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Effects of inorganic minerals replaced by complexed glycinates on growth, blood profiles, immune responses, intestinal morphology, and mineral excretion in piglets

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Abbreviations: ITM = inorganic trace mineral, GCM = glycine-complexed trace mineral, OTM = organic trace mineral, BW = body weight, TiO_2 = titanium dioxide, ATTD = apparent total tract digestibility, T1 = treatment 1, and so on, ALP = alkaline phosphatase, Alb = albumin, Fer = ferritin, TIBC = total iron binding capacity, TP = total protein, CP = ceruloplasmin, TRF = transferrin, Ig = immunoglobulin, C3 = complement 3, and so on, VH = villous height, CD = crypt depth, sIgA = secretory immunoglobulin A

Abstract:

BACKGROUND: The effects of inorganic trace minerals (ITM) replaced by low-dose glycine-complexed trace minerals (GCM) on growth, serum parameters, immunity, intestinal morphology, and mineral excretion in piglets were investigated. One hundred and twenty-eight weaned piglets (14.18 ± 0.33kg body weight (BW)) were randomly assigned to 4 treatments with 4 replicates, 8 piglets per replicate. Treatments consist of: (T1) basal diet + 100% inorganic trace mineral (ITM) as the control group (20 ppm Cu, 150 ppm Fe, 150 ppm Zn, and 30 ppm Mn from sulfates); (T2) basal diet + 50% ITM (Cu, Fe, Zn, and Mn from sulfates, 50% of control) + 50% organic trace minerals (OTM, Cu, Fe, Zn, and Mn from glycine complexed trace minerals (GCM), 50% of control); (T3) basis diet + 50% OTM from GCM; (T4) basal diet + 70% OTM from GCM. The feeding period lasted 28 d and was divided into 2 stages (0 to 14 d and 15 to 28 d). After feeding trial, 6 pigs per treatment were randomly selected to slaughter for sampling.

RESULTS: Average daily gain, feed intake, and G:F were not affected by dietary treatments during the overall period. During the second, and the overall feeding phases, the digestibility of Zn and Fe in T3 and T4 was higher than that of T1 ($P < 0.05$). The concentration of serum ferritin in T2 was significantly higher than T3 and T4. Serum immunoglobulin A concentration in the ileal mucosa of T2 was higher than that of T1 ($P < 0.05$), and the higher duodenum villus height was observed in T4 compared with the rest treatments ($P < 0.05$). The lowest trace mineral excretion was overserved in T3 ($P < 0.01$); in addition, the urinary concentrations of Zn and Fe in T2 were lower than that in T1 ($P < 0.05$).

CONCLUSION: These results indicate that GCM have higher bioavailability than ITM, and that supplementation of low-dose GCM to replace full dose ITM could reduce mineral excretion without affecting performance, blood profiles, immune responses, and intestinal morphology in piglets.

Key words: trace minerals, piglets, bioavailability, metal pollution

Introduction

Microminerals fulfill a central role in many metabolic processes throughout the body. Supplementing adequate microminerals in swine diets requirements is crucial for growth, reproduction, immune system development, and antioxidant capacity [1,2]. Even though NRC (2012) further states that the innate portion of the diet is included within the listed requirements, it is a common practice that micromineral premixes and diets are formulated while neglecting the contribution of these innate microminerals. This is mainly due to the unknown bioavailability and variable concentrations in grains and other feedstuffs [3]. In practice, most commercial premixes exceed the NRC (2012) requirements, and consequently, microminerals in many diets often exceed the requirements of pigs.

One apparent disadvantage of over supplying microminerals to the animals is the environmental consequence of elevated minerals excretions. The excess trace minerals emitted are difficult to collect for centralized treatment, and could leach to the soil and water sources around the farm and potentially cause heavy metal pollution. It is rather urgent to find solutions to reduce the amount of trace minerals used from the dietary sources, which may offer a direct and effective method to reduce heavy metal pollution from livestock production.

Without affecting the growth and health of the animals, the most plausible solution to reduce the excretion of trace minerals is to use mineral sources with a superior bioavailability, which reduces dietary mineral supplementation. So far, previous studies have shown that the bioavailability of organic trace minerals (OTM) as complexed glycinate form is higher than that of frequently-used inorganic trace minerals [4-7]; however, most studies focused on replacing one or two elements, and data on totally replacing ITM (i.e., Fe, Cu, Mn and Zn) by its respective OTM at different levels in diets are limited. This study was designed to evaluate the effects of ITM replaced by lower-dose glycine-complexed trace minerals (GCM) on growth performance, serum biochemical

parameters, immunity, intestinal morphology, and fecal mineral excretion in piglets.

Materials and methods

Experiment Design and Animal Management

The site and piglets of the feeding trial were provided by Anji Zhengxin Breeding Farm (Huzhou, China). A total of 128 pigs with an initial BW of 14.18 kg (SEM = 0.33) were allotted to 4 dietary treatments in a randomized complete block design with 4 replicates (pens) and 8 animals per pen. All piglets were 35 days old and weaned at 23 days of age. Pigs were acclimatized for 5 days. The formal feeding trial started when pigs were 40 days old and lasted for 28 days, which was divided into two phases. Phase I was 0 to 14 d and phase II was 14 to 28 d. The basal diet (Table 1) was formulated according to the common-used trace mineral dosage of 15-30 kg piglets in Chinese intensive pig farms (above NRC (2012) recommendation) (Table 2). The specific mineral supplemental doses and analytical values of trace minerals in 4 treatments were provided in Table 2. Titanium dioxide (TiO_2) was added to the diet as an indicator for determining the apparent total tract digestibility (ATTD) of trace minerals. Inorganic trace minerals (Cu, Fe, Zn, and Mn from $CuSO_4 \cdot 5H_2O$, $FeSO_4 \cdot H_2O$, $ZnSO_4 \cdot H_2O$, and $MnSO_4 \cdot H_2O$) were used in treatment 1 (T1, as control), and the respective mineral supplementation levels match commercially recommended concentrations of 20 mg Cu, 150 mg Fe, 150 mg Zn, 30 mg Mn per kg feed, respectively. Treatment 2 (T2) consists of 50% ITM of control and 50% OTM including Cu, Fe, Zn and Mn provided by GCM (BASF SE, Germany) at concentrations of 10, 75, 75, 15 mg kg^{-1}, respectively. Treatment 3 (T3) and treatment 4 (T4) used lower levels of GCM (50% and 70% of control, respectively), which was described in T2 as mineral sources for the diet.

