Journal of Animal Science, 2021, Vol. 99, No. 9, 1–9

https://doi.org/10.1093/jas/skab252
Advance Access publication August 27, 2021
Received: 27 January 2021 and **Accepted:** 26 August 2021
Ruminant Nutrition

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Effect of bis-glycinate bound zinc or zinc sulfate on zinc metabolism in growing lambs

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Abstract

To assess the efficacy of bis-glycinate bound Zn, 36 crossbred wethers (34 ± 2 kg) were sorted by body weight into three groups and stagger started on a Zn-deficient diet (18 mg Zn/kg dry matter [**DM**]; 22.5% neutral detergent fiber [**NDF**]) for 45 d prior to a 15-d metabolism period (10 d adaptation and 5 d collection). On day 46, lambs were randomly assigned to dietary treatments (four lambs treatment^{-1}group^{-1}): no supplemental Zn (**CON**) or 15 mg supplemental Zn/kg DM (**ZINC**) as Zn sulfate (**ZS**) or bis-glycinate (**GLY**; Plexomin Zn, Phytobiotics). Blood was collected from all lambs on days 1, 44, 56, and 61. Liver, jejunum, and longissimus dorsi samples were collected after euthanasia on day 61. Gene expression was determined via quantitative real-time polymerase chain reaction. Data were analyzed using ProcMixed of SAS (experimental unit = lamb; fixed effects = treatment, group, and breed) and contrast statements assessed the effects of supplemental Zn concentration (ZINC vs. CON) and source (GLY vs. ZS). After 15 d of Zn supplementation, plasma Zn concentrations were greater for ZINC vs. CON and GLY vs. ZS ($P \leq 0.01$); tissue Zn concentrations were unaffected ($P \geq 0.27$). Liver Cu concentrations were lesser for ZINC vs. CON ($P = 0.03$). Longissimus dorsi Mn concentrations were greater for ZINC vs. CON ($P = 0.05$) and tended to be lesser for GLY vs. ZS ($P = 0.09$). Digestibility of DM, organic matter (**OM**), and NDF was lesser for ZINC vs. CON ($P \leq 0.05$); acid detergent fiber digestibility tended to be greater for GLY vs. ZS ($P = 0.06$). Nitrogen retention (g/d) tended to be greater for GLY vs. ZS ($P = 0.10$), and N apparent absorption was lesser for ZINC vs. CON ($P = 0.02$). Zinc intake, fecal output, retention, and apparent absorption were greater for ZINC vs. CON ($P \leq 0.01$). Apparent absorption of Zn was –5.1%, 12.8%, and 15.0% for CON, ZS, and GLY, respectively. Nitrogen and Zn retention and apparent absorption were not correlated for CON ($P \geq 0.14$) but were positively correlated for ZINC (retention: $P = 0.02$, $r = 0.52$; apparent absorption: $P < 0.01$, $r = 0.73$). Intestinal expression of Zn transporter ZIP4 was lesser for ZINC vs. CON ($P = 0.02$). Liver expression of metallothionein-1 (**MT1**) tended to be greater for GLY vs. ZS ($P = 0.07$). Although Zn apparent absorption did not differ between sources ($P = 0.71$), differences in post-absorptive metabolism may be responsible for greater plasma Zn concentrations and liver MT1 expression for GLY-supplemented lambs, suggesting improved bioavailability of GLY relative to ZS.

Key words: amino acid chelate, bioavailability, cattle, sheep, trace mineral

Abbreviations

ADF	acid detergent fiber
BW	body weight
DM	dry matter
ICP-OES	inductively coupled plasma optical emission spectrometry
NDF	neutral detergent fiber
OM	organic matter
RT-qPCR	quantitative real-time polymerase chain reaction
Zn–Met	Zn–Methionine

Introduction

Zinc is critical to support animal growth through protein synthesis and bone metabolism with muscle and bone containing the majority of Zn in the body (Suttle, 2010). As feedstuffs may not contain enough Zn to meet the requirement of animal (30–32 mg Zn/kg dry matter [**DM**] for sheep and beef cattle; NRC, 2007; NASEM, 2016) or the Zn in feedstuffs is unavailable to the animal, livestock are often provided supplemental Zn. Several sources of supplemental Zn are available, including inorganic sources such as Zn sulfate ($ZnSO_4$) as well as organic sources such as amino acid chelates. A newly available amino acid chelate is bis-glycinate bound Zn (Plexomin Zn, Phytobiotics, Eltville, Germany), which consists of two equivalents of glycine bound to one equivalent of Zn. Organic Zn sources have been shown to be more bioavailable than inorganic Zn sources when supplemented to ruminants (Wright and Spears, 2004; Pal et al., 2010; Ma et al., 2020), but little research has been conducted utilizing bis-glycinate bound Zn. Unlike other trace minerals, such as Cu, tissue concentrations of Zn do not readily change in response to dietary supplementation. Thus, a widely accepted method to assess the bioavailability of supplemental Zn sources in ruminants has been to measure Zn retention in the body. Because Zn metabolism is highly conserved across species, small ruminants, such as sheep, serve as a useful experimental model for larger ruminants, such as beef cattle. The objective of the current study was to assess the bioavailability of bis-glycinate Zn compared with $ZnSO_4$ based on apparent absorption and retention of Zn by lambs. Additionally, in this study, we sought to determine the effects of supplemental Zn source on nutrient digestibility, plasma and tissue trace mineral concentrations, as well as gene expression of proteins involved in Zn transport and storage. It was hypothesized that bis-glycinate Zn would be more bioavailable than $ZnSO_4$, resulting in greater Zn retention by lambs supplemented with the organic Zn source.

