nocek *et al.*, 2006; Sharman *et al.*, 2008; Siciliano-Jones *et al.*, 2008) or several supplemental levels of different sources (Virden *et al.*, 2004; Nocek *et al.*, 2006). In this study, only the source effect of Mn was considered. All treatments received 29.11 ppm of Mn in the basal diet and 14 ppm in mineral-vitamin premixe. In this way, we avoid the possible system error introduced by the variance of absorption rate introduced by dietary Mn levels and other possible antagonists. What is worth mentioning of the design, we can estimate the interaction effect of $(HMTBA)_2$-Mn and Mn sulfate without sedulous statistical analysis. For example, if SM effects better than M and S, then synergy exist.

Lactation performance

Milk production: In this study, the average daily milk yield in cows received Mn as Mn-$(HMTBA)_2$ (SM and M) were higher than those received Mn as M_nSO_4 (S). Previous researches suggested that the main role of Mn played was involved in reproductive performance (Bentley and Phillips, 1951; Rojas *et al.*, 1965; De Carvalho *et al.*, 2010) and bone development in cows. In this reason, there is little information regarding the effect of the level or source of Mn on lactation performance of cows. Data comparing organic and inorganic sources of supplemental Mn are also lack for lactation cows. If any, the dietary Mn was generally studied in the model of mingling with the compound of other minerals. We failed in searching for comparable results in previous study; nevertheless, we tried to lay out a logical explanation through a series of related research. Weiss and Socha (2005) concluded that about 1.6 times higher than NRC (2001) estimated Mn was needed for lactation cows to meet inevitable fecal losses. All cows received 14 ppm Mn, just 1 time requirement level of NRC (2001) in our study. In another words, the dietary Mn can only ensure the basic health of the cows in our study but still cannot meet the needs for maximizing the lactation performance. An explanation for the increased milk yield is that the bioavailability of Mn from organic was better than inorganic sources. This has been repeatedly confirmed in chickens (Henry *et al.*, 1989, 1992; Smith *et al.*, 1995; Yan and Waldroup, 2006). On the other hand, bioavailability of trace minerals is not static and is affected by stress (Nockels *et al.*, 1993; Scaletti *et al.*, 2003). Based on the theory of Ravagnolo and Misztal (2000) that cows will encounter the challenge of heat stress when the THI become above 72, cows were in the heat-stress state during day 45 to 115. We hypothesized that heat stress highlighted the bioavailability advantage of $(HMTBA)_2$-Mn in our study. Accordingly, it is feasible to replace Mn sulfate with $(HMTBA)_2$-Mn to avoid possible loss when cows challenged by heat-stress.

Milk compositions: Although, improvements in milk yield and components have been reported previously, in general, milk composition did not appear to be affected by source of trace mineral supplementation (Ballantine *et al.*, 2002; Kellogg *et al.*, 2003). However, Kincaid and Socha (2004) reported greater milk protein content for cows supplement with AA complexes of Zn, Mn and Cu. In our study, the percentage of milk fat, milk protein, MUN and non-fat solids of milk were not affected by the source of dairy Mn. Unlikely, improvements were observed in the yields of 4% FCM and milk fat for cows fed with Mn-$(HMTBA)_2$ (SM and M) compared with cows in control (S). Three nutrition modes were reported affecting the milk fat synthesis (1) change the rumen fermentation type; (2) effect on the fat mobilization through insulin-glucose pathway; (3) effect of the fat acids and isomers on milk fat synthesis. Manganese happened to be involved in all the three modes

(Arelovich *et al.*, 2000; Lu *et al.*, 2007). As above, the supplement of Mn in organic forms before or during the peak-lactation period may retard the drop of milk fat resulting from the increase of milk production and profit raisers in the sell of raw milk talking payment on quality grade.

