Electronic Supplementary Information

Selenium deficiency decreases the development of spleen by deactivating the PI3K/Akt/mTOR pathway

Jianfa Wang,‡ Shuai Lian,‡ Xianjing He, Debin Yu, Dongbo Sun* and Rui Wu*

College of Animal Science and Veterinary Medicine in Heilongjiang Bayi Agricultural University; Heilongjiang Provincial Key Laboratory of Prevention and Control of Bovine Diseases, Daqing 163319, P.R. China. E-mail: fuhewu@126.com; dongbosun@126.com

‡ These authors contributed equally to the work.

Supplementary Tables

Table S1 Selenium concentration of control group and SD group chicken in the whole blood and spleen.

Sample	Group	7d	21d	35d
Whole blood	SD	0.0750±0.0031*	0.0433±0.0027**	0.0220±0.0030**
	Control	0.0822±0.0024	0.1433±0.0013	0.1545±0.0021
Spleen	SD	0.1516±0.0023**	0.0763±0.0027**	0.0634±0.0009**
	Control	0.2375±0.0009	0.2936±0.0040	0.3107±0.0073

* Indicates a significant difference from the control group at the $P < 0.05$ level;

** Indicates a significant difference from the control group at the $P < 0.01$ level.

Table S2 Effect of dietary Se on spleen weights, organ coefficients of spleen and splenocytes numbers.

Index	Group	7d	21d	35d
Spleen weight (g)	SD	0.0647±0.0165	0.1176±0.0281**	0.2373±0.0494**
	Control	0.0839±0.0051	0.3294±0.0409	0.8124±0.0387
organ coefficients of spleen (g/kg)	SD	0.6753±0.1474	0.7797±0.0981	0.8541±0.0143**
	Control	0.7740±0.0107	0.8787±0.0247	1.4250±0.0432
splenocytes number ($\times 10^8$)	SD	0.1525±0.0130*	0.2354±0.0271**	0.3617±0.0368**
	Control	0.2824±0.0224	0.4084±0.0329	0.4661±0.0437

* Indicates a significant difference from the control group at the $P < 0.05$ level;

** Indicates a significant difference from the control group at the $P < 0.01$ level.

Table S3 List of the vendors of the kits used in serum GH and IGF-I detection.

Kits Name	Product Number	Vendors Name
Chicken GH ^{125}I-labelled RIA Kit	XFFM1695	Shanghai Xinfan Biological Technology co., LTD
Chicken IGF-I ELISA Kit	YM-AQ2649	Shanghai Guduo Biological Technology co., LTD

Note:RIA= radioimmunoassay; ELISA= enzyme-linked immunosorbent assay

Table S4 List of primary and secondary antibodies used in immunohistochemistry and Western blotting.

	Antibody Name	Product Number	Dilution
PI3K	P: Rabbit Anti-PI3K antibody	Bioss(bs-2067R)	WB=1:1000;IHC=1:100
	S1: HRP-labeled goat anti-rabbit IgG for IHC	Bioss(bs-0295G -HRP)	1:1000
	S2: Goat Anti-rabbit IgG for WB	Bioss(bs-0295G)	1:5000
AKT	P: Goat anti-AKT1 antibody	Santa(sc-7126)	1:1000
	S: Donkey anti-goat IgG	Abcam (ab-6885)	1:5000
p-AKT	P: Rabbit anti-p-Akt (Ser473) antibody	Santa(sc-7985-R)	1:200
	S: Goat anti-rabbit IgG	Bioss(bs-0295G)	1:5000
β-actin	P: Rabbit anti-β-actin antibody	Bioss(bs-10966R)	1:2000
	S: Goat anti-rabbit IgG	Bioss(bs-0295G)	1:5000
mTOR	P:Rabbit Anti-mTOR antibody	Abcam (ab-2732)	WB=1:500;IHC=1:100
	S1: HRP-labeled goat anti-rabbit IgG for IHC	Bioss(bs-0295G -HRP)	1:1000
	S2: Goat Anti-rabbit IgG for WB	Bioss(bs-0295G)	1:5000
P70S6K	P: Rabbit anti-S6K1 antibody	Abcam (ab-32359)	1:200
	S: Goat anti-rabbit IgG	Bioss(bs-0295G)	1:5000
p- P70S6K	P: Rabbit Anti-S6K1 (phospho T412) antibody	Abcam (ab-194521)	1:1000
	S: Goat anti-rabbit IgG	Bioss(bs-0295G)	1:5000

