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Effects of high nutrient intake on the growth performance, intestinal morphology and immune function of neonatal intra-uterine growth-retarded pigs

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Abstract

Intra-uterine growth-retarded (IUGR) neonates have shown an impairment of postnatal intestinal development and function. We hypothesised that the immune function of IUGR neonates might be affected by increased nutrient intake (NI) during the suckling period. Therefore, we investigated the effects of high NI (HNI) on the growth performance, intestinal morphology and immunological response of IUGR and normal-birth weight (NBW) piglets. A total of twelve pairs of IUGR and NBW piglets (7 d old) were randomly assigned to two different nutrient-level formula milk groups. After 21 d of rearing, growth performance, the composition of peripheral leucocytes, serum cytokines and intestinal innate immune-related genes involved in the Toll-like receptor (TLR)-4–myeloid differentiation factor 88–NF-κB pathway were determined. The results indicated that IUGR decreased the average daily DM intake (ADMI) and the average daily growth (ADG). However, the ADMI and ADG were increased by HNI, irrespective of body weight. Likewise, serum cytokines (TNF-α and IL-1β) and ileal gene expressions (*TLR-4*, *TLR-9*, *TRAF-6* and *IL-1β*) were lower in IUGR piglets, whereas HNI significantly increased blood lymphocyte percentage and serum IL-10 concentrations, but decreased neutrophil percentage, serum IL-1β concentrations and ileal gene expressions (*NF-κB* and *IL-1β*). Furthermore, IUGR piglets with HNI exhibited lower serum concentrations of TNF-α and IL-1β than NBW piglets, and these alterations in the immune traits of IUGR piglets receiving HNI were accompanied by decreasing ileal gene expressions of *TLR-4*, *TLR-9*, *NF-κB* and *IL-1β* that are related to innate immunity. In conclusion, the present findings suggest that increased NI during the suckling period impaired the immune function of neonatal piglets with IUGR.

Key words: Intra-uterine growth-retarded pigs: Nutrient intakes: Immune function: Intestines

Intra-uterine growth retardation (IUGR) is usually defined as impaired growth and development of the embryo and/or its organs during gestation[1]. In humans, IUGR has been observed in about 23·8 % of newborns and approximately thirty million babies worldwide suffer from IUGR every year[2]. Previous studies have shown that IUGR neonates are associated with higher postnatal morbidity and mortality[3,4]. Due to developmental and growth restriction, IUGR neonates often appear immature with regard to the digestive and immune systems compared with their normal counterparts[5,6]. For example, changes in histopathology and thymus size have been observed[7,8], and lower numbers of T cells in the thymus[9] as well as an abnormal cytokine profile in serum[10] and the intestine[5,10,11] have been reported in IUGR neonates.

In order to achieve catch-up growth, human IUGR neonates are generally fed a high-protein formula[12] or a special formula containing a high density of nutrients[13]. However, catch-up growth in the first few weeks of postnatal life renders IUGR neonates to an increased risk of the metabolic syndrome such as obesity or other obesity-related diseases in later life[14]. In addition, evidence in poultry has shown that high nutrient density could decrease the immune function[15], whereas decreased feed intake can optimise the immune system[16]. Although it has been widely reported that IUGR impairs intestinal development and function, the intestinal innate immune response and the role of early high nutrient intake (HNI) in regulating innate immunity are still vague. Because of the physiological and genomic similarities between pigs and humans[17], the pig has been recognised as an ideal

Abbreviations: ADMI, average daily DM intake; ANI, adequate nutrient intake; BW, body weight; HNI, high nutrient intake; IUGR, intra-uterine growth retardation; NBW, normal birth weight; NI, nutrient intake; TLR, Toll-like receptor; VCR, villous height:crypt depth ratio.

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model for the study of clinical nutrition. Moreover, as a multifetal domestic animal, pigs display severe naturally occurring IUGR due to uteroplacental insufficiency[18].

Therefore, studying the immunological response of IUGR piglets to HNI may provide useful information on IUGR human infants fed a nutrient-enriched formula. The aim of the present study was to assess the difference in growth and immune function between IUGR and normal-birth weight (NBW) piglets in response to HNI during the suckling period.

Materials and methods

Animal care and formula milk

The animal use and care protocol was approved by the Animal Care and Use Committee of Sichuan Agricultural University. The basic formula milk powder (Table 1) was formulated according to previous studies[11,19]. The basic nutrient-level formula milk was prepared by mixing 1 kg of formula powder (DM 87·5 %) with 4 litres of water to a milk solution, which was similar to sow milk composition. The high nutrient-level formula milk was prepared by mixing 1·73 kg of formula powder with 4 litres of water to the milk solution, whose nutrient contents were about 1·5-fold those of the former.

Animal housing and experimental design

Piglets with a birth weight near the mean litter birth weight (SD 0·5) were identified as NBW, whereas those with at least 1·5 SD lower birth weight were defined as IUGR according to our previous study[20]. The average birth weights of NBW and IUGR piglets (Duroc × (Landrace × Yorkshire)) used in the present study were 1·52 (SD 0·06) and 0·87 (SD 0·04) kg, respectively. Piglets were fed with liquid diets at 50 ml/kg body weight (BW) per meal with a feeding bottle seven times per d at 3 h intervals between 06.00 and 24.00 hours. Therefore, piglets receiving the basic nutrient-level formula milk had adequate nutrient intake (ANI), whereas those receiving the high nutrient-level formula milk had HNI. A total of twelve pairs of IUGR and NBW piglets, regardless of sex, at 7 d of age from twelve sows were selected and allotted to one of the two dietary groups. This produced four experimental groups (birth weight/nutrient intake (NI)): IUGR/ANI, NBW/ANI, IUGR/HNI and NBW/HNI (n 6 per group). All pigs were housed individually in metabolism cages (0·8 m × 0·7 m × 0·4 m) at an ambient temperature of 30°C in an environmentally controlled room. Room humidity was controlled between 50 and 60 % during the experimental period of 21 d. Piglets had free access to water. The BW and the formula milk intake of pigs were recorded daily. The average daily DM intake (ADMI) was calculated by multiplying the average daily intake of formula milk by its corresponding DM content. Formula milk intake was calculated as the difference between the offered amounts and the refusals.

