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**The role of zinc chelate of hydroxy analogue of methionine in cadmium toxicity: effects on cadmium absorption on intestinal health in piglets**

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Shot title: The role of zinc on cadmium toxicity in piglets

**Supplementary Material S1** The procedure of real-time PCR

A total volume of 10 μL assay solution was prepared for real-time PCR, which contained: 1 μL complementary DNA template, 5 μL SYBR Green mix, 0.2 μL Rox, 3 μL deionized water, and 0.4 μmol/L each of forward and reverse primers.

The following protocol was performed:

(i) pre-denaturation (10 s at 95 °C); (ii) amplification and quantification, repeated 40 cycles (5 s at 95 °C, 20 s at 60 °C); (iii) melting curve construction (60–99 °C with heating rate of 0.1 °C per second and fluorescence measurements). The relative level of a target gene was expressed as a ratio of the target gene to the control gene using the formula 2−(ΔΔCt), whereΔΔCt = (CtTarget − Ctβ-actin)treatment − (CtTarget − Ct β-actin)control. The relative expression of target genes in the control group was set to be 1.0.



**Supplementary Figure S1.** The molecular structure of zinc chelate of hydroxy analogue of methionine fed to piglets.

**Supplementary Table S1** *Concentration of Cd and Zn in diets (mg/kg) and drinking water for piglets (mg/L) (mean ± SEM, n=5).*

|  |  |  |
| --- | --- | --- |
| Item | Cd | Zn |
| Con | 0.18±0.01 | 150.34±9.85 |
| Cd  | 29.24±2.03 | 153.43±6.19 |
| Cd+100 mg Zn/kg | 32.91±2.91 | 256.59±6.67 |
| Cd+200 mg Zn/kg | 31.87±3.47 | 360.92±9.80 |
| Drinking water | ND | 0.05±0.01 |

ND = Not detected; Cd = cadmium; Zn = zinc; Zn-HMTB = zinc chelate of hydroxy analogue of methionine

The dietary treatments included a basal control diet (CON) and three diets with 30 mg Cd per kg from CdCl2 and 0 (Cd), 100 (Cd + 100 mg Zn/kg) and 200 (Cd + 200 mg Zn /kg) mg Zn from Zn-HMTB per kg of diet, respectively

**Supplementary Table S2** *The precision of the analytical method for the metal element determination in tissues and feces of piglets. (%, n=5)*

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Item | Cd | Zn | Mg | Fe | Cu |
| Stomach | 13.03 | 4.03 | 3.27 | 4.25 | 8.43 |
| Jejunum | 13.80 | 4.29 | 2.00 | 3.71 | 13.78 |
| Ileum | 7.22 | 6.26 | 6.84 | 3.67 | 8.14 |
| Cecum | 14.62 | 7.92 | 5.64 | 5.18 | 8.93 |
| Colon | 14.52 | 4.04 | 7.94 | 8.95 | 11.42 |
| Feces | 11.24 | 8.74 | 7.35 | 12.57 | 10.18 |
| Liver | 10.10 | 5.11 | 0.12 | 5.96 | 9.51 |
| Kidney | 4.45 | 5.15 | 3.40 | 8.29 | 12.84 |
| Heart | 19.08 | 5.13 | 4.60 | 13.00 | 15.34 |
| Spleen | 12.44 | 8.65 | 4.01 | 9.27 | 22.92 |
| Lung | 17.12 | 3.70 | 4.38 | 7.51 | 13.66 |
| Muscle | 20.72 | 4.56 | 7.94 | 9.69 | 17.11 |