Reproduction and ovarian function: Many experiments observed OTM effects on fertility, either in addition to inorganic minerals or in substitution for inorganic minerals. Cows fed OMT had fewer days to first estrus (Campbell *et al.*, 1999; Rojas *et al.*, 1965) fewer days to conception (Ballantine *et al.*, 2002; Kellogg *et al.*, 2003) and increased percentage of cows pregnant (Rojas *et al.*, 1965). Also, there were experiments showed that the addition of organic trace elements has little or even effect on the reproductive performance (Ballantine *et al.*, 2002). Toni *et al.* (2007) observed minimal effects of replacing inorganic minerals with OTM on fertility. Ballantine *et al.* (2002) have reported no significant results were found on time to first ovulation or conception rate to first service even though numerical improvement for both parameters was observed when replacing inorganic sulfates of Cu, Zn or Mn and Co with OTM. Whether the results were positive or not, they generally deal with the mixtures of trace elements in dry period or around parturition of cows. Unlikely, we conducted the experiment from 45 to 165 day in milk, in order to investigate the effects of Mn-$(HMTBA)_2$ on dairy cow during peak-and mid-lactation stage. The time arrangement was very embarrassing for reproductive performance detection basing on the report of Hall *et al.* (2007). They did not evaluate reproductive performance as part of their study and believed that an 8 week feeding study starting 30 to 110 days post-partum was not appropriate for evaluating reproductive performance. We agreed partially but not completely with them. We also consider that was unscientific to evaluate reproductive performance, such as days to first estrus, services per conception and days to conception during the mid-lactation period of cows. However, we believed it is both practical and logical to evaluate follicular development in this period, as the follicular development was regulated by neural and hormonal fluctuations. Unlike previous reports, the fertility performance and follicular development was hampered slightly instead of enhanced in our study. In addition, increased persistent corpus luteum and ovarian cysts (4/10 persistent corpus luteum and 2/10 ovarian cysts) were also observed in cows of M group. The total dietary Mn in was only 43.11 ppm (supplied + the basic) in this study and obviously did not exceed the tolerance dose. We now have not grasp the interpretation of this result yet. We inferred that the sources of dietary Mn effected on the follicular development through the regulating of blood Mn concentration, nerve and hormone. Mn is necessary for cholesterol synthesis which is requires for synthesis of the estrogen, steroids, progesterone and testosterone (Keen and Zidenberg-Cherr, 1990). What is more, the corpus luteum has a high Mn content and may be affected by the dietary manganese level (Brown and Casillas, 1986). Perhaps, the progesterone (P4) secretion from fresh corpora luteum were greatly promoted for cows of M (almost of the cows have just ovulated when the formal experiment started). Therefore, the luteolysis was inhibited, the incidences of durable luteinizing, ovarian cyst and follicle atresia tend to increase and follicular development was hampered. We did not collect the data of serum hormones, to avoid the effect of intensive-blood-sampling stress (intensive samplings were necessary for cows in estrus) on evaluation of lactation performance. If conditions permit, hormones and trace minerals concentrations in blood and follicular fluid should be evaluated in the further studies and more detailed outline of the regulatory mechanism may be formed during the comprehensive analysis of the data. Accordingly, feedstuffs should pay attention to the supplemental level and period of Mn-$(HMTBA)_2$. Only appropriate level during the right period will be payment. For the whole colony of lactating cows, No more than 0.5 time of NRC

(2001) requirement of Mn in form of Mn-$(HMTBA)_2$ was recommended in the process of extensions. Perhaps the Mn-$(HMTBA)_2$ supplemental proportion could be raised in the period of gestation, especially in dry period and before parturition.

Blood analysis: Since Mn collaborative with choline and biotin, it was involved in fat metabolism, also affect the lipid metabolism (Lu *et al*., 2007). Many studies have shown that manganese has a specific anti-fatty liver function and can promote fat utilization and prevent the occurrence of liver fatty degeneration (Curran and Azarnoff, 1961; Kawano *et al*., 1987; Davis *et al*., 1990). The lack of manganese can cause disorder of lipid metabolism in rats. However, In this study, The contents of TC, HDL-C, LDL-C and TG of SM and M group were not only did not reduce but increase to some extent compared with S group but the difference among the three groups were not significant. Other researchers have reported similar results previously. Hansen *et al*. (2006) reported that there the cow serum cholesterol were no significantly effected by dietary manganese in cow. Lassiter and Morton (1968) reported that serum cholesterol did not differ significantly on sheep after 16 weeks fed with manganese content 0.8 or 29.9 mg kg^{-1}.

Many reports indicate that blood manganese content is not sensitive to the dietary manganese level change. Weiss and Socha (2005) reported that there was no significant difference on whole blood manganese from cows feeding Mn 43 or 200 mg kg^{-1}. Bentley and Phillips (1951) reported that the whole blood manganese from cows fed with manganese 7-10 or 30 mg kg^{-1} diet up to 3 years did not differ significantly. Hansen *et al*. (2009) showed that dietary manganese concentration has no effect on serum manganese levels. It suggested the existence of manganese concentration self-balancing mechanism in cow, even in the long-term dietary manganese concentration fluctuations, it still able to maintain the blood manganese concentration not affected. In this study, the results were in conflict with this theory, mainly because that previous experiments were all testing the manganese concentration at fasting. Unlikely, four time points (04:00, 06:00, 07:00 and 09:00) in this test were monitored. In addition, there was also no significant difference between the groups at fasting, similarly to the previous reports.

CONCLUSION

The present study suggested that replacing a portion of dietary manganese sulfate with methionine hydroxy manganese during the peak-and mid-lactation periods will improve the lipid metabolism and milk fat secretion and ensure the profits of dairy farmers. However, the follicular development may be hampered for post-partum cows with dietary Mn totally supplemented as Mn-$(HMTBA)_2$.

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