Table 1. Composition and nutrient level of the basal formula milk powder (87·5 % DM basis, %)

Ingredients	%
Whole-milk powder (24 % CP)	58·00
Whey protein concentrate (34 % CP)	25·00
Casein	5·70
Coconut oil	10·00
CaH_2PO_4	0·10
Choline chloride (50 %)	0·10
Vitamin premix*	0·10
Mineral premix†	0·50
L-Arg (98·5 %)	0·06
DL-Met (98·5 %)	0·06
L-Lys·HCl (78·5 %)	0·30
L-Thr (98 %)	0·03
L-Trp (98 %)	0·05
Total	100·00
Nutrient content	
Digestible energy (kJ/kg)	18 390
CP (%)	25·30
Ca (%)	1·02
Total P (%)	0·81
Available P (%)	0·67
Digestible Lys (%)	1·93
Digestible Met (%)	0·63
Digestible Arg (%)	0·86

CP, crude protein.
* Vitamin premix provided per kg powder diet: vitamin A, 0·94 mg; vitamin D_3, 0·01 mg; vitamin E, 20 mg; vitamin K_3, 1 mg; vitamin B_{12}, 0·04 mg; riboflavin, 5 mg; niacin, 20 mg; pantothenic acid, 15 mg; folic acid, 1·5 mg; thiamin, 1·5 mg; pyridoxine, 2 mg; biotin, 0·1 mg.
† Mineral premix provided per kg powder diet: Zn, 90 mg; Mn, 4·0 mg; Fe, 90 mg; Cu, 6·0 mg; I, 0·2 mg; Se, 0·3 mg.

Blood sampling and analyses

Blood samples were collected by venepuncture on the morning (08.00 hours) of days 14 and 21 after an overnight fast. A part of the sample was injected into Eppendorf tubes containing sodium heparin for the examination of leucocytes. The rest were allowed to coagulate for 40 min before centrifugation (3500 ***g***, 10 min). Eppendorf tubes were immediately placed on ice until they arrived at the veterinary hospital for leucocyte determination (within 2 h). The isolated serum samples were then stored at −80°C until analysis. Leucocyte examination (neutrophil, lymphocyte and monocyte counts) was done through an automatic blood analyser. Serum TNF-α, IL-1β and IL-10 were assayed using corresponding commercially available porcine ELISA kits (R&D Systems). The minimum detectable concentrations of TNF-α, IL-1β and IL-10 were 7, 30 and 8 pg/ml, respectively.

Tissue sample collection

At the end of the experiment, all piglets were anaesthetised with an intravenous injection of pentobarbital sodium (15 mg/kg BW) and slaughtered. The liver, spleen, kidney and pancreas of each piglet were weighed immediately. The length and weight of the small intestine were measured after the removal of luminal contents. Duodenal, jejunal and ileal samples of approximately 2 cm in length were stored in 4 % methanal solution for histological analyses. The rest of the ileum was frozen in liquid N_2, and then stored at −80°C.

Small-intestinal morphology

Duodenal, jejunal and ileal samples stored in 4 % methanal solution were prepared after staining with haematoxylin and eosin

using standard paraffin embedding procedures. A total of five intact, well-oriented crypt–villus units were selected in triplicate for each intestine of piglets. Villous heights and crypt depths were measured using an image processing and analysis system (Optimus software version 6.5; Media Cybergenetics).

Total RNA extraction and real-time RT-PCR

Total RNA was isolated from ileal samples using TRIzol (catalogue no. 15 596-026; Invitrogen). RNA quality was verified by both agarose gel (1 %) electrophoresis and spectrometry (A260/A280, Beckman DU-800; Beckman Coulter, Inc.). Real-time RT-PCR was performed in duplicate to amplify the target gene and the reference gene of the ileum using the one-step SYBR® PrimeScript™ RT-PCR kit II (catalogue no. DRR086A; Takara). Briefly, the reaction mixture (10·0 μl) contained 5·6 μl of a freshly premixed one-step SYBR Green RT-PCR Master mix and a PrimeScript™ Enzyme Mix, 0·8 μl of the primer pair and 3·6 μl RNA template that contained about 150 ng RNA. PCR consisted of one cycle at 42°C for 5 min, one cycle at 95°C for 10 s and forty cycles at 95°C for 5 s and 60°C for 34 s, followed by a dissociation step at 95°C for 15 s, 60°C for 60 s and 95°C for 15 s. To confirm specific amplification, melt curve analysis was performed (ABI 7900HT; Applied Biosystems).