Cd = cadmium; Zn = zinc; Mg = magnesium; Fe = iron; Cu = copper

**Supplementary Table S3** *Primers used for real-time PCR*

|  |  |
| --- | --- |
| Gene | Sequence 5’-3’  |
| *DMT1* | F: CGCGCTTCGCCCGAGTGAT |
|  | R: TGGAAGACGGCCACCAGCAGA |
| *FPN1* | F: TGTGGCAATGGGTCACATCA |
|  | R: AGACACCCAGCCATTTATTGGA |
| *TRPV6* | F: CACTTTAGGAGAGGCTTGCTG |
|  | R: ATGACTTTATTGGAAGGTAGGAGG |
| *ZIP4* | F: TGCTGAACTTGGCATCTGGG |
|  | R: CGCCACGTAGAGAAAGAGGC |
| *ZIP8* | F: TTGCTGGAGGCATGTTCCTT |
|  | R: AGAATGGCTATGAAGCCGGT |
| *ZIP14* | F: GAACGCTGCTCTCTAATGCG |
|  | R: TGGCTGTGTCCATGATGGTG |
| *ZnT1* | F: CCAGGGGAGCAGGGAACCGA |
|  | R: TCAGCCCGTTGGAGTTGCTGC |
| *ZnT2* | F: GACAGCGCCAGCCAGCATCA |
|  | R: GGCAGCCACCAAAACGCCCA |
| *ZnT5* | F: ACCAGTCTCAGTTGGAGGGCTGA |
|  | R: TCCATGGGTATGGGTGTGGGCA |
| *LAT1* | F: GAGCAGGTGAAGCTGAAGAAGG |
|  | R: CCCAAAGACGGAGAAGAGGC |
| *LAT2* | F: ACAGGAGTGCCCGTCTATT |
|  | R: GCTCACCAGGGTCAACAAC |
| *y+LAT1* | F: CTCGGGCATCTTCGTCT |
|  | R: CCCAGTTCCGCATAACA |
| *y+LAT2* | F: CTGTGCCTATGTCAAGTGG |
|  | R: GCAGAGTAGAGGGCGAGA |
| *B0AT1* | F: AGGAACCGCCAGAGTAAC |
|  | R: CATCAGGAAGAAATAGCCAC |
| *PEPT1* | F: GAGTAAGAAGTTTCCCAAGAG |
|  | R: TTGATTTGACAGATGAGCC |
| *IL-2* | F: TGCACTAACCCTTGCACTCA |
|  | R: CAACTGTAAATCCAGCAGCAA |
| *IL-4* | F: CCCGAGTGTCAAGTGGCTTA |
|  | R: TGATGATGCCGAAATAGCAG |
| *IL-6* | F: TCCAGCATCATTGCATCATC |
|  | GGCTCCACTCACTCCACAAG |
| *IL-8* | F: TGAGAAGCAACAACAACAGCA |
|  | R: CAGCACAGGAATGAGGCATA |
| *IL-10* | F: GGGCTATTTGTCCTGACTGC |
|  | R: GGGCTCCCTAGTTTCTCTTCC |
| *IL-12* | F: ATCTCGGTTGGTGTTGTTCC |
|  | R: GGGTATCTCGTCCTCTGTCC |
| *IFN-γ* | F: TTCAGCTTTGCGTGACTTTG |
|  | R: GGTCCACCATTAGGTACATCTG |
| *MCP-1* | F: CCTCATCCTCCAGCATGAAGGTCTCTGC |
|  | R: GGTGGAGTCAGGCTTCAAGGCTTCGG |
| *TGF-β1* | F: AAGCGGCAACCAAATCTATG |
|  | R: CCCGAGAGAGCAATACAGGT |
| *TNF-α* | F: ACAGGCCAGCTCCCTCTTAT |
|  | R: CCTCGCCCTCCTGAATAAAT |
| *ZO-1* | F: CCTGCTTCTCCAAAAACTCTT |
|  | R: TTCTATGGAGCTCAACACCC |
| *Claudin-1* | F: TCGACTCCTTGCTGAATCTG |
|  | R: TTACCATACCTTGCTGTGGC |
| *Occludin* | F: ACGAGCTGGAGGAAGACTGGATC |
|  | R: CCCTTAACTTGCTTCAGTCTATTG |
| *β-actin* | F: CGTTGGCTGGTTGAGAATC |
|  | R: CGGCAAGACAGAAATGACAA |