Materials and Methods

Animals and experimental design

All procedures and protocols for this experiment were approved by the Iowa State University Animal Care and Use Committee (#19-223). A total of 45 weaned, crossbred wether lambs were purchased from a single source and housed at the Iowa State University Sheep Teaching Farm (Ames, IA). To accommodate room in the metabolism facility, lambs were sorted into three groups (14 lambs/group) based on initial body weight (**BW**; 34 ± 2 [SD] kg) and stagger started (18–19 d between groups) on a Zn-deficient diet (Table 1) for 45 d. The average Zn concentration of the diet for the three groups was 18 ± 1.4 (SD) mg Zn/kg DM. Lambs were weighed weekly to monitor growth and adjust feed delivery to maintain feed intake at 4% BW (DM basis). On day 44 of the depletion period, lambs were weighed (group 1 = 44 ± 2 [SD] kg; group 2 = 43 ± 3 [SD] kg; group 3 = 46 ± 3 [SD] kg) and transported (6 km) to the metabolism facility at Iowa State University (Ames, IA) where they were housed in two pens. On the following day, all 14 lambs were placed in individual metabolism crates (123.2 × 41.9 × 93.4 cm) for at least a 2-h acclimation period. Lamb disposition and feed intake during the acclimation period were used to determine the final 12 lambs that would be enrolled in the experiment. Lambs were then randomly assigned to one of the three dietary treatments (n = 4 lambs treatment^{-1}group^{-1}): no supplemental Zn (**CON**) or 15 mg supplemental Zn/kg DM (**ZINC**) as inorganic $ZnSO_4$ (**ZS**) or organic bis-glycinate bound Zn (**GLY**; Plexomin Zn, Phytobiotics, Eltville, Germany). Beginning on day 46, Zn treatments were mixed with 50 g of finely ground corn and delivered on top of a small portion of the diet; the remainder of the diet was delivered once all fine ground corn was consumed. Lambs were fed once daily (~0800 hours) via stainless steel feeders and provided ad libitum water via plastic waterers. Lambs were acclimated to metabolism crates for 10 d, during which time feed was offered at 105% of the previous day's intake. Following the adaptation period, total feces and urine were collected for 5 d, during which time lambs were limited to 95% of their adaptation period intake to minimize feed refusals.

Table 1. Ingredient composition and nutrient analysis of diet fed to lambs throughout the experiment

Dry matter (DM), %	87
Ingredient, % DM basis	
Cracked corn	29
Beet pulp	18
Corn starch	14
Hay	14
Corn gluten meal	10
Premix[1]	10
Molasses	5
Analyzed composition[2], DM basis	
Crude protein, %	16.0
Neutral detergent fiber, %	22.5
Ether extract, %	1.9
Ca, %	0.76
P, %	0.27
Cu, mg/kg DM	6.0
Fe, mg/kg DM	430
Mn, mg/kg DM	60
Zn, mg/kg DM	18

[1]Premix formulated to provide 0.4 mg/kg Co (cobalt carbonate hydrate), 78.1 mg/kg Mn (manganese sulfate monohydrate), 110 mg/kg Se (selenium selenite), 8.1 mg/kg I (calcium iodine), 0.52 IU/kg vitamin A, 0.10 IU/kg vitamin D, and 30 IU/kg vitamin E; Bovatec (Zoetis, Parsippany-Troy Hills, NJ) was included at 0.015% DM.
[2]Average composition of total mixed ration samples from all groups; analyses, excluding Zn, were performed by Dairyland Laboratories (Arcadia, WI).

Sample collection and analytical procedures

Metabolism collection period

For each of the three groups, 50 g of the diet was collected each day. Feed refusals were collected daily (~0700 hours) and approximately 200 g of discarded feed was placed into a labeled bag. Feces were collected in a fecal pan lined with a labeled bag

each day and weighed to determine the total fecal output by each lamb. Feces were then mixed, and a 10% aliquot was collected. Daily samples of the diet, feed refusals, and feces were dried at 70 °C, ground through a 2-mm screen (Retsch ZM 100; Retsch GmbH, Haan, Germany), and stored in plastic bags at room temperature for later analysis. Urine was collected in plastic containers beneath metabolism crates and acetic acid (200 mL; 6 M) was added to each urine collection container to ensure urine pH was <3, thus limiting N volatilization. Urine samples were weighed, and a 10% aliquot was stored at –20 °C for later analysis. A 100-mL volumetric flask was brought to volume with urine and weighed to determine specific gravity and subsequently calculate the total volume of urine produced by each lamb. Composites of diet, feed refusal, and fecal samples from the 5-d collection period were analyzed for DM, organic matter (**OM**), neutral detergent fiber (**NDF**), acid detergent fiber (**ADF**), nitrogen (N), and Zn; composites of urine samples from the 5-d collection period were analyzed for N and Zn. True DM was determined by drying in a forced-air oven for 24 h at 105 °C. OM was determined by ashing in a muffle furnace for 4 h at 600 °C. NDF and ADF were analyzed sequentially utilizing an ANKOM^{200} Fiber Analyzer (ANKOM Technology, Macedon, NY; Goering and Van Soest, 1970). Nitrogen was determined by combustion utilizing a Leco Tru-Mac (Leco Corporation, St. Joseph, MI). The Zn concentration of samples was determined utilizing inductively coupled plasma optical emission spectrometry (**ICP-OES**; Optima 7000 DV, Perkin Elmer, Waltham, MA; Pogge et al., 2014). Nutrient and Zn intake, retention, digestibility, and apparent absorption were calculated as described in the study of Pogge et al. (2014).