Relative mRNA abundance was determined using the Δ cycle threshold (ΔC_t) method, as outlined in the protocol of Applied Biosystems. In brief, a ΔC_t value is the C_t difference between the target gene and the reference gene ($\Delta C_t = C_t^{\text{target}} - C_t^{\text{reference}}$). For each of the target genes, the $\Delta\Delta C_t$ values of all the samples were calculated by subtracting the average ΔC_t of the corresponding IUGR/ANI group. The $\Delta\Delta C_t$ values were then converted to fold differences by raising 2 to the power $-\Delta\Delta C_t$ ($2^{-\Delta\Delta C_t}$). Further details on relative gene expression analysis have been described previously[21]. Primers (Table 2) for the assayed genes and the reference gene were designed using Primer Express 3.0 (Applied Biosystems).

Statistical analysis

Data of blood leucocytes and serum cytokines were analysed as repeated measures using the MIXED procedure of Statistical Product and Service Solutions 17.0 (SPSS, Inc.) according to the following model:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + U_k + \omega_l + (\alpha\omega)_{il} + (\beta\omega)_{jl} + (\alpha\beta\omega)_{ijl} + \varepsilon_{ijkl},$$

where μ is the mean; α_i is the effect of BW (i = IUGR, NBW); β_j is the effect of NI (j = ANI, HNI); $\alpha\beta_{ij}$ is the interaction between BW and NI; U_k is the litter ($k = 1, 2, \ldots, 12$); ω_l is the time (days 14 and 21), $\alpha\omega_{il}$ is the interaction between BW and time; $\beta\omega_{jl}$ is the interaction between NI and time; $\alpha\beta\omega_{ijl}$ is the interaction between BW, NI and time; $\varepsilon_{ijkl} \sim N(0, \sigma^2)$ represents the random error. Data of intestinal morphology were also analysed as repeated measures according to the model; however, ω_l here refers to the segment (duodenum, jejunum and ileum), $\alpha\omega_{il}$ refers to the interaction between BW and segment, $\beta\omega_{jl}$ refers to the interaction between NI and segment and $\alpha\beta\omega_{ijl}$ refers to the interaction between BW, NI and segment. Data on growth performance, organ indices and gene expressions were analysed according to the model, but omitting the effect of time and the interaction between time, BW and NI. Results are presented as means with their standard errors. Differences between groups were analysed using the general linear model procedure followed by Duncan's test. $P < 0{\cdot}05$ was considered as statistically significant.

Table 2. Primer sequences of the target and reference genes

Genes	Primer sequence (5′–3′)	Product (bp)	GenBank accession
TLR-2	Forward: TCGAAAAGAGCCAGAAAACCAT Reverse: CTTGCACCACTCGCTCTTCA	58	NM213761
TLR-4	Forward: AGAAAATATGGCAGAGGTGAAAGC Reverse: CTTCGTCCTGGCTGGAGTAGA	64	GQ304754
TLR-9	Forward: AATCCAGTCGGAGATGTTTGCT Reverse: GACCGCCTGGGAGATGCT	79	AY859728
MyD88	Forward: GTGCCGTCGGATGGTAGTG Reverse: TCTGGAAGTCACATTCCTTGCTT	65	NM001099923
TRAF-6	Forward: GCTGCATCTATGGCATTTGAAG Reverse: CCACAGATAACATTTGCCAAAGG	70	AJ606305.1
NF-κB	Forward: TGCTGGACCCAAGGACATG Reverse: CTCCCTTCTGCAACAACACGTA	60	AK348766.1
SIGIRR	Forward: ACCTGGGCTCCCGAAACTAC Reverse: GTCATCTTCTGACACCAGGCAAT	62	AK239384.1
TOLLIP	Forward: CCCGCGCTGGAATAAGG Reverse: CATCAAAGATCTCCAGGTAGAAGGA	74	AK239879.1
IL-1β	Forward: TCTGCCCTGTACCCCAACTG Reverse: CCAGGAAGACGGGCTTTTG	64	NM214055.1
β-Actin	Forward: GGCGCCCAGCACGAT Reverse: CCGATCCACACGGAGTACTTG	66	DQ845171.1

TLR, Toll-like receptor; *MyD88*, myeloid differentiation factor 88; *TRAF-6*, TNF receptor-associated factor 6; *SIGIRR*, single Ig IL-1-related receptor; *TOLLIP*, Toll-interacting protein.

Table 3. Effects of the level of nutrient intake on the growth performance of intra-uterine growth-retarded (IUGR) and normal-birth weight (NBW) neonates

(Mean values with their standard errors)

	ANI		HNI			*P*		
Parameters	IUGR	NBW	IUGR	NBW	SEM	BW	NI	BW×NI
Initial weight (kg)	1·68^{a}	2·76^{b}	1·68^{a}	2·80^{b}	0·19	<0·001	0·742	0·758
Final weight (kg)	5·34^{a}	7·74^{c}	6·32^{b}	9·01^{d}	0·64	<0·001	0·001	0·620
Net weight gain (kg)	3·66^{a}	4·99^{b}	4·62^{b}	6·14^{c}	0·55	<0·001	0·001	0·722
ADG (g/d)								
Days 0–7	152^{a}	225^{b}	180^{a}	233^{b}	33	<0·001	0·261	0·538
Days 7–14	152	163	146	174	40	0·327	0·902	0·669
Days 14–21	218^{a}	324^{b}	335^{b}	496^{c}	61	<0·001	<0·001	0·311
Days 0–21	174^{a}	237^{b}	221^{b}	292^{c}	26	<0·001	0·001	0·722
ADMI (g/d)								
Days 0–7	114^{a}	150^{b}	146^{b}	196^{c}	20	<0·001	<0·001	0·416
Days 7–14	108^{a}	153^{b}	164^{b}	247^{c}	17	<0·001	<0·001	0·021
Days 14–21	201^{a}	258^{b}	318^{c}	377^{d}	35	0·001	<0·001	0·928
Days 0–21	142^{a}	187^{b}	210^{b}	272^{c}	22	<0·001	<0·001	0·393
FCR*								
Days 0–7	0·77^{a}	0·67^{a}	0·83^{a,b}	0·94^{b}	0·12	0·932	0·006	0·073
Days 7–14	0·76^{a}	0·96^{a,b}	1·17^{b}	1·44^{c}	0·18	0·007	<0·001	0·637
Days 14–21	0·96	0·81	0·99	0·77	0·17	0·046	0·947	0·685
Days 0–21	0·82^{a,b}	0·79^{a}	0·97^{c}	0·93^{b,c}	0·08	0·466	0·003	0·920