**Supplementary Table S4** *Effect of dietary supplementation with 100 and 200 mg /kg Zn from Zn-HMTB on concentrations (mg/kg) of Cd, Zn, Mg, Fe, and Cu in gastrointestinal tissue of piglets fed with 30 mg/kg Cd.*

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Iterms | Con | Cd | Cd+100 mg Zn/kg | Cd+200 mg Zn/kg | SEM | *P*-value |
| Stomach |  |  |  |  |  |  |
| Cd | 0.00c | 0.47a | 0.42a | 0.21b | 0.02 | < 0.001 |
| Zn | 15.17 | 17.06 | 15.48 | 16.70 | 0.58 | 0.109 |
| Mg | 191 | 184 | 182 | 181 | 15.8 | 0.973 |
| Fe | 9.17a | 5.85b | 3.26bc | 4.87b | 0.72 | < 0.001 |
| Cu | 0.46 | 0.98 | 0.72 | 0.53 | 0.22 | 0.494 |
| Jejunum |  |  |  |  |  |  |
| Cd | 0.11b | 8.06a | 7.14a | 8.04a | 0.50 | < 0.001 |
| Zn | 17.36b | 19.2b | 19.46b | 23.96a | 0.87 | < 0.001 |
| Mg | 170b | 165b | 184ab | 201a | 8.09 | 0.029 |
| Fe | 36.47 | 25.90 | 28.99 | 41.82 | 4.56 | 0.153 |
| Cu | 1.53 | 1.35 | 1.39 | 2.14 | 0.32 | 0.536 |
| Ileum |  |  |  |  |  |  |
| Cd | 0.02b | 2.17a | 2.22a | 0.58b | 0.33 | 0.024 |
| Zn | 14.98 | 16.13 | 15.46 | 17.01 | 0.61 | 0.435 |
| Mg | 150 | 155 | 147 | 147 | 6.11 | 0.275 |
| Fe | 10.11a | 5.73b | 5.15b | 4.47b | 1.19 | 0.168 |
| Cu | 0.44 | 0.79 | 0.62 | 0.33 | 0.18 | 0.284 |
| Cecum |  |  |  |  |  |  |
| Cd | 0.00c | 0.33a | 0.32a | 0.21b | 0.02 | < 0.001 |
| Zn | 15.59 | 14.87 | 16.43 | 15.69 | 0.91 | 0.609 |
| Mg | 124 | 124 | 128 | 123 | 6.78 | 0.834 |
| Fe | 20.25a | 13.74b | 11.89b | 26.29a | 2.92 | < 0.001 |
| Cu | 0.90 | 0.87 | 0.90 | 0.55 | 0.14 | 0.491 |
| Colon |  |  |  |  |  |  |
| Cd | 0.00c | 0.47a | 0.46a | 0.23b | 0.03 | < 0.001 |
| Zn | 12.68 | 13.06 | 13.77 | 14.26 | 0.52 | 0.201 |
| Mg | 115 | 120 | 129 | 124 | 5.30 | 0.376 |
| Fe | 15.24 | 10.73 | 10.73 | 22.43 | 2.56 | 0.052 |
| Cu | 1.40 | 1.37 | 1.56 | 1.47 | 0.11 | 0.672 |

Cd = cadmium; Zn = zinc; Mg = magnesium; Fe = iron; Cu = copper; Zn-HMTB = zinc chelate of hydroxy analogue of methionine.

The dietary treatments included a basal control diet (CON) and three diets with 30 mg Cd per kg from CdCl2 and 0 (Cd), 100 (Cd + 100 mg Zn/kg) and 200 (Cd + 200 mg Zn /kg) mg Zn from Zn-HMTB per kg of diet, respectively.

Values are mean of six replicates (n=6). abcMean values with different letters were significantly different (*P* < 0.05).