Blood and tissue collection and analysis

Blood was collected from all lambs prior to feeding via jugular venipuncture at the beginning and end of the depletion period (days 1 and 44) as well as the beginning and end of the collection period (days 56 and 61). Blood was collected into trace element K_2ethylenediaminetetraacetic acid (EDTA) blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, NJ) and transported to the laboratory on ice prior to centrifugation (1,000 × *g* for 20 min at 4 °C). Plasma was then aliquoted into microcentrifuge tubes and stored at –20 °C until preparation for Zn analysis via ICP-OES (Pogge and Hansen, 2013). Liver, small intestine (jejunum), and muscle (longissimus dorsi) samples were collected after lambs were humanely euthanized via intravenous injection of sodium pentobarbital on day 61. Muscle was excised between the 12th and 13th rib on the left side of the carcass. A segment of the jejunum was excised approximately 0.5 m proximal from the ileocecal junction. The jejunum segment was then cut open, cleaned of digesta, and scraped with a chilled microscope slide to collect intestinal mucosa. Tissue samples were stored at –20 °C for trace mineral analysis. An additional sample was collected from the liver and jejunum, flash frozen in liquid N, and stored at –80 °C for gene expression analysis.

Tissue trace mineral concentrations were determined via ICP-OES after drying in a forced-air oven and acid digestion (Pogge and Hansen, 2013). Gene expression of metallothionein-1 (**MT1**) in the liver and jejunum, as well as Zn transporter ZIP4 (**ZIP4**), metal cation symporter ZIP14 (ZIP14), and Zn transporter 1 (**ZNT1**) in the jejunum was determined via quantitative real-time polymerase chain reaction (**RT-qPCR**) analysis as described in the study of McGill et al. (2016). Briefly, messenger ribonucleic acid was extracted using the TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA; 15596026) and the RNeasy Mini Kit (Qiagen, Hilden, Germany; 74104/74106) and then reverse transcribed into complementary deoxyribonucleic acid using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Thermo Fisher Scientific; 11904018). Reactions were performed in a QuantStudio3 Real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA) and relevant primer information is presented in Table 2. Relative gene expression was determined utilizing the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with 40S ribosomal protein S9 (**RPS9**) serving as the reference housekeeping gene. Variation within and between plates for liver RPS9 was 2.0% and 0.8%, respectively; intra- and inter-plate variation for small intestine RPS9 was 5.3% and 2.7%, respectively.

Statistical analysis

Data were analyzed using the Mixed Procedure of SAS 9.4 (SAS Institute, Cary, NC). The model included the fixed effects of treatment, metabolism group, and breed code (1 = ¾ Suffolk × Texel; 2 = Suffolk × Texel × Polypay; 3 = South African Meat Merino cross, Suffolk × Polypay, Texel × Polypay). Lamb served as the experimental unit for all variables of interest (n = 12 lambs/treatment). Orthogonal contrast statements were constructed to determine the effects of supplemental Zn concentration (ZINC [GLY and ZS] vs. CON) and supplemental Zn source (GLY vs. ZS). Plasma Zn from the start of the depletion period (day 1) was used as a covariate in the analysis of plasma Zn at the end of depletion (day 44). As dietary treatments did not start until day 46, day 44 plasma Zn concentrations were used as a covariate in analysis of plasma Zn at the start and end of collection (days 56 and 61). For gene expression data, delta cycle threshold (**CT**) values (delta CT = CT of target gene – CT of housekeeping gene [RPS9]) were analyzed using the Mixed Procedure of SAS as described above. Pearson correlations between N and Zn retention and apparent absorption were determined using the Corr Procedure of SAS. Data were tested for normality using the Shapiro–Wilks

Table 2. Description of primers used for quantitative real-time polymerase chain reactions

Protein	Gene	NCBI[1] reference no.	Primer sequence[2]	Reference
Metallothionein-1	*MT1*	NM_001040492.2	F: 5′-ATGGACCCGAACTGCTCCTGC-3′ R: 5′-GCGCAGCAGCTGCACTTGTCCG-3′	Fry et al. (2013)
Zn transporter ZIP4	*SLC39A4*	NM_001046067.1	F: 5′-CTCTTGCTGCCCCTGGAC-3′ R: 5′-CCACCAGATCTGCGCGAG-3′	Ma et al. (2020)
Metal cation symporter ZIP14	*SLC39A14*	NM_001098036.1	F: 5′-AGGCTCCTGCTCTACTTC-3′ R: 5′-AGCGTCTCAGAGGTATAATG-3′	Hansen et al. (2009)
Zn transporter 1 ZNT1	*SLC30A1*	NM_001205893.2	F: 5′-CCAGACAGATCCAGAAAAGT CCA-3′ R: 5′-ACTGAACCCAAAGCATCTCCA-3′	Ma et al. (2020)
40S ribosomal protein S9	*RPS9*	NM_001101152.1	F: 5′-CGCCTCGACCAAGAGCTGAAG-3′ R: 5′-CCTCCAGACCTCACGTTTCTTCC-3′	Janovick-Guretzky et al. (2007)

[1]National Center for Biotechnology Information (U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda MD, 20894).
[2]F, forward; R, reverse.

test and outliers were assessed using Cook's D statistic. Data are reported as least square means ± SEM. Significance was declared at $P \leq 0.05$ and tendencies from $0.05 < P \leq 0.10$.