ANI, adequate nutrient intake; HNI, high nutrient intake; BW, body weight; NI, nutrient intake; ADG, average daily gain; ADMI, average daily DM intake; FCR, feed conversion ratio.

^{a,b,c,d} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

* FCR was calculated by dividing the ADMI by its corresponding ADG.

Results

Growth performance

In the present study, regardless of the NI, the initial BW, final BW and BW gain of IUGR piglets were lower ($P<0·001$) than those of NBW neonates (Table 3). However, relative to NBW piglets with ANI, IUGR piglets receiving HNI had a comparable BW gain, but the final BW was still lower (−18·3%, $P<0·05$). Regardless of the NI, IUGR piglets had a lower ($P<0·001$) average daily gain during the 1st and 3rd weeks compared with NBW piglets; as a result, the overall average daily gain was lower ($P<0·001$) in IUGR piglets relative to NBW piglets. HNI increased ($P=0·001$) the final BW and BW gain of piglets and increased the average daily gain ($P=0·001$), the ADMI ($P<0·001$) and the feed conversion ratio ($P=0·003$) throughout the experimental period. BW and NI had no interaction effect on growth performance, except for the ADMI during the 2nd week ($P=0·021$). Furthermore, IUGR piglets with HNI had a similar average daily gain to NBW piglets receiving ANI due to the similar ADMI throughout the experimental period.

Organ indices

As shown in Table 4, BW ($P<0·001$) and NI ($P=0·005$) had a significant effect on the intestinal length:BW ratio in piglets. HNI decreased ($P=0·005$) the relative intestinal length but increased ($P=0·096$) the relative liver weight. The relative intestinal weight ($P=0·002$), intestinal length ($P<0·001$), liver weight ($P<0·001$) and pancreas weight ($P=0·023$) of IUGR piglets were significantly higher than those of NBW piglets. The relative intestinal weight, intestinal length and liver weight of IUGR piglets with HNI were increased ($P<0·05$) by 27·8, 15·3 and 29·3% than those of NBW piglets with

Table 4. Effects of the level of nutrient intake on the organ indices of intra-uterine growth-retarded (IUGR) and normal-birth weight (NBW) neonates

(Mean values with their standard errors)

	ANI		HNI			*P*		
Parameters	IUGR	NBW	IUGR	NBW	SEM	BW	NI	BW×NI
Intestinal weight:BW (%)	6·08^{b}	4·75^{a}	6·07^{b}	5·03^{a}	0·75	0·002	0·687	0·659
Intestinal length:BW (cm/kg)	170^{c}	124^{a}	143^{b}	112^{a}	13	<0·001	0·005	0·235
Liver weight:BW (%)	3·26^{b}	2·63^{a}	3·40^{b}	2·82^{a}	0·21	<0·001	0·096	0·774
Spleen weight:BW (%)	0·22	0·19	0·19	0·21	0·05	0·801	0·671	0·181
Kidney weight:BW (%)	0·40	0·37	0·40	0·40	0·06	0·368	0·596	0·669
Pancreas weight:BW (%)	0·25	0·20	0·24	0·20	0·04	0·023	0·903	0·799

ANI, adequate nutrient intake; HNI, high nutrient intake; BW, body weight; NI, nutrient intake.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Table 5. Effects of the level of nutrient intake on the count and percentage of blood leucocytes, neutrophils, lymphocytes and monocytes in intra-uterine growth-retarded (IUGR) and normal-birth weight (NBW) neonates
(Mean values with their standard errors)

	ANI		HNI			*P*			
Parameters	IUGR	NBW	IUGR	NBW	SEM	BW	NI	BW×NI	Time
Leucocytes (10^9/litre)									
Day 14	18·30	12·16	10·65	13·48	4·83	0·766	0·976	0·064	0·005
Day 21	9·70	8·71	11·83	8·70	3·50				
Neutrophils (10^9/litre)									
Day 14	8·13[b]	3·10[a]	2·07[a]	1·31[a]	2·18	0·111	0·250	0·264	0·006
Day 21	2·99	1·80	2·14	2·95	1·92				
Lymphocytes (10^9/litre)									
Day 14	9·83	8·96	9·29	11·06	4·18	0·204	0·476	0·142	0·003
Day 21	6·62	6·72	9·42	5·57	2·95				
Monocytes (10^9/litre)									
Day 14	0·15	0·12	0·09	0·07	0·06	0·240	0·186	0·384	0·002
Day 21	0·17	0·31	0·30	0·21	0·15				
Neutrophils (%)									
Day 14	41·90[b]	27·05[a,b]	17·53[a]	15·05[a]	12·89	0·115	0·015	0·237	0·003
Day 21	29·35	19·63	19·18	29·03	16·59				
Lymphocytes (%)									
Day 14	56·53[a]	72·02[a,b]	80·30[b]	84·32[b]	12·55	0·161	0·018	0·180	0·003
Day 21	69·45	78·33	77·83	68·88	17·52				
Monocytes (%)									
Day 14	0·80	0·93	0·77	0·65	0·26	0·145	0·198	0·259	0·003
Day 21	1·63	3·30	3·00	2·08	1·31				

ANI, adequate nutrient intake; HNI, high nutrient intake; BW, body weight; NI, nutrient intake.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

ANI, respectively. However, the relative weights of the spleen and kidney were not affected by IUGR or NI. No interaction was found between BW and NI for any of the relative weights of the organs.