Table 2. Supplemental levels and analytical values of trace minerals in four treatments

Item	Treatment 1	Treatment 2	treatment 3	Treatment 4
	100% ITM	50% ITM+50% GCM	50% GCM	70% GCM

Supplemental levels, mg kg^{-1}				
Cu	20	20	10	14
Fe	150	150	75	105
Zn	150	150	75	105
Mn	30	30	15	21
Analytical values (0 to 14 d), mg kg^{-1}				
Cu	27.74	27.04	17.36	21.42
Fe	256.56	253.93	179.45	211.07
Zn	183.24	182.11	106.78	139.04
Mn	42.72	43.74	28.08	34.79
Analytical values (15 to 28d), mg kg^{-1}				
Cu	28.83	28.21	18.35	22.67
Fe	214.83	216.22	142.06	172.87
Zn	179.97	179.84	103.57	134.74
Mn	42.73	43.72	28.43	34.12

Pens used in the feeding trial had hard concrete slotted flooring and were equipped with nipple drinkers and stainless-steel feeders. All piglets were allowed ad libitum access to feed and water. Room temperature and ventilation were controlled automatically according to the standard procedures of the farm. Pigs were weighed on at day 1, 15, and 28 of the trial and the feed intake was also recorded at the last day of each phase. Upon finishing the feeding trial, 6 pigs per treatment (at least one pig per pen) were randomly selected for slaughtering and sampling. Average daily gain (ADG), average daily feed intake (ADFI), and gain to feed ratio (G:F) were calculated at the end of the trial.

Sample Collecting

Fresh feces collection was carried out on the last three days of each phase on the pen basis, mixed and stored feces sample of each phase at -80 ℃ for later analysis. Before slaughtering, blood samples were collected by syringe from the precaval vein of selected pigs. Serum was obtained by centrifuging at 1260×g at 4 ℃ for 10 min and was frozen at −80 ℃ until the analysis. After slaughter, urine was collected from the bladder using a

syringe and stored at -80 °C. The small intestine was separated and dissected. The segments of middle duodenum and jejunum were separated for a length of 2 cm with flushing by sterile saline solution and stored in 4% paraformaldehyde under 4 °C. Additional jejunum segment was isolated and stored in 2.5% malondialdehyde under 4 ℃. Duodenum, jejunum and ileum mucosa were scraped and collected using the clean glass slides and stored at -80 °C.

Serum Parameters and Immune Response Analysis

Serum alkaline phosphatase (ALP), albumin (Alb), serum ferritin (Fer), total iron binding capacity (TIBC), total protein (TP), and urea were determined by an automated biochemical analyzer (Olympus AU5831, Tokyo, Japan). The concentrations of ceruloplasmin (CP) and transferrin (TRF) were measured using the commercial kits (Jiancheng Bioengineering Institute, Nanjing, China) and ELISA kits (Jiancheng Bioengineering Institute, Nanjing, China), respectively. To assess the immune function, serum immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), complement 3 (C3) and complement 4 (C4) concentrations were detected with immunoturbidimetry by commercial kits (Jiancheng Bioengineering Institute, Nanjing, China). Concentrations of serum inflammatory factors IL-6, IL-10, and TNF-α were determined by ELISA kits (Beijing 4A Biotech Co., Ltd, Beijing, China). The jejunum and ileum mucosa were ground on ice by a glass grinder to make a 10% homogenate for the detection of sIgA concentration, and the procedure was also carried out according to the instruction of commercial kit (Jiancheng Bioengineering Institute, Nanjing, China).

Mineral Measurement and ATTD Calculation

The trace mineral concentrations in feed, feces and urine samples were determined by atomic flame absorption spectrometry (ICE-3500, Thermo Crop., USA). Before the analysis, feces and feed samples were dried at 65 ℃ for 48 h, and then pulverized by a grinder and passed through a 60 mesh sieve (0.25 mm) to

achieve homogenous samples. The ground feces and feed sample were digested using the method described as follows: 1 g of sample was placed in a crucible and ashed at 550 ℃ for 4 h in muffle furnace until it became off-white powder, and 10 ml of 10% hydrochloric acid was added for dissolving. After filtration, the filtrate was diluted to 100 ml with distilled water. Urine sample was thawed and treated by microwave wet digestion as follows: 1 ml sample was mixed with 6 ml nitric acid for 3 minutes at 120 ℃ in a microwave digestion system, 120 ℃ for 8 minutes, and kept at 230 ℃ for 4 minutes until a pale yellow solution appeared, then removed the residual acid, and finally dilute with distilled water to 50 ml. The absorption wavelengths of Cu, Zn, Fe and Mn in the flame of air-acetylene combustion were 324.8 nm, 213.9 nm, 248.3 nm and 279.5 nm, respectively. The ATTD of trace minerals was calculated as follows: ATTD (%) = $100*(1-I_r*N_f/(I_f*N_r))$; I_r: the content of indicator in feed (%); I_f: the content of indicator in feces (%); N_r: the content of trace minerals in feed (%); N_f: the content of trace minerals in feces (%).

Intestinal Morphology Analysis and Scanning Electron Microscopy (SEM)

After adequate fixation, small intestine samples were embedded in paraffin, cut cross-section of 5 μm and then stained with hematoxylin and eosin (H&E, Solarbio Science & Technology Co., Ltd, Beijing, China). Images were acquired using a DM3000 microscope (Leica, Wetzlar, Germany). Villous height (VH) and crypt depth (CD) were measured using Image-Pro software (Media Cybernetics, MD, USA) as previously reported [8,9]. All images and measurements were performed by the same person using the same microscope. The jejunum segment in glutaraldehyde was cut into a small rectangle before osmic acid fixation. Trimmed jejunum samples were rinsed three times with PBS and then fixed with 1% OsO_4 for 1.5 h, then osmic acid was discarded and dehydrated in a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%) for 20 min at each step and then transferred into a mixture of alcohol and iso-amyl acetate (v:v = 1:1) for 30 min and iso-amyl acetate for 1 h. The specimens were then dehydrated in a Hitachi Model HCP-2 critical point dryer with liquid

CO_2. The dehydrated specimens were coated with gold-palladium and visualized using a Philips Model SU8010 FASEM (HITACHI, Japan).