Note:P=primary antibody; S=secondary antibody; IHC=immunohistochemistry; WB=Western blotting

Supplementary Figures

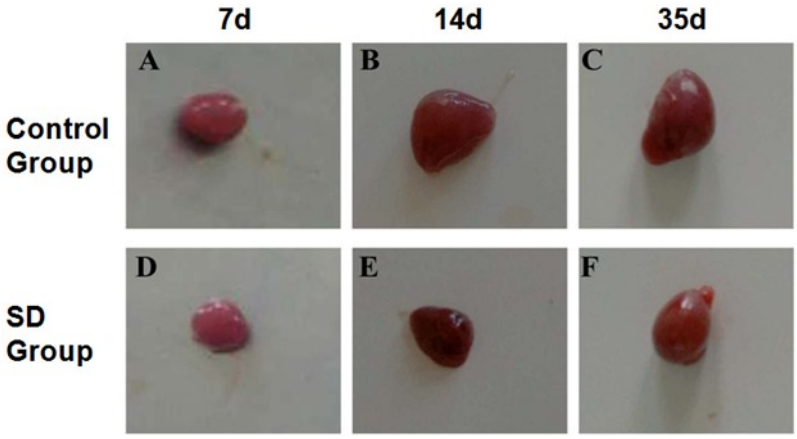

Fig.S1 The representative pictures of spleens from chicken in control and SD groups at the 7, 21, and 35 day.

Supplementary Methods

Animal and diet: The protocol was approved by the Heilongjiang Bayi Agricultural University Animal Care and Use Committee. Briefly, 54 1-day-old specific pathogen free male Sanhuang broiler chickens were purchased from Harbin Veterinary Research Institute, and randomly allocated to Se-deficient and control groups (27 chickens per group). The basal diet (corn 62.47%, soybean 24.50%) was composed of corn and soybean meal produced in the Se-deficient area (Keshan County) of Heilongjiang Province, China, and was not supplemented with Se. Se concentration of the corn and soybean was 0.00542, and 0.02625mg/kg, respectively. Se concentration of the Se-deficient diet was 0.01mg/kg. The control diet was supplemented with Se to 0.4mg/kg. Chicks were housed in battery brooder cages with raised wire floors, and the room temperature was maintained at 30, 28, and 25°C for the first, second, and subsequent weeks. Chickens were provided free access to the designated diets in plastic trough sand deionized water in stainless steel troughs.

Se concentration detection: Se concentration in chicken whole blood and spleen were detected by Agilent SpectrAA-880 Zeeman graphite furnace atomic absorption spectrometer (GFAAS) according to previous study.[1] Briefly, spleen samples were decomposed with the full automatic microwave, and then analyzed by the GFAAS. The prepared samples (500 μL) were diluted 1+2 with 0.1% *V/V* nitric acid and 0.1% Triton X-100 were introduced into graphite furnace whit appropriate volume of palladium modifier and iridium modifier. The calibration curves were prepared in nitric acid-Triton X-100 mixture spiked with known amount of selenium standard solution and palladium or iridium solution as matrix modifier.

Western blotting: The translation level of PI3K, AKT, p-AKT, mTOR, p70S6K, and p-p70S6K in spleen were detected by Western blotting. Spleen samples were collected and were lysed with a lysis buffer (20 mM HEPES, 10 mM NaF, 1 mM Na_3VO_4, 500 mM NaCL, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 1% protease inhibitor cocktail). Then, the protein concentration were determined by the method of Bradford (Biyotime, Shanghai, China). The protein samples were mixed in 1:1 ratio with the sample. electrophoresed using

SDS-polyacrylamide gel standard loading buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris HCl, check the pH and bring it to pH 6.8). Samples were run on 4% to 12% Tris-Glycine precast gel (BeyoGel™ SDS-PAGE Precast Gel) in SDS-PAGE running buffer (192 mM glycine, 25 mM Tris-base and 0.1% SDS). After SDS-PAGE electrophoresis, the proteins were transferred to PVDF membranes using a Bio-Rad Tans-Blot Tansfer cell (Bio-Rad Laboratories, Hercules, CA). After transfer, PVDF membranes were blocked by incubation with blocking buffer (PBS containing 0.2% Triton X-100 and 15% skim milk). Membranes were incubated with the appropriate primary antibody and secondary antibody (Tab. S4). The immunoreactive bands were detected using