Composition of peripheral leucocytes

No effect of BW or the interaction between BW and NI was observed on the count or percentage of neutrophils, lymphocytes and monocytes (Table 5). HNI significantly decreased ($P=0·015$) neutrophil percentage, but increased ($P=0·018$) lymphocyte percentage. The counts of leucocytes ($P=0·005$), neutrophils ($P=0·006$) and lymphocytes ($P=0·003$) were decreased on day 21. However, the count ($P=0·002$) and percentage ($P=0·003$) of monocytes were increased on day 21. In addition, the neutrophil count of IUGR piglets with ANI was increased ($P<0·05$) by 163 % compared with that of NBW piglets receiving HNI on day 14.

Table 6. Effects of the level of nutrient intake on the concentrations of TNF-α, IL-1β and IL-10 in intra-uterine growth-retarded (IUGR) and normal-birth weight (NBW) neonates
(Mean values with their standard errors)

	ANI		HNI			*P*			
Parameters	IUGR	NBW	IUGR	NBW	SEM	BW	NI	BW×NI	Time
TNF-α (pg/ml)									
Day 14	92·3[a,b]	99·5[b]	91·4[a]	97·4[a,b]	5·3	0·002	0·072	0·53	0·011
Day 21	100·6[a]	110·1[b]	95·3[a]	99·9[a]	5·5				
IL-1β (pg/ml)									
Day 14	215·1[a]	237·6[b]	212·0[a]	223·8[a]	10·0	<0·001	0·045	0·711	0·010
Day 21	245·3[a]	269·2[b]	237·0[a]	258·7[b]	10·1				
IL-10 (pg/ml)									
Day 14	113·0[b]	98·4[a]	115·0[b]	121·9[b]	9·9	0·202	<0·001	0·002	0·004
Day 21	121·6[b]	107·1[a]	124·9[b]	130·6[b]	9·4				
TNF-α:IL-10									
Day 14	0·82[a]	1·02[b]	0·80[a]	0·80[a]	0·07	<0·001	<0·001	<0·001	0·002
Day 21	0·83[a]	1·03[b]	0·77[a]	0·77[a]	0·05				
IL-1β:IL-10									
Day 14	1·92[a]	2·43[b]	1·86[a]	1·85[a]	0·19	<0·001	<0·001	<0·001	0·002
Day 21	2·04[a]	2·53[b]	1·91[a]	1·99[a]	0·20				

ANI, adequate nutrient intake; HNI, high nutrient intake; BW, body weight; NI, nutrient intake.
[a,b] Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Table 7. Effects of the level of nutrient intake on the intestinal morphology of intra-uterine growth-retarded (IUGR) and normal-birth weight (NBW) neonates

(Mean values with their standard errors)

	ANI		HNI			*P*			
Parameters	IUGR	NBW	IUGR	NBW	SEM	BW	NI	BW×NI	Segment
Villous height (μm)									
Duodenum	708^{a,b}	617^{a}	665^{a,b}	759^{b}	70	0·112	0·741	0·001	0·002
Jejunum	406^{a}	487^{c}	471^{b,c}	431^{a,b}	44				
Ileum	407^{a}	525^{c}	491^{b,c}	452^{b}	76				
Crypt depth (μm)									
Duodenum	172^{a,b}	160^{a}	189^{b}	158^{a}	37	0·477	0·353	0·077	0·013
Jejunum	93^{a}	102^{a,b}	109^{b}	98^{a,b}	19				
Ileum	112^{a}	117^{a}	135^{b}	108^{a}	27				
VCR									
Duodenum	4·32^{a,b}	4·02^{a}	3·66^{a}	4·82^{b}	1·20	0·038	0·388	0·779	0·002
Jejunum	4·49	4·85	4·40	4·47	0·97				
Ileum	3·86^{a}	4·70^{b}	3·78^{a}	4·23^{a,b}	0·91				

ANI, adequate nutrient intake; HNI, high nutrient intake; BW, body weight; NI, nutrient intake; VCR, villous height:crypt depth ratio.
^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

Serum concentrations of TNF-α, IL-1β and IL-10

As shown in Table 6, IUGR decreased serum concentrations of TNF-α ($P=0·002$) and IL-1β ($P<0·001$), as well as the ratios of TNF-α:IL-10 ($P<0·001$) and IL-1β:IL-10 ($P<0·001$) in piglets. HNI increased the concentration of IL-10 ($P<0·001$) but decreased IL-1β concentration ($P=0·045$), as well as the ratios of TNF-α:IL-10 ($P<0·001$) and IL-1β:IL-10 ($P<0·001$). BW and NI had significant interaction effects on IL-10 concentration ($P=0·002$), as well as on the ratios of TNF-α:IL-10 ($P<0·001$) and IL-1β:IL-10 ($P<0·001$). The concentrations of TNF-α ($P=0·011$), IL-1β ($P=0·010$) and IL-10 ($P=0·004$) as well as the IL-1β:IL-10 ratio ($P=0·002$) were higher on day 21 compared with those on day 14. However, the TNF-α:IL-10 ratio ($P=0·002$) decreased on day 21.