Results

Plasma and tissue trace mineral concentrations

Plasma Zn concentrations at the start of the depletion period (day 1) did not differ among treatments ($P \geq 0.14$; Table 3). At the end of the depletion period (day 44), plasma Zn concentrations tended to be greater for ZINC compared with CON ($P = 0.08$). There was no effect of supplemental Zn concentration or source on plasma Zn concentrations at the start of the collection period (day 56; $P \geq 0.24$), but by the end of the collection period (day 61), ZINC-supplemented lambs had greater plasma Zn compared with CON ($P < 0.01$) and GLY had greater plasma Zn compared with ZS ($P = 0.01$). Liver, jejunum, and longissimus dorsi Zn concentrations were not affected by treatment ($P \geq 0.27$). Liver Cu concentrations were lesser for ZINC compared with CON ($P = 0.03$) but did not differ due to supplemental Zn source ($P = 0.57$). Copper concentrations in the jejunum and longissimus dorsi were not affected by treatment ($P \geq 0.48$) nor were Mn concentrations in the jejunum ($P \geq 0.15$). Manganese concentrations in the longissimus dorsi were greater for ZINC compared with CON ($P = 0.05$) and concentrations tended to be lesser for GLY compared with ZS ($P = 0.09$).

Nutrient digestibility

Daily intake of DM, OM, NDF, and ADF did not differ for ZINC compared with CON ($P \geq 0.19$; Table 4). Daily intake of DM, OM, and ADF was greater for GLY compared with ZS ($P \leq 0.05$) and daily intake of NDF tended to be greater for GLY compared with ZS ($P = 0.06$). Daily fecal output of DM, OM, NDF, and ADF did not differ due to source ($P \geq 0.11$). However, fecal output of DM, OM, and NDF was greater for ZINC compared with CON ($P \leq 0.05$), and fecal output of ADF tended to be greater for ZINC compared with CON ($P = 0.09$). Digestibility of DM, OM, and NDF was lesser for ZINC compared with CON ($P \leq 0.05$) but did not differ between supplemental Zn sources ($P \geq 0.39$). Digestibility of ADF tended to be greater for GLY compared with ZS ($P = 0.06$).

Nitrogen and zinc retention and apparent absorption

Apparent absorption and retention of N and Zn by lambs are reported in Table 5. Daily urine output (L/d) did not differ among treatments ($P \geq 0.38$). Daily N intake was greater for GLY compared with ZS ($P = 0.05$); ZINC did not differ from CON ($P = 0.20$). Fecal N output was greater for ZINC compared with CON ($P = 0.03$), while GLY did not differ from ZS ($P = 0.26$). Urinary N output was not affected by treatment ($P \geq 0.61$). Nitrogen retention (g/d) tended to be greater for GLY compared with ZS ($P = 0.10$), while ZINC was not different from CON ($P \geq 0.66$). As a percent of intake, N retention was not affected by treatment ($P \geq 0.32$). Apparent absorption of N was lesser for ZINC compared with CON ($P = 0.02$) but did not differ between sources ($P = 0.31$).

Zinc intake, fecal output, retention (mg/d and as a percent of intake), and apparent absorption were greater for ZINC compared with CON ($P < 0.01$) but did not differ between supplemental Zn sources ($P \geq 0.14$). Urinary Zn output tended to be greater for GLY compared with ZS ($P = 0.09$) but did not differ for ZINC compared with CON ($P = 0.97$). For CON lambs, N and Zn retention (as a percent of intake) were not correlated ($P = 0.51$) nor were N and Zn apparent absorption ($P = 0.14$). However, N and Zn retention (as a percent of intake) were positively correlated ($P = 0.02$, $r = 0.53$) for ZINC lambs, as was apparent absorption of N and Zn ($P < 0.01$, $r = 0.73$).

Table 3. Effect of supplemental Zn concentration and source on plasma (mg/L) and tissue (mg/kg dry matter) trace mineral concentrations of lambs

	Treatment[1]				Contrast P-value	
	CON	ZS	GLY	SEM[2]	ZINC vs. CON[3]	GLY vs. ZS
Plasma Zn						
Initial (day 1)	0.88	0.84	0.92	0.040	0.93	0.14
End of depletion[4] (day 44)	0.86	0.96	0.95	0.045	0.08	0.82
Start of collection[5] (day 56)	0.96	0.98	1.03	0.037	0.24	0.29
End of collection[5] (day 61)	0.90	0.97	1.08	0.031	<0.01	0.01
Liver[6]						
Cu	459	363	389	32.4	0.03	0.57
Zn	121	128	123	4.7	0.34	0.46
Jejunum[6]						
Cu	18	19	17	3.3	0.95	0.75
Mn	7.5	9.6	7.8	0.89	0.23	0.15
Zn	119	119	115	3.2	0.53	0.40
Longissimus dorsi[6]						
Cu	4.2	4.4	4.3	0.23	0.48	0.69
Mn	0.51	0.64	0.55	0.037	0.05	0.09
Zn	102	111	105	4.2	0.27	0.36

[1]CON, no supplemental Zn; ZS, 15 mg supplemental Zn/kg dry matter as Zn sulfate; GLY, 15 mg supplemental Zn/kg dry matter as bis-glycinate bound Zn (Plexomin Zn, Phytobiotics, Eltville, Germany).
[2]Highest SEM of any treatment reported.
[3]ZINC vs. CON = GLY and ZS vs. CON.
[4]Initial (day 1) plasma Zn utilized as a covariate in analysis (covariate $P = 0.15$).
[5]End of depletion (day 44) plasma Zn utilized as a covariate in analysis (covariate $P < 0.01$); Zn repletion started on day 46.
[6]Samples collected after lambs were euthanized on day 61.