Metal Transporter mRNA Expression in Duodenum

An analysis of duodenum metal transporter gene expression was conducted as previously described by Huang [10]. The duodenal mucosa was ground by liquid nitrogen and then added to the TRIzol reagent (Invitrogen, Carlsbad, California, USA) for RNA extraction. The RNA quantity and quality were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The cDNA was synthesized with the PrimeScript RT reagent kit with gDNA Eraser (TAKARA, Dalian China) according the following procedure: 2 μg RNA was used to erase gDNA at 42℃ for 2 min, and the reverse transcription was conducted at 37 ℃ for 15 min and 85 ℃ for 5 s. Real-Time PCR was performed on a CFX96TM Real-Time system (BioRad, Hercules, CA, USA) in a total volume of 25 μL, which consists of 12.5 μL SYBR Premix EX Taq (TAKARA), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 2 μL cDNA and 9.5 μL double-distilled water. The primer sequences for ZnT1, Fpn1, Ctr1, DMT1 and β-actin were designed with primer 5.0 (Table 3) and synthesized in Tsingke Biological Technology Co., Ltd. The protocol of PCR consisted of 30 s at 95 ℃ and followed 40 cycles of 95 ℃ for 5 s and 58 ℃ for 30 s. β-actin was used as a housekeeping gene and the relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method [11].

Table 3. Primers for gene expression using real-time PCR

Gene	Accession number	Primer sequence (5′ to 3′)	Size(bp)
ZnT1	NM_001139470.1	F: ATCACAGCGGCTTCGGCAAC R: GGTCTCCTCCTGGTCGGTTCC	155
Fpn1	NM_001128440.1	F: GAGCAGCAGCAGCGATAGCAG	121

		R: GAGGTCAGGTAGTCGGCCAAGG	
Ctr1	NM_214100.3	F: TCGTGGACATCACAGAGCATTGC R: AAGGAGACGAGGAGGCGAAGAC	126
DMT1	NM_001128440.1	F: TGGAGGATCGCAGGCGGTATC R: AGCCACCACATACAACACCACATG	108
β-actin	DQ845171.1	F: CCAGGTCATCACCATCGGCAAC R: CAGCACCGTGTTGGCGTAGAG	163

Statistical Analysis

Data were analyzed by using the one-way analysis of variance (ANOVA) to test homogeneity of variances *via* Levene's test and followed with LSD test to determine statistically significant differences among treatments (SPSS 20.0), and dietary treatment was defined as the independent variables. Each pen served as the experimental unit for growth performance and trace minerals fecal retention data, and the individual pig was the experimental unit for other indices. Data are expressed as the mean ± SEM. A significant level was set at $P < 0.05$.

Results

Growth Performance

The average daily gain in each phase was similar among the treatments (Table 4). In phase 1, ADFI of T2 was significantly higher than that of T4. In phase 2, the G:F of piglets in 100% inorganic trace minerals treatment and the mixture of 50% glycine complexed trace minerals with 50% inorganic trace minerals treatment (T1 and T2) were significantly higher than that of piglets in low-dose groups (T3 and T4).

Table 4. Effect of inorganic trace minerals replaced by glycine complexed trace minerals on growth performance in piglets[†]

Item	100%ITM	50%ITM+ 50%GCM	50%GCM	70%GCM	SEM	P - value
Initial weight, kg	14.60	14.94	13.66	13.53	0.33	0.374
Middle weight, kg	21.97	22.82	21.50	20.78	0.39	0.331
Final weight, kg	31.25	32.19	30.16	29.16	0.55	0.242
ADG, g						
0 to 14 d	526.79	562.50	560.27	517.86	10.33	0.321
15 to 28 d	662.95	669.64	618.30	598.22	13.11	0.149
0 to 28 d	594.87	616.07	589.29	558.04	9.74	0.212
ADFI, g						
0 to 14 d	845.54^{ab}	883.97^{a}	861.92^{ab}	819.42^{b}	8.78	0.043
15 to 28 d	1198.63	1211.67	1176.22	1134.49	21.72	0.656
0 to 28 d	1022.08	1047.82	1019.07	976.96	14.50	0.414
G:F, g kg^{-1}						
0 to 14 d	623.09	635.92	650.03	631.51	8.71	0.779
15 to 28 d	553.30^{a}	552.94^{a}	525.27^{b}	528.18^{b}	4.47	0.012
0 to 28 d	581.98	588.02	578.26	571.33	3.79	0.509

[†]Data are expressed as means of the replicates (n = 4). In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).

Apparent Total Tract Digestibility of Trace Minerals

As shown in Table 5, no differences in ATTD of tested minerals were detected in the first period (0 to 14 d); in the second period and the overall feeding period, the ATTD of zinc and iron in the low-dose GCM treatments (T3 and T4) were higher than that in the 100% inorganic treatment (T1).

Table 5. Effect of different levels of trace minerals on minerals apparent total tract digestibility in piglets[†]

Item	100% ITM	50%ITM+50%GCM	50%GCM	70%GCM	SEM	P - value
0 to 14 d						
Cu （%）	28.60	31.04	33.60	34.97	1.71	0.612
Zn （%）	53.50	57.23	61.07	61.55	1.47	0.175
Fe （%）	35.81	38.98	45.59	46.14	2.11	0.235
Mn （%）	28.71	28.79	33.02	37.50	1.80	0.272
15 to 28 d						
Cu （%）	26.31	31.84	31.61	34.62	1.21	0.087
Zn （%）	49.57^{b}	56.17^{ab}	60.23^{a}	60.72^{a}	1.58	0.024
Fe （%）	28.30^{b}	37.90^{ab}	45.91^{a}	47.80^{a}	2.51	0.006

Mn （%）	26.42	29.87	34.76	36.72	2.07	0.299
0 to 28 d						
Cu （%）	27.46	31.44	32.60	34.79	1.26	0.222
Zn （%）	51.54^{b}	56.70^{ab}	60.65^{a}	61.13^{a}	1.33	0.017
Fe （%）	32.05^{b}	38.44^{ab}	45.75^{a}	46.97^{a}	2.14	0.025
Mn （%）	27.57	29.33	33.89	37.11	1.62	0.138

†Data are expressed as means of the replicates (n = 4). In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).