Intestinal morphology

Irrespective of the NI, IUGR decreased ($P=0·038$) the intestinal villous height:crypt depth ratio (VCR) in piglets (Table 7). No effect of NI was observed on intestinal morphology. BW and NI had a significant interaction effect on villous height ($P=0·001$). The villous height ($P=0·002$), the crypt depth ($P=0·013$) and the VCR ($P=0·002$) were significantly affected by the segment in the small intestine, with the duodenum having the highest villous height and the deepest crypt depth and the jejunum having the highest VCR. Furthermore, compared with NBW piglets receiving ANI, the duodenal and ileal crypt depths were deeper (15–18 %, $P<0·05$), but the ileal VCR was higher (− 20 %, $P<0·05$) in IUGR piglets receiving HNI, respectively.

Gene expression in the ileum

The mRNA abundance of Toll-like receptor (*TLR*)-*4* ($P=0·039$), *TLR-9* ($P=0·003$), TNF receptor-associated factor 6 (*TRAF-6*, $P=0·034$), *IL-1β* ($P=0·021$) and Toll-interacting protein (*TOLLIP*, $P=0·053$) was decreased in the ileum of IUGR piglets relative to NBW piglets (Table 8). HNI decreased

Table 8. Effects of the level of nutrient intake on the mRNA abundance of innate immune-related genes in the ileum of intra-uterine growth-retarded (IUGR) and normal-birth weight (NBW) neonates

(Mean values with their standard errors)

	ANI		HNI			*P*		
Genes	IUGR	NBW	IUGR	NBW	SEM	BW	NI	BW×NI
TLR-2	1·02	1·25	1·31	1·14	0·44	0·864	0·580	0·226
TLR-4	1·04^{a,b}	1·42^{b}	0·76^{a}	1·23^{a,b}	0·38	0·039	0·231	0·841
TLR-9	1·26^{a}	1·28^{a}	1·16^{a}	3·96^{b}	1·26	0·003	0·005	0·003
MyD88	1·10	1·09	0·72	1·25	0·46	0·214	0·585	0·201
TRAF-6	1·09	2·09	1·23	1·90	0·97	0·034	0·938	0·651
NF-κB	1·07^{a,b}	1·23^{b}	0·78^{a}	0·85^{a,b}	0·30	0·421	0·034	0·763
SIGIRR	1·16	1·08	0·81	0·83	0·47	0·891	0·112	0·780
TOLLIP	1·07^{a}	1·59^{b}	1·03^{a}	1·13^{a}	0·46	0·053	0·114	0·182
IL-1β	1·04	1·37	0·71	1·02	0·26	0·021	0·015	0·937

ANI, adequate nutrient intake; HNI, high nutrient intake; BW, body weight; NI, nutrient intake; *TLR*, Toll-like receptor; *MyD88*, myeloid differentiation factor 88; *TRAF-6*, TNF receptor-associated factor 6; *SIGIRR*, single Ig IL-1-related receptor; *TOLLIP*, Toll-interacting protein.
^{a,b} Mean values within a row with unlike superscript letters were significantly different ($P<0·05$).

the mRNA abundance of *NF-κB* ($P=0{\cdot}034$) and *IL-1β* ($P=0{\cdot}015$), but increased *TLR-9* ($P=0{\cdot}005$) mRNA expression in the ileum. BW and NI had no interaction effect on the mRNA abundance of these genes in the ileum, except for *TLR-9* ($P=0{\cdot}003$). Moreover, IUGR piglets receiving HNI had a lower ($P<0{\cdot}05$) mRNA abundance of *TLR-4*, *NF-κB* and *TOLLIP* in the ileum than NBW piglets receiving ANI.

Discussion

The present study was one of the rare studies documenting the effect of HNI during the suckling period on the growth and immune function of IUGR piglets reared in well-controlled conditions. The intriguing findings were that IUGR piglets exhibited a differential immune response to HNI compared with NBW piglets. Particularly, the increased NI during the 21 d of the suckling period impaired the systematic immune response of IUGR piglets by decreasing the number of leucocytes, altering the serum cytokine profile and the intestinal expression of innate immune-related genes.

The lower BW gain in IUGR pigs could be resulting from the inadequate intake of nutrients, as indicated by the markedly decreased average DM intake in IUGR pigs. However, IUGR pigs are able to exert a similar growth rate to normal pigs when receiving a similar DM intake. The present results showed that there was a comparable BW gain between the IUGR pigs with HNI and NBW pigs with ANI. These findings indicate that IUGR piglets receiving HNI achieved catch-up growth. The difference in the BW of piglets was due to the ADMI and the corresponding different nutrient contents of the formula milk.

IUGR could lead to a relatively longer intestine in neonates, as previously described in pigs[22], rabbits[23] and sheep[24]. In the present study, consistently, IUGR piglets exhibited a relatively longer intestine and a heavier liver and pancreas, indicating the potential metabolic priority over key organs relative to whole-body growth[25,26]. The liver plays a major role in the metabolism of dietary nutrients and other substances[27]. The higher relative liver weight in IUGR piglets with HNI may presumably be due to the compensatory hypertrophy of the liver, which is in accordance with the report of Rompala *et al.*[28] showing that rams with a high level of feed intake resulted in a greater liver weight:empty BW ratio.