Table 4. Effect of supplemental Zn concentration and source on dry matter (DM), organic matter (OM), neutral detergent fiber (NDF), and acid detergent fiber (ADF) intake, output, and digestibility by lambs

	Treatment[1]				Contrast *P*-value	
	CON	ZS	GLY	SEM[2]	ZINC vs. CON[3]	GLY vs. ZS
Intake, kg/d						
DM	0.89	0.89	1.00	0.040	0.19	0.05
OM	0.84	0.84	0.94	0.038	0.19	0.05
NDF	0.20	0.20	0.23	0.010	0.23	0.06
ADF	0.09	0.09	0.10	0.004	0.20	0.04
Fecal output, kg/d						
DM	0.17	0.18	0.21	0.011	0.05	0.11
OM	0.14	0.15	0.17	0.009	0.04	0.13
NDF	0.08	0.09	0.09	0.005	0.02	0.28
ADF	0.04	0.05	0.05	0.003	0.09	0.56
Digestibility, %						
DM	80.9	79.5	79.6	0.60	0.05	0.94
OM	83.5	82.0	82.1	0.59	0.03	0.92
NDF	61.6	56.5	58.3	1.50	0.01	0.39
ADF	52.2	47.3	51.9	1.74	0.17	0.06

[1]CON, no supplemental Zn; ZS, 15 mg supplemental Zn/kg DM as Zn sulfate; GLY, 15 mg supplemental Zn/kg DM as bis-glycinate bound Zn (Plexomin Zn, Phytobiotics, Eltville, Germany).
[2]Highest SEM of any treatment reported.
[3]ZINC vs. CON = GLY and ZS vs. CON.

Table 5. Effect of supplemental Zn concentration and source on apparent absorption and retention of nitrogen and Zn by lambs

	Treatment[1]				Contrast *P*-value	
	CON	ZS	GLY	SEM[2]	ZINC vs. CON[3]	GLY vs. ZS
Urine output, L/d	1.15	1.31	1.07	0.201	0.87	0.38
Nitrogen						
Intake, g/d	20.9	20.9	23.5	0.96	0.20	0.05
Fecal output, g/d	5.9	6.5	7.0	0.37	0.03	0.26
Urine output, g/d	8.9	8.8	9.2	0.56	0.83	0.61
Retained, g/d	6.1	5.6	7.2	0.72	0.66	0.10
Retained, % of intake	29.0	26.3	30.2	2.91	0.79	0.32
Apparent absorption, %	71.7	68.8	70.0	0.92	0.02	0.31
Zn						
Intake, mg/d	15.9	37.5	39.1	0.76	<0.01	0.14
Fecal output, mg/d	16.7	32.7	33.1	1.35	<0.01	0.84
Urinary output, mg/d	0.32	0.24	0.41	0.068	0.97	0.09
Retained, mg/d	−1.0	4.6	5.6	1.32	<0.01	0.56
Retained, % of intake	−6.3	11.9	13.9	4.59	<0.01	0.74
Apparent absorption, %	−5.2	12.8	15.0	4.51	<0.01	0.71

[1]CON, no supplemental Zn; ZS, 15 mg supplemental Zn/kg dry matter as Zn sulfate; GLY, 15 mg supplemental Zn/kg dry matter as bis-glycinate bound Zn (Plexomin Zn, Phytobiotics, Eltville, Germany).
[2]Highest SEM of any treatment reported.
[3]ZINC vs. CON = GLY and ZS vs. CON.

Gene expression

Intestinal (jejunum) gene expression of MT1 did not differ among treatments ($P \geq 0.42$; Figure 1A). However, liver MT1 expression tended to be greater for GLY compared with ZS ($P = 0.07$; Figure 2). Jejunum ZIP4 expression was lesser for ZINC compared with CON ($P = 0.02$) but did not differ between Zn sources ($P = 0.80$; Figure 1B). There was no effect of treatment on jejunum ZIP14 ($P \geq 0.32$; Figure 1C) or ZNT1 expression ($P \geq 0.12$; Figure 1D).

Discussion

Zinc is an essential trace element required to support the structure and function of numerous enzymes and transcription factors. Critical roles for Zn in livestock health and production include nucleic acid synthesis, protein metabolism, and antioxidant defense (Suttle, 2010). Classical signs of Zn deficiency include impaired growth and immune function. To prevent clinical deficiency, it is currently recommended that sheep and beef cattle diets contain 30 to 32 mg Zn/kg DM (NRC, 2007; NASEM, 2016). However, additional Zn may be necessary to support optimal growth and carcass quality (Spears and Kegley, 2002). Zinc supplements can be classified based on the chemical nature of the ligand associated with the mineral. Inorganic sources of Zn include $ZnSO_4$ and Zn oxide (ZnO), whereas organic sources of Zn include Zn–proteinates, Zn–amino acid complexes, and Zn–amino acid chelates (Spears, 1996). It has been suggested that organic Zn sources are more