Hematological Response

Table 6. Effect of inorganic trace minerals replaced by glycine complexed trace minerals on serum parameters in piglets†

Item	100% ITM	50%ITM+ 50%GCM	50%GCM	70%OGCM	SEM	P - value
ALP (U/L)	243.80	231.17	251.67	248.17	10.09	0.907
Alb (g/L)	20.02	21.60	22.30	18.98	0.67	0.288
Fer (μg/L)	4.00^{ab}	5.67^{a}	3.50^{b}	3.75^{b}	0.33	0.050
TIBC (μmol/L)	92.24	93.42	101.57	89.93	3.01	0.562
TP (g/L)	58.06	59.38	54.38	58.15	0.90	0.215
Urea (mmol/L)	3.72	4.04	3.94	3.72	0.18	0.911
Cp (U/L)	46.10	44.76	37.88	39.98	2.74	0.715
TRF (mg/mL)	2.67	3.58	2.63	3.30	0.23	0.409

†Data are expressed as means of the replicates (n = 6). In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).

Table 6 shows the results of serum biochemical indicators tested. The concentration of serum ferritin in T2 was significantly higher than that of T3 and T4 and numerically higher than T1 as well; however, supplemental glycine complexed trace minerals to replace inorganic trace minerals did not affect other serum parameters tested.

Immune Response

There were no significant differences on the concentration of serum IgA, IgG, IgM, C3 and C4 among the

four treatments (Fig. 1A). The similar results were also found in serum inflammatory factors (TNF-α, IL-6 and IL-10) as shown in Fig. 1B. However, the sIgA concentration of ileum in T2 was significantly higher than that of T1 (Fig. 1C).

Intestinal Morphology

The duodenal VH in 70% GCM treatment was significantly higher than that in other 3 treatments; similarly, the v/c of T4 was also numerically higher than the other three treatments (Fig. 2D). In the jejunal morphology, VH and CD of T4 was higher than that of T1, and T3 had the highest v/c though it did not reach the significant difference (Fig. 2E). The SEM images of the jejunum showed that intestinal villi from T3 and T4 were more intact than that of T1 and T2 (Fig. 2C). Under 20,000 times magnification, the jejunum microvilli of T3 and T4 were more serried than T1 and T2 (Fig. 2C).

Relative Expression of Metal Transporter mRNA

In Fig. 3, the relative expression of ZnT1 mRNA in T4 was higher ($P < 0.05$) than that in the other three treatments. The relative expression of Fpn1, Ctr1 and DMT1 mRNA were not affected by dietary micromineral treatment.

Minerals Excretion

Table 7 shows that the amount of trace minerals excreted in urine and feces were positively correlated with the dietary supplemental mineral level. The trace minerals excretion in urine and feces of low-dose treatments (T3 and T4) were lower ($P < 0.05$) than the commercial level minerals treatments (T1 and T2), and the trace minerals excretion in T3 was also lower ($P < 0.05$) than T4. When 50% of dietary ITM replaced by OTM from Glycinates, the excretion amount of Zn and Fe in urinary sample of T2 was lower ($P < 0.05$) than that of T1.

Table 7. Effect of inorganic trace minerals replaced by glycine complexed trace minerals on minerals excretion in urine and feces[†]

Item	100%ITM	50%ITM+50%GCM	50%GCM	70%GCM	SEM	P - value
Urine						
Cu, μg/L	0.86[a]	0.79[a]	0.44[c]	0.53[b]	0.04	0.000
Zn, μg/L	1.99[a]	1.83[b]	0.86[d]	1.01[c]	0.11	0.000
Fe, μg/L	0.50[a]	0.42[b]	0.24[d]	0.30[c]	0.02	0.000
Mn, μg/L	0.18[a]	0.14[ab]	0.10[c]	0.12[b]	0.01	0.000
Feces						
Cu, mg kg^{-1}	249.60[a]	244.93[a]	137.57[c]	186.82[b]	12.15	0.000
Zn, mg kg^{-1}	1181.45[a]	1123.11[a]	550.61[c]	783.81[b]	68.97	0.000
Fe, mg kg^{-1}	1663.33[a]	1597.89[a]	775.43[c]	1081.81[b]	97.91	0.000
Mn, mg kg^{-1}	358.67[a]	383.95[a]	198.87[c]	266.19[b]	19.61	0.000

[†]Data are expressed as means of the replicates (n = 4). In the same row, values with no letter or the same letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$).

Discussion

The trace minerals required for growth and development of piglets after weaning are mainly from innate reserves and diets. Due to the incomplete development of intestinal and low feed intake within a period of time after weaning, the trace minerals taken by piglets from the diet are limited. Therefore, providing the piglets with higher bio-available trace minerals may be an effective method to reduce the amount of trace minerals used. In our study, we evaluated the effects of low-dose glycine-complexed trace minerals replacing ITM in piglets.