Growth rate was increased in IUGR piglets receiving HNI. However, it seems that catch-up growth would impair the immune system according to the results of serum cytokines. The neonatal period was the intense period of changes in the expression of molecules involved in the recognition of bacteria by epithelial and immune cells, such as TLR and cytokines[11]. Cytokine concentrations and their ratios were sharply changed in IUGR piglets with HNI relative to NBW piglets, which suggested the compromised immune function of piglets.

In addition, during the neonatal period, cells of the innate immune system, predominantly neutrophils, macrophages and natural killer cells, are mainly responsible for the clearance of foreign antigens. In neonates, cells involved in triggering innate immunity are functional, but they are present in lower numbers and have lower enzyme activity than their adult counterparts[29]. In the present study, we did not test macrophages and natural killer cells, but the number and percentage of neutrophils were decreased along with the increase of lymphocyte percentage in IUGR piglets receiving HNI on day 14. As the events in the neonatal period allow the maturation of the immune system, peripheral lymphocyte subsets also showed certain changes. Comans-Bitter *et al.*[30] and de Vries *et al.*[31] found that an increase in T- and B-lymphocytes occurred during the 1st weeks of life, while natural killer cells declined after birth[30,31].

Small-intestinal morphology containing the villous height, the crypt depth and the VCR of the duodenum, jejunum and ileum is one of the major indicators reflecting gut health in piglets. The increasing villous height implied the increased surface area for nutrient absorption[32], whereas the deeper crypt suggested a fast new villous tissue turnover in response to normal sloughing or inflammation from a pathogen[33]. In the present study, IUGR piglets with HNI had higher jejunal and ileal villous heights, compared with piglets with ANI, which could be an important reason for the catch-up growth. However, the jejunal and ileal crypt depths in IUGR piglets receiving HNI were deeper. The present results are consistent with a previous study which suggested that piglets with a high level of feed intake had a higher villous height and a deeper crypt depth[34].

Moreover, the gastrointestinal tract is the largest immune organ in the body, and, as such, is the location for the majority of lymphocytes and immune effector cells with pattern recognition receptors[35], which sense luminal antigens and mediate the inflammatory response[36]. TLR are typical pattern recognition receptors in mediating mucosal innate host defence and in maintaining mucosal and commensal homeostasis[36]. MyD88, TRAF-6 and NF-κB are downstream signalling molecules and transcription factors shared by TLR-2, -4 and -9[37], while single Ig IL-1-related receptor and TOLLIP are crucial negative regulators[38]. It has been demonstrated that the TLR-4–Myd88–NF-κB signal pathway is involved in inflammation[39]. Nenci *et al.*[40] suggested that the down-regulation of *NF-κB* at the mRNA level might be a regulatory mechanism to augment long-term inflammatory responses. The decreased expressions of *TLR-4*, *TLR-9*, *NF-κB* and *IL-1β* in IUGR piglets receiving HNI suggested that HNI during the suckling period would reduce the intestinal innate immunity of IUGR piglets.

In summary, the present results suggest that HNI during the suckling period would lead to an abnormal immune function of neonatal piglets with IUGR. Further investigations are warranted to determine whether IUGR pigs with HNI would have a persistent impact on the immune system.