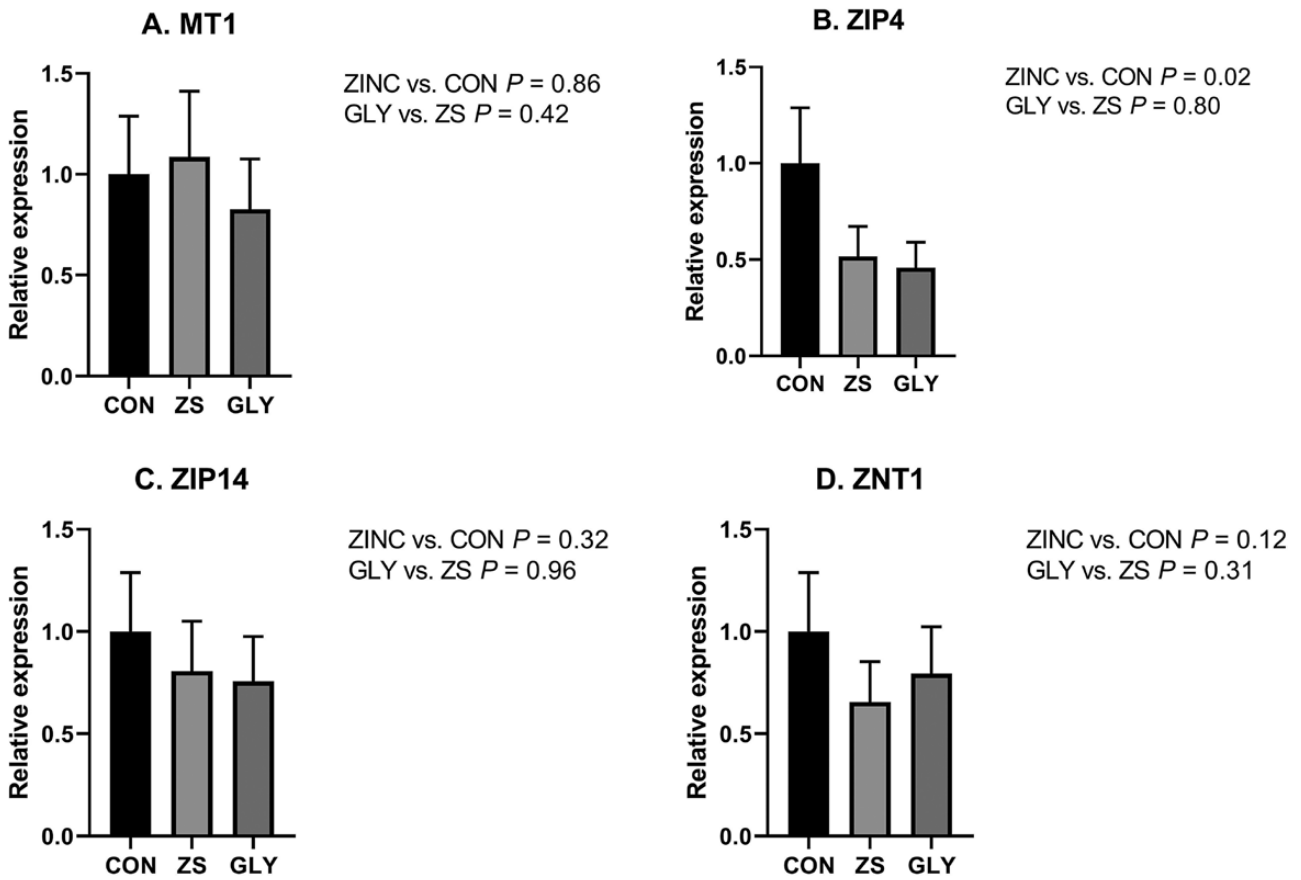

Figure 1. Intestinal (jejunum) expression of genes involved in Zn storage (metallothionein-1 [MT1]; A), luminal Zn import (Zn transporter ZIP4 [ZIP4]; B), basolateral Zn import (metal cation symporter ZIP14 [ZIP14]; C), and basolateral Zn export (Zn transporter 1 [ZNT1]; D). Gene expression of ZS and GLY is expressed relative to CON (CON, no supplemental Zn; ZINC, 15 mg supplemental Zn/kg dry matter as Zn sulfate [ZS] or bis-glycinate bound Zn [GLY; Plexomin Zn, Phytobiotics, Eltville, Germany]). Intestine samples were collected after lambs were euthanized on day 61, 15 d after the start of Zn supplementation.

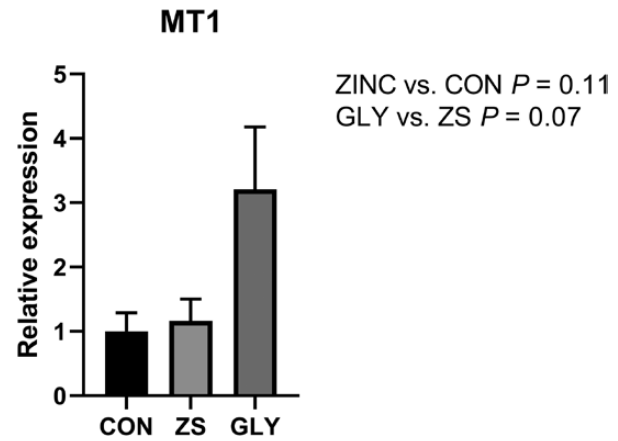

Figure 2. Liver gene expression of metallothionein-1 (MT1), a Zn storage protein. Gene expression of ZS and GLY is expressed relative to CON (CON, no supplemental Zn; ZINC, 15 mg supplemental Zn/kg dry matter as Zn sulfate [ZS] or bis-glycinate bound Zn [GLY; Plexomin Zn, Phytobiotics, Eltville, Germany]). Liver samples were collected after lambs were euthanized on day 61, 15 d after the start of Zn supplementation.

bioavailable than inorganic Zn sources due to their ability to remain complexed or chelated in the rumen, preventing them from interacting with dietary antagonists and competing with other minerals for absorption (Goff, 2018). Comparing the bioavailability of Zn sources can be particularly challenging due to the lack of reliable biomarkers of Zn status. Plasma and tissue concentrations of Zn are commonly used biomarkers but are not sensitive to small changes in Zn intake (Hambidge, 2003). Thus, the current study also assessed Zn apparent absorption and retention to compare the bioavailability of ZS and GLY when supplemented to lambs. These measures are best assessed under low Zn supplementation conditions to ensure that absorption of Zn is limited by Zn availability from the two sources rather than homeostatic control mechanisms (Spears, 1989).

Zinc homeostasis is tightly controlled at the point of absorption which occurs throughout the small intestine (see Maares and Haase, 2020). Lee et al. (1989) observed the highest rate of Zn absorption in the jejunum followed by the duodenum and ileum. Under conditions of low dietary Zn, ZIP4 expression is increased and the transporter is localized to the apical membrane of the enterocyte to facilitate Zn absorption (Liuzzi et al., 2004). Not surprisingly, after consuming a Zn deplete diet (analyzed 18 mg Zn/kg DM) for 61 d, ZIP4 expression in the jejunum was greater for CON compared with lambs receiving ZINC for the 15 d immediately prior to tissue collection. The lack of difference in intestinal ZIP4 expression between supplemental Zn sources could be a result of bis-glycinate bound Zn becoming dissociated in the abomasum or small intestine (Cao, 2000), allowing free mineral to interact with ion-specific transporters and influence cellular uptake mechanisms in a

similar fashion as ZS. In congruence with ZIP4 expression, no differences in Zn apparent absorption were observed between ZS (12.8%) and GLY (15.0%). VanValin et al. (2018) reported similar Zn apparent absorption values for lambs supplemented 40 mg Zn/d from ZS or Zn–Methionine (**Zn–Met**). Spears (1989) observed no difference in apparent absorption of Zn when lambs were fed a semi-purified diet (analyzed 2.8 mg Zn/kg DM) and supplemented 15 mg Zn/kg DM from ZnO or Zn–Met, but Zn retention was greater for Zn–Met compared with ZnO. In a separate experiment conducted by Spears (1989), plasma Zn concentrations were greater 12 and 24 h after lambs were given a 300 mg oral dose of Zn–Met compared with those dosed with ZnO. Results of these two experiments led the author to hypothesize that if Zn–Met is absorbed and transported in the blood without modification, tissue uptake, and utilization of Zn may differ from ZnO. Indeed, there is in vitro evidence showing Zn bound to amino acids can be absorbed by intestinal cells via amino acid transporters (Sauer et al., 2017).

Under conditions of adequate dietary Zn, intestinal expression of MT1 has been shown to increase (Liuzzi et al., 2004). However, Zn supplementation did not induce changes in MT1 expression or Zn concentrations in the jejunum. Consistent with this finding, MT1 expression and Zn concentrations in the jejunum were similar for piglets fed a low (57 mg/kg DM) or normal (164 mg/kg DM) Zn diet but were greater for piglets fed a high (2,425 mg/kg DM) Zn diet. Wright and Spears (2004) observed no effect of supplemental Zn on duodenal Zn concentrations in Holstein calves until supplementation was increased from 20 to 500 mg Zn/kg DM, suggesting that pharmacological concentrations of dietary Zn may be required to induce changes in intestinal MT1 gene expression and Zn accumulation. Zinc bound to MT1 in the enterocyte may be excreted when intestinal cells are sloughed or released to enter the bloodstream via the basolateral Zn exporter, ZNT1. Expression of ZNT1 in the intestine has also been shown to be regulated by dietary Zn (McMahon and Cousins, 1998). Thus, it is unclear why the expression of ZNT1 in the current study was not increased by Zn supplementation. The study by Nishito and Kambe (2019) demonstrated how post-translational regulation of ZNT1 controls cellular Zn concentrations; under Zn-sufficient conditions, ZNT1 protein accumulates on the plasma membrane, while under Zn-deficient conditions, ZNT1 protein is endocytosed and degraded. Based on this evidence, abundance and cellular location of proteins involved in Zn homeostasis (chaperones, transporters, etc.) are likely more informative than gene expression. Unfortunately, antibodies specific for many of these proteins in livestock species are currently unavailable. Also located on the basolateral membrane of enterocytes is ZIP14 (Guthrie et al., 2015). Gene expression of this Zn importer is increased in response to inflammatory stimuli which facilitates cellular Zn uptake during the acute-phase response to infection (Liuzzi et al., 2005). As lambs in the current study displayed no signs of illness, it is unsurprising that no differences in ZIP14 expression were observed.

After 15 d of Zn supplementation, plasma Zn concentrations were greater for ZINC compared with CON, driven by greater plasma Zn for GLY compared with ZS. Few studies have observed changes in plasma Zn concentrations due to source unless supplemented at high concentrations. For example, plasma Zn was not affected when Holstein calves were supplemented ZS, Zn–proteinate, or a 50:50 blend of these sources at 20 mg Zn/kg DM for 98 d; however, when supplementation was increased to 500 mg Zn/kg DM for 14 d, plasma Zn was greater for Zn–proteinate and the blend relative to ZS (Wright and Spears, 2004). At high levels, inorganic Zn may trigger downregulation of absorptive mechanisms to maintain Zn homeostasis, whereas organic Zn may not elicit these suppressive effects if Zn remains bound to its ligand in circulation. While several studies have reported no effects of Zn source on plasma Zn concentrations (Spears, 1989; Spears and Kegley, 2002; VanValin et al., 2018), Zn source has been shown to influence tissue Zn concentrations. Liver Zn concentrations after 42 d of supplementation (20 mg Zn/kg DM) were greatest for steers supplemented Zn–Glycine compared with those supplemented ZS or Zn–Met (Spears et al., 2004). Although liver Zn concentrations were not affected by supplemental Zn source in the current study, gene expression of MT1 in the liver was approximately 2-fold greater for GLY compared with ZS. Functions of MT1 include intracellular metal metabolism and/or storage, donation of metals to target proteins or enzymes, as well as metal detoxification and protection against oxidative stress (Davis and Cousins, 2000). Similar to the results observed herein, Carmichael (2019) found liver MT1 expression to be more sensitive to different Zn sources than liver Zn concentrations in steers. Greater plasma Zn concentrations and liver MT1 expression for GLY-supplemented lambs further support the hypothesis proposed by Spears (1989) that organic and inorganic Zn sources are metabolized differently after absorption. Additionally, these data support the hypothesis proposed by authors of the current study that GLY is more bioavailable than ZS.

Previous research has identified a positive relationship between dietary Zn and protein metabolism in rats (Oberleas and Prasad, 1969; Greeley et al., 1980). More recently, Carmichael et al. (2018) observed a positive correlation (r = 0.46) between Zn and N retention in feedlot steers; N retention (as a percent of intake) was greater for steers supplemented 120 mg Zn/kg DM compared with unsupplemented steers (basal diet analyzed 32 mg Zn/kg DM). Regardless of source, Zn and N retention were positively correlated (r = 0.53) for Zn-supplemented lambs in the current study; apparent absorption of Zn and N was also positively correlated (r = 0.73). Although not significant (P = 0.14), apparent absorption of Zn and N displayed a negative relationship in CON. This relationship is likely driven by the extremely low Zn apparent absorption rates (–5.2%) for unsupplemented lambs. Lesser apparent absorption despite greater intestinal ZIP4 expression for CON suggests that the availability of Zn in the basal diet may have been limiting Zn absorption. Dietary factors known to negatively affect Zn absorption include high concentrations of Ca, P, Cu, and Fe. The diet fed in the current study was formulated to meet or slightly exceed Ca, P, and Cu recommendations (NRC, 2007), and analyzed concentrations of these minerals do not overtly suggest a Zn antagonism. Given the grain components of the diet, it is likely a portion of dietary P was in the form of phytate, which is usually not a concern for ruminants but can hinder Zn absorption if phytate bypasses microbial phytases in the rumen and reaches the small intestine intact (Suttle, 2010). Iron concentrations were relatively high (analyzed 430 mg/kg DM) in this diet and could have been competing with Zn for intestinal absorption (Solomons, 1986).

In addition to interacting with other dietary constituents in the digestive tract, differences in chemical characteristics (i.e., solubility and chelation strength) between Zn sources may affect ruminal fermentation and subsequent nutrient digestibility. Digestibility of DM, OM, and NDF was lesser for Zn supplemented compared with control lambs in the current study. Alternatively, Zn supplemented at 20 mg Zn/kg DM from ZS or Zn–Met did not affect DM, OM, or NDF digestibility in lambs (Garg et al., 2008). Although DM, OM, and NDF digestibility did not differ due to source in the current study, ADF digestibility tended to be lesser for ZS compared with GLY. Others have also reported

improvements in ADF digestibility when an organic (Zn–Met and Zn–proteinate) source was compared with an inorganic (ZS) source (Garg et al., 2008; Alimohamady et al., 2019). High concentrations of Zn have been shown to lessen the rate and extent of cellulose digestion in vitro potentially due to inhibition of cellulolytic enzymes (Eryavuz and Dehority, 2009). However, more work is needed to determine the effect of physiologically relevant concentrations of dietary Zn on fiber digestibility as well as how the amount and physical form of fiber in the diet interact with Zn in the ruminant gastrointestinal tract.

Although tissue concentrations of Zn were unresponsive to moderate changes in dietary Zn, tissue concentrations of other trace minerals were affected by Zn supplementation. Liver Cu concentrations were lesser for ZINC compared with CON. A similar relationship has been observed by others (Genther-Schroeder et al., 2016; Carmichael, 2019) and could be a function of Zn inducing MT1 synthesis. MT has a greater affinity for Cu than Zn (Richards, 1989) so increased MT1 in circulation could be binding Cu and making it unavailable for uptake by tissues such as the liver, a major storage depot for Cu in ruminants. Longissimus dorsi Mn concentrations were greater for ZINC compared with CON, driven by a tendency for greater concentrations in ZS compared with GLY. Zinc and Mn are known to use similar transport mechanisms (Garrick et al., 2006; He et al., 2006; Girijashanker et al., 2008), so Zn from different sources may interact with these transporters differently in the intestine as well as peripheral tissues such as the muscle. Unfortunately, the roles of trace minerals in muscle are poorly understood and are an area of growing research interest (Vest et al., 2018; Gordon et al., 2019).

Livestock require Zn for optimal health and production. As feedstuffs may not contain enough Zn to meet the requirement of animals or dietary antagonists limit the availability of Zn, livestock are often provided supplemental Zn. Several sources of supplemental Zn are available, including inorganic and organic sources. In previous research, authors have sought to compare the bioavailability of different Zn sources but little research has been conducted utilizing bis-glycinate bound Zn. Although Zn apparent absorption and retention were not affected by supplemental Zn source in the current experiment, lambs supplemented bis-glycinate bound Zn had greater concentrations of Zn in circulation and liver expression of MT1, a key regulator of Zn homeostasis. Collectively, these data suggest that Zn from GLY is more available for biological processes than Zn from ZS. Future research should seek to further understand post-absorptive metabolism of bis-glycinate bound Zn as well as effects of this Zn source on immune function and growth performance of livestock.

Acknowledgment

This study was partially supported by Phytobiotics (Eltville, Germany).

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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