Nowadays, livestock is generally fed diets that are formulated to provide an excess of trace minerals to maximize growth performance. The growth-promoting mechanism of trace minerals as Cu and Zn is mainly that a high concentration of metal ions could inhibit the growth of intestinal microbes and stimulate the release of neuropeptide Y, which results in increase of feed intake, maintain the body's immune function and antioxidant capacity [12-15]. In our study, the ADFI of the low-dose GCM treatment (T4) was numerically lower than that of

T1 with commercial level of supplemental ITM, and the feed intake of the partial replacement treatment (T2) was numerically higher than that of other 3treatments in phase 1. Thus, higher feed intake and Cu, Zn concentration may be the reason for the greater ADG and G:F in T2 compared with other 3 treatment groups. However, during the overall feeding period, low doses of glycine-complexed trace minerals did not have a significant impact on piglet performance, which is similar to Feng's findings [16]. The vulnerable intestinal mucosa of piglets is susceptible to damage due to conversion from milk to solid feed after weaning. The pigs treated with glycine-complexed trace minerals had more intact intestinal villi in that the jejunum microvilli of pigs in T3 and T4 were more serried than that in T1. We speculated that this may be due to the fact that glycine from complex can promote protein synthesis in intestinal epithelial cells and reduce epithelial cell apoptosis [17].

It is obviously biased to evaluate the health status of piglets with growth performance. Thus, a commercial level of supplemental ITM was used as a control to explore whether low doses of OTM have an impact on the health of piglets from serum biochemical parameters and immunity. Serum total protein is composed of albumin and globulin, and their levels are important indicators of serological examinations, and they are generally affected by the concentration of dietary protein and disease. In our study, supplemental low-dose OTM from GCM did not cause significant changes in serum total protein and albumin, which may indicate that metal elements have less effect on protein digestion and absorption. Serum urea can reflect the utilization of protein, compared to trace mineral sulfates, glycine-complexed trace minerals provide glycine while providing trace elements, but this seems to be negligible for nitrogen metabolism. Alkaline phosphatase is one of the biochemical indicators for evaluating the inflammation in the liver and kidneys [18]. The study of its structure found that ALP contains 2 binding sites of Zn^{2+}, and Zn^{2+} are not free to enter and exit the ALP due to the presence of Glu49 residue [19]. That may explain that the difference of zinc ion concentration did not affect the level of serum ALP in this study. It also indicated that the low doses of GCM are sufficient to maintain the

normal function of the liver and kidney. After absorption by intestinal epithelial cells, the iron in the diet enters the plasma and binds with transferrin to participate in the transport [20]. The total iron-binding capacity reflects the maximum amount of iron that can be bound by transferrin, which actually indicates the level of serum transferrin. In our study, there was no significant difference of transferrin and total iron binding capacity among the 4 treatments, indicating that the amount of iron involved in body transport was not affected by dietary iron concentrations. However, in serum ferritin, which implicates serum iron storage capacity, we found that T2 had significantly higher serum ferritin concentration than T3 and T4. This indicates that the total amount of iron entering the serum is still affected by the trace minerals concentration of the diet, while low-dose treatments had no significant difference in serum ferritin compared to the control treatment, which may prove that glycine complexed iron has a higher digestibility than the ferrous sulfate. Ceruloplasmin is a ferroxidase that contains greater than 95% of the copper found in plasma. It appears to have more than one functional role in mammalian metabolism such as copper transport and antioxidant defense [21]. It is also considered to be a positive acute phase protein in pigs, so the activity of ceruloplasmin can be used to reflect whether the animal is in a normal physiological state [22,23]. In our study, the activity of ceruloplasmin was not affected by different concentrations of Cu^{2+} in the diet, whereas in Feng's study [24], the high copper diet significantly increased the activity of ceruloplasmin, which may be related to the considerable difference in the amount of copper added in the two experiments. It is worth mentioning that our results are consistent with the findings of Creech's study [25], which has the similar copper doses.

Serum antibodies are the essential component of innate and adaptive immunity and immunological memory. They also contribute significantly to immunopathology [26]. In this study, serum immunoglobulins (IgA, IgG, and IgM) and complements 3 and 4 were not affected by the level and source of trace minerals, which is similar to the results of Feng's research [27] in which piglets were fed with iron glycine chelate. This indicates that the

minimum treatment dose in this study was not sufficient to affect humoral immunity. The weaning process of piglets is often accompanied by inflammation, which can trigger up-regulation of pro-inflammatory cytokines that can damage the mucosal barrier and intestinal permeability, such as TNF-α, IL-6. In Pu's study [28], supplementation iron dextran to newborn piglets reduced the expression of inflammatory factors TNF-α and IL-6 mRNA, confirming that iron supplementation can alleviate the increase in inflammatory factors caused by iron deficiency. In present study, we found that serum concentrations of pro-inflammatory factors (TNF-α, IL-6) and anti-inflammatory factor (IL-10) were not affected by different treatments, indicating that the doses used in this experiment were not low enough to cause any significant difference in cytokines. Secretory IgA (sIgA) serves as the first line of defense in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms. Through a process known as immune exclusion, sIgA promotes the clearance of antigens and pathogenic microorganisms from the intestinal lumen by blocking their access to epithelial receptors, entrapping them in mucus, and facilitating their removal by peristaltic and mucociliary activities [29,30]. In Fig. 1C, only ileal sIgA exhibited significant differences between the treatments, and the concentration of sIgA in mucosa of T2 was significantly higher than that of T1, suggesting that glycine complexed trace minerals can improve intestinal mucosal immunity in piglets. A similar study by Levkut did not find that jejunal sIgA concentrations in broilers were affected by dietary concentrations of $ZnSO_4$ and Gly-Zn [31].

The intestinal mucosa is formed by monolayer columnar epithelium cells, performing the primary functions in digesting, absorbing of nutrients, and preventing luminal pathogens and toxic substances cause any damage. Early weaning stress syndrome usually leads to a decrease in VH and intestinal dysfunction in piglets. Impaired intestinal epithelial function disrupts immune homeostasis and increases inflammation, disturbing the intestinal barrier function, unbalancing absorptive-secretory electrolytes, and leading to fluid and diarrhea [32]. Payne reported that jejunum VH of weanling pigs from sows fed $ZnSO_4$ or Zn-AA was greater than those from sows

fed the control diet [33]. Ma also found that feeding 90 mg kg^{-1} Zn-Gly increased the VH of the jejunal mucosa of broilers [34]. In our results, the duodenum VH of the piglets in the 70% GCM treatment was increased, indicating that the higher concentrations of GCM are positive for the intestinal epithelium cells. The results of optical microscopy and electron microscopy (×150) also showed that the jejunum villi morphology in T3 and T4 were more intact. At 20,000× magnification, the jejunum villi of T3 and T4 were also denser. This may be related to the nutritional function of glycine in the GCM discussed above.

The intact intestinal villus morphology not only reduces local inflammation and diarrhea in the intestine, but also facilitates the absorption of nutrients. In the apparent total tract digestibility of trace minerals, glycine complexed trace minerals exhibited higher digestibility than inorganic trace minerals, and there were significant differences in iron and zinc. ZnT1, Fpn1 and Ctr1 are specific transporters of zinc, iron and copper, respectively. ZnT1 is located in the basolateral membrane of intestinal epithelial cells, transporting Zn^{2+} absorbed into intestinal epithelial cells into capillaries [35,36]. Fpn1 is also located in the basolateral membrane of small intestinal epithelial cells, oxidized Fe^{2+} into Fe^{3+} in coordination with ferrous oxidase (HP), and transported to plasma for binding with transferrin [37,38]. Ctr1 is located in the cell membrane of small intestinal epithelial cells, and Cu^{2+} first binds it and then enter into intestinal epithelial cells [39-41]. DMT1 is a divalent metal transporter, participating in the absorption of dietary divalent metal ions (Fe^{2+}, Mn^{2+}, etc.) in the small intestine epithelium, and transporting metal ions from the digestive tract into the blood [42]. By further measuring the expression of metal transporter mRNA in the duodenal mucosa, we found that the increase expression of ZnT1 and Fpn1 mRNA in T4 was consistent with the aforementioned high apparent digestibility. These two results demonstrate that the digestibility of glycine complexed iron and zinc is higher than that of inorganic iron and zinc. In the two treatments supplemented with 100% trace minerals, there was no significant difference in ATTD of trace minerals, but in the results of trace mineral emissions from urine and feces, T2 had lower trace mineral

emissions than T1 (except Mn), the concentration of urine zinc and urine iron in T2 was significantly lower than that in T1. Since the digestibility of glycine complexed trace minerals is higher than that of inorganic trace minerals, compared with 100% ITM treatment, 50% ITM+50% GCM treatment has more metal ions absorbed into the body, while the excretions were also lower. This indicates that glycine complexed trace minerals have higher bioavailability and are more easily selected for storage. Although the metal excretions of T3 and T4 were lower, it is difficult to conclude whether this was due to the difference in the amount of addition or bioavailability.

Conclusion

Under the current trial conditions, our results indicate that glycine-complexed minerals have higher bioavailability than inorganic trace minerals; supplementation of low-dose GCM to replace ITM could reduce fecal minerals excretion without affecting performance, blood profiles, immune responses and intestinal morphology in piglets.

Ethics approval and consent to participate

All procedures applied in this study were approved by the Zhejiang University Institution Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health guidelines for the care and use of experimental animals.

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

No conflict of interest exists.

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Author contributions

Minqi Wang conceived and designed the whole scheme of the experiments. Xun Pei, Geng Wang conducted the experiments. Lujie Liu and Wanjing Sun participated in the determination of experimental indexes. Zhiping Xiao and Wenjing Tao helped analyze and interpret the experimental data. Mingyan Huai provided glycine complexed trace minerals. Xun Pei wrote the manuscript. Mingyan Huai, Lily Li and Wolf Pelletier revised and copy-edited the manuscript.

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469 **Table 1.** Composition and nutrient levels of the basal diet (as-fed basis)

Ingredients / nutrient level (%)	Phase I (0-14d)	Phase II (15-28d)
Corn	43.0	64.5
Expanded soybean	7.5	0
Expanded rice	8.0	0
Fermented soybean meal	7.5	7.5
Soybean meal	0	11.0
Fish meal	3.0	3.0
Wheat flour	5.0	5.0
Cheese powder	2.5	2.5
Spray dried blood meal	2.0	0
Intestinal membrane protein	2.5	0
Dried whey	7.5	0
Sucrose	7.5	2.5
Calcium citrate	1.2	1.2
$CaHPO_4$	0.6	0.6
NaCl	0.2	0.2
Titanium dioxide	0.3	0.3
L-Lysine HC	0.45	0.45
DL-Methionine	0.05	0.05
L-Threonine	0.2	0.2
L- Tryptophan	0.05	0.05
Vitamine premix	0.04	0.04
Trace minerals premix†	0.5	0.5
Enzymes‡	+	+
Antibiotics§	+	+
Total	100	100
Nutrient level¶ %		
CP	18.10	17.70
EE	4.04	3.49
CF	1.63	2.23
Lys	1.42	1.32
Met	0.36	0.36
DE（MJ Kg^{-1}）	12.42	12.93

Continued

Ca	0.73	0.69
Total P	0.56	0.54

[†]Provided as experimental design.

[‡]Natuphos E 10000, 50mg ton^{-1} feed.

[§]Antibiotics supplemented in feed per ton：Enramycin 12 mg，Terramycin calcium 200 mg, Macleaya cordata premix 500 mg, Gallotannic acid premix 500 mg；

[¶]Analyzed data except DE.

476 **Figure Legends**

477 **Figure 1. Effects of different levels of glycine complexed trace minerals on Serum and intestinal mucosal**
478 **immunity of weaned piglets.** A. Serum immunoglobulin and complement levels; B. Serum TNF-α, IL-6, and
479 IL-10 levels; C. Jejunum and ileum mucosal sIgA levels. Data are expressed as means ± SEM (n = 6). Values
480 with "*" are significantly different ($P < 0.05$), whereas those with "**" are extremely significant different ($P <$
481 0.01).

482

483 **Figure 2. Microscopic observation of intestinal villus morphology and Measurements of villus height,**
484 **crypt depth, and villus height/crypt depth (V/C) in duodenum, jejunum.** A. Histological photomicrographs
485 of duodenum section with optical microscopy, ×400; B. Histological photomicrographs of jejunum section with
486 optical microscopy, ×400; C. Photomicrographs of jejunum villus with scanning electron microscope (SEM),
487 The above is ×150, the bottom is ×20000; D. Villus height, crypt depth, and V/C in duodenum; E. Villus height,
488 crypt depth, and V/C in jejunum. Data are expressed as means ± SEM (n = 6). Values with "*" are significantly
489 different ($P < 0.05$), whereas those with "**" are extremely significant different ($P < 0.01$).

490

491 **Figure 3. Effects of different levels of glycine complexed trace minerals on relative expression of metal**
492 **transporter.** Data are expressed as means ± SEM (n = 6). Values with "*" are significantly different ($P < 0.05$),
493 whereas those with "**" are extremely significant different ($P < 0.01$).

Figures

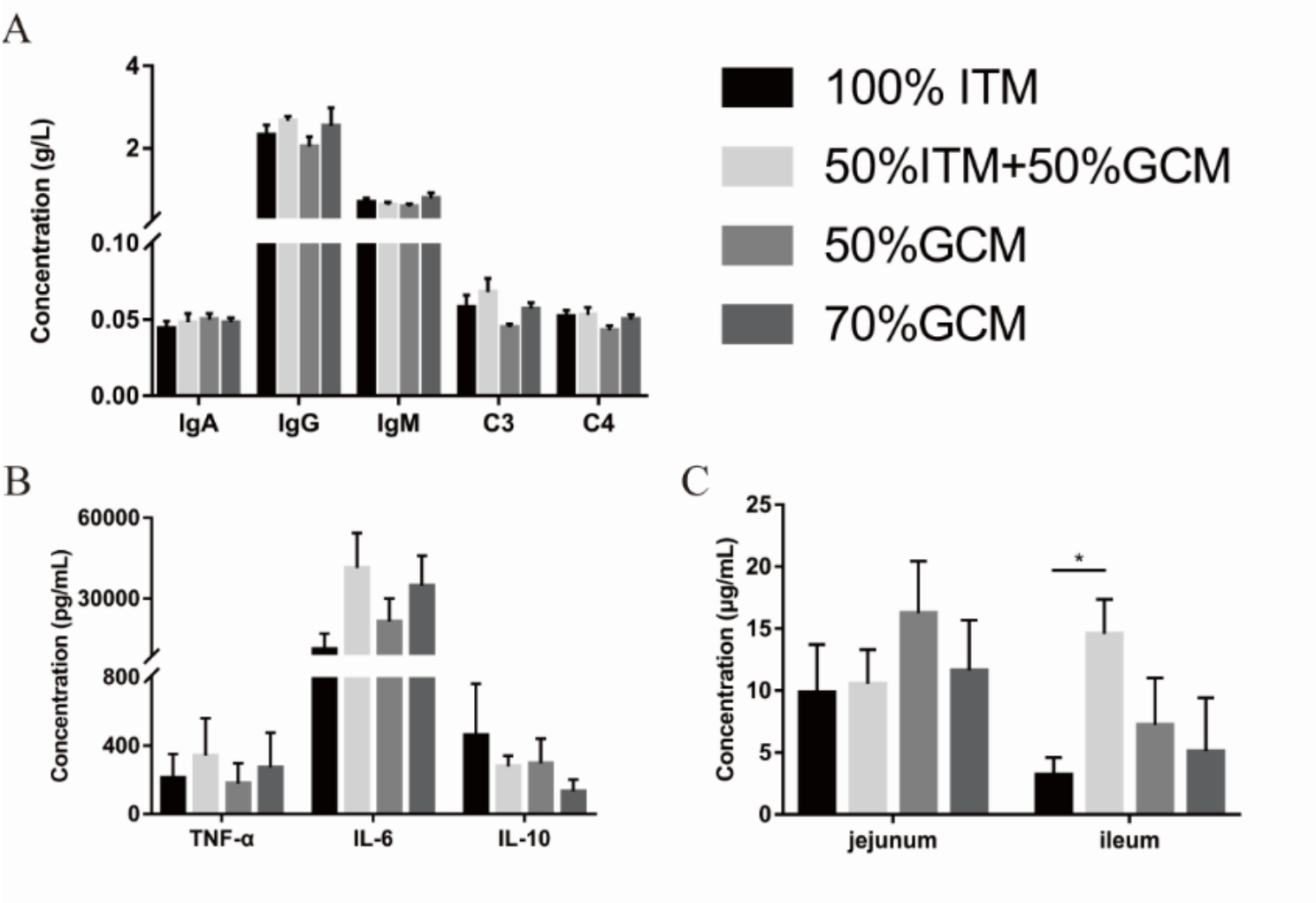

Figure 1

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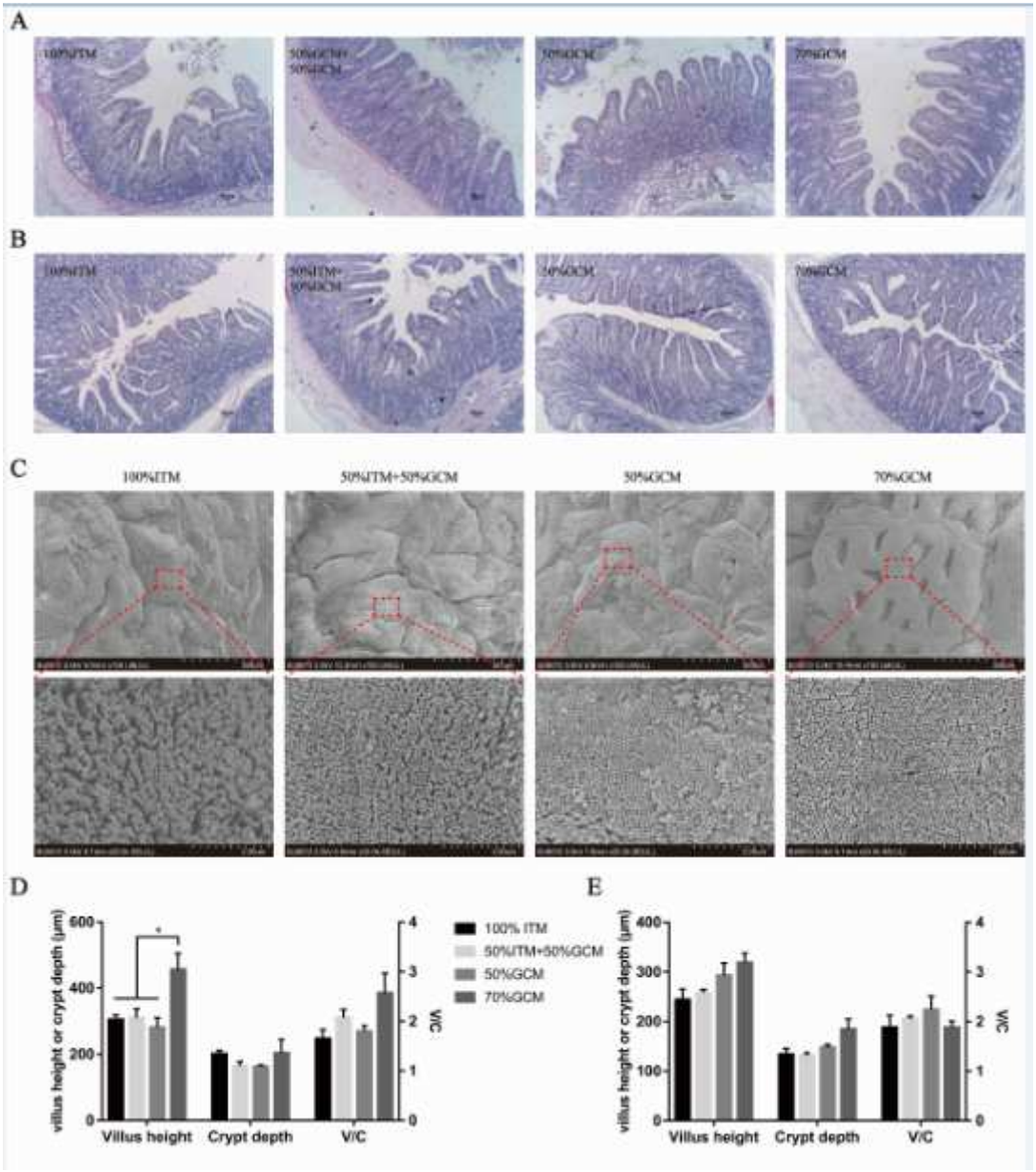

Figure 2

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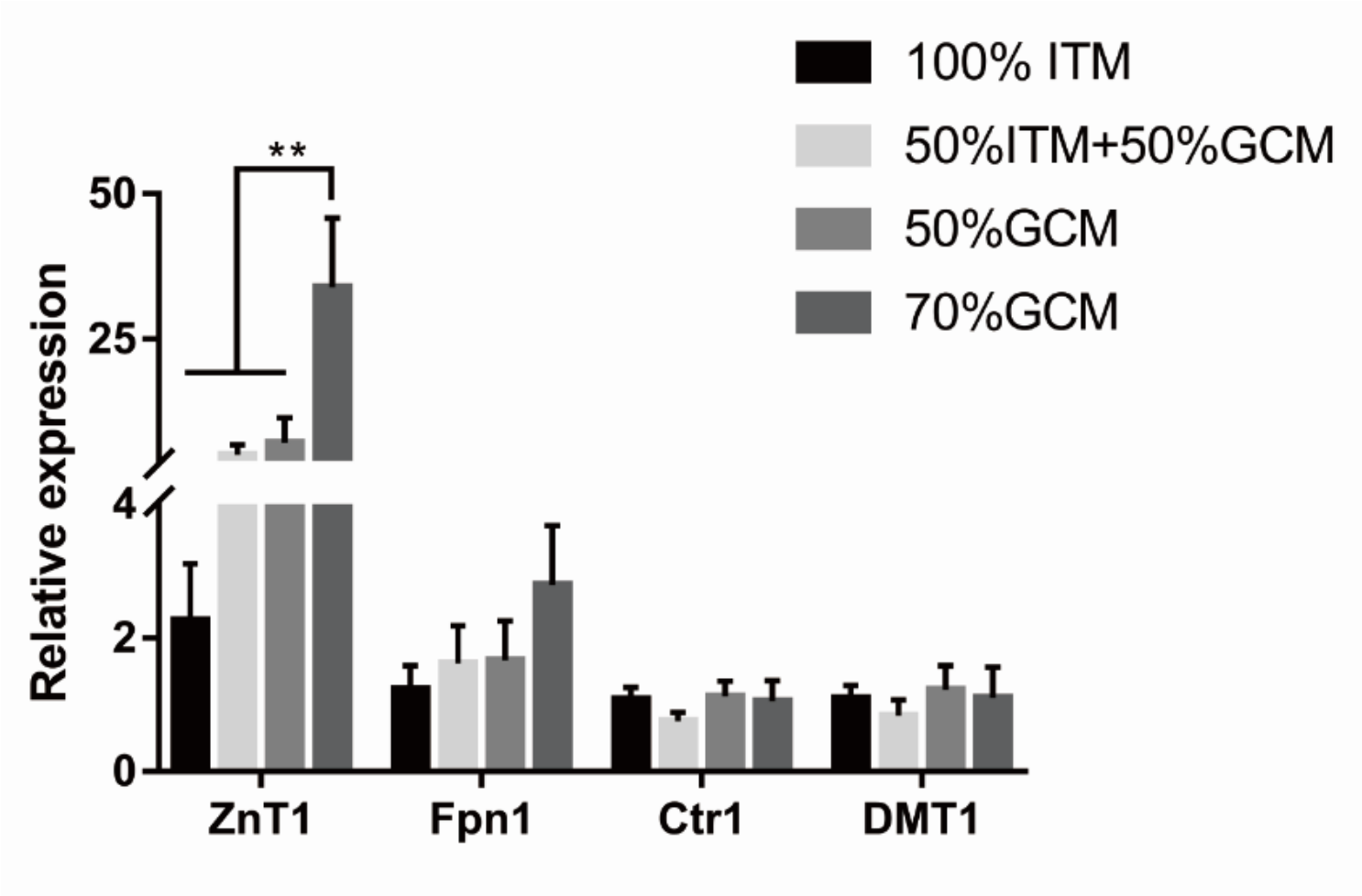

Figure 3

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