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References

1. Wu G, Bazer FW, Cudd TA, *et al.* (2004) Maternal nutrition and fetal development. *J Nutr* **134**, 2169–2172.
2. de Onis M, Blossner M & Villar J (1998) Levels and patterns of intrauterine growth retardation in developing countries. *Eur J Clin Nutr* **52**, Suppl. 1, S5–S15.
3. Aucott SW, Donohue PK & Northington FJ (2004) Increased morbidity in severe early intrauterine growth restriction. *J Perinatol* **24**, 435–440.
4. Garite TJ, Clark R & Thorp JA (2004) Intrauterine growth restriction increases morbidity and mortality among premature neonates. *Am J Obstet Gynecol* **191**, 481–487.
5. D'Inca R, Gras-Le Guen C, Che L, *et al.* (2011) Intrauterine growth restriction delays feeding-induced gut adaptation in term newborn pigs. *Neonatology* **99**, 208–216.
6. D'Inca R, Kloareg M, Gras-Le Guen C, *et al.* (2010) Intrauterine growth restriction modifies the developmental pattern of intestinal structure, transcriptomic profile, and bacterial colonization in neonatal pigs. *J Nutr* **140**, 925–931.
7. Cromi A, Ghezzi F, Raffaelli R, *et al.* (2009) Ultrasonographic measurement of thymus size in IUGR fetuses: a marker of the fetal immunoendocrine response to malnutrition. *Ultrasound Obstet Gynecol* **33**, 421–426.
8. Lang U, Baker RS, Khoury J, *et al.* (2000) Effects of chronic reduction in uterine blood flow on fetal and placental growth in the sheep. *Am J Physiol Regul Integr Comp Physiol* **279**, R53–R59.
9. Contreras YM, Yu X, Hale MA, *et al.* (2011) Intrauterine growth restriction alters T-lymphocyte cell number and dual specificity phosphatase 1 levels in the thymus of newborn and juvenile rats. *Pediatr Res* **70**, 123–129.
10. Zhong X, Li W, Huang X, *et al.* (2012) Impairment of cellular immunity is associated with overexpression of heat shock protein 70 in neonatal pigs with intrauterine growth retardation. *Cell Stress Chaperones* **17**, 495–505.
11. Chatelais L, Jamin A, Gras-Le Guen C, *et al.* (2011) The level of protein in milk formula modifies ileal sensitivity to LPS later in life in a piglet model. *PLoS One* **6**, e19594.
12. Premji S, Fenton T & Sauve R (2006) Higher versus lower protein intake in formula-fed low birth weight infants. *The Cochrane Database of Systematic Reviews* issue 1, CD003959.
13. Young L, Morgan J, McCormick FM, *et al.* (2012) Nutrient-enriched formula versus standard term formula for preterm infants following hospital discharge. *The Cochrane Database of Systematic Reviews* issue 3, CD004696.
14. Stettler N, Stallings VA, Troxel AB, *et al.* (2005) Weight gain in the first week of life and overweight in adulthood: a cohort study of European American subjects fed infant formula. *Circulation* **111**, 1897–1903.
15. Guo YL, Li WB & Chen JL (2010) Influence of nutrient density and lighting regime in broiler chickens: effect on antioxidant status and immune function. *Br Poult Sci* **51**, 222–228.
16. Jang I, Kang S, Ko Y, *et al.* (2009) Effect of qualitative and quantitative feed restriction on growth performance and immune function in broiler chickens. *Asian-Aust J Anim Sci* **22**, 388–395.
17. Humphray SJ, Scott CE, Clark R, *et al.* (2007) A high utility integrated map of the pig genome. *Genome Biol* **8**, R139.
18. Wu G, Bazer FW, Wallace JM, *et al.* (2006) Board-invited review: intrauterine growth retardation: implications for the animal sciences. *J Anim Sci* **84**, 2316–2337.
19. Dourmad JY, Noblet J & Etienne M (1998) Effect of protein and lysine supply on performance, nitrogen balance, and body composition changes of sows during lactation. *J Anim Sci* **76**, 542–550.
20. Che L, Thymann T, Bering SB, *et al.* (2010) IUGR does not predispose to necrotizing enterocolitis or compromise postnatal intestinal adaptation in preterm pigs. *Pediatr Res* **67**, 54–59.
21. Livak KJ & Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta *C*(T)) method. *Methods* **25**, 402–408.
22. Xu RJ, Mellor DJ, Birtles MJ, *et al.* (1994) Impact of intrauterine growth retardation on the gastrointestinal tract and the pancreas in newborn pigs. *J Pediatr Gastroenterol Nutr* **18**, 231–240.
23. Cellini C, Xu J, Arriaga A, *et al.* (2004) Effect of epidermal growth factor infusion on fetal rabbit intrauterine growth retardation and small intestinal development. *J Pediatr Surg* **39**, 891–897.
24. Avila CG, Harding R, Rees S, *et al.* (1989) Small intestinal development in growth-retarded fetal sheep. *J Pediatr Gastroenterol Nutr* **8**, 507–515.
25. Bauer R, Walter B, Hoppe A, *et al.* (1998) Body weight distribution and organ size in newborn swine (sus scrofa domestica) – a study describing an animal model for asymmetrical intrauterine growth retardation. *Exp Toxicol Pathol* **50**, 59–65.
26. Mostyn A, Litten JC, Perkins KS, *et al.* (2005) Influence of size at birth on the endocrine profiles and expression of uncoupling proteins in subcutaneous adipose tissue, lung, and muscle of neonatal pigs. *Am J Physiol Regul Integr Comp Physiol* **288**, R1536–R1542.
27. Jobgen WS, Fried SK, Fu WJ, *et al.* (2006) Regulatory role for the arginine–nitric oxide pathway in metabolism of energy substrates. *J Nutr Biochem* **17**, 571–588.
28. Rompala RE, Johnson DE, Rumpler WV, *et al.* (1991) Energy utilization and organ mass of Targhee sheep selected for rate and efficiency of gain and receiving high and low planes of nutrition. *J Anim Sci* **69**, 1760–1765.
29. Kovarik J & Siegrist CA (1998) Immunity in early life. *Immunol Today* **19**, 150–152.
30. Comans-Bitter WM, de Groot R, van den Beemd R, *et al.* (1997) Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. *J Pediatr* **130**, 388–393.
31. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, *et al.* (2000) Longitudinal survey of lymphocyte subpopulations in the first year of life. *Pediatr Res* **47**, 528–537.
32. Caspary WF (1992) Physiology and pathophysiology of intestinal absorption. *Am J Clin Nutr* **55**, 299S–308S.
33. Yason CV, Summers BA & Schat KA (1987) Pathogenesis of rotavirus infection in various age groups of chickens and turkeys: pathology. *Am J Vet Res* **48**, 927–938.

34. van Beers-Schreurs HM, Nabuurs MJ, Vellenga L, *et al.* (1998) Weaning and the weanling diet influence the villous height and crypt depth in the small intestine of pigs and alter the concentrations of short-chain fatty acids in the large intestine and blood. *J Nutr* **128**, 947–953.
35. Kelly D & Coutts AG (2000) Early nutrition and the development of immune function in the neonate. *Proc Nutr Soc* **59**, 177–185.
36. Newburg DS & Walker WA (2007) Protection of the neonate by the innate immune system of developing gut and of human milk. *Pediatr Res* **61**, 2–8.
37. Takeda K & Akira S (2004) TLR signaling pathways. *Semin Immunol* **16**, 3–9.
38. Shibolet O & Podolsky DK (2007) TLRs in the gut. IV. Negative regulation of toll-like receptors and intestinal homeostasis: addition by subtraction. *Am J Physiol Gastrointest Liver Physiol* **292**, G1469–G1473.
39. Kawai T & Akira S (2009) The roles of TLRs, RLRs and NLRs in pathogen recognition. *Int Immunol* **21**, 317–337.
40. Nenci A, Becker C, Wullaert A, *et al.* (2007) Epithelial NEMO links innate immunity to chronic intestinal inflammation. *Nature* **446**, 557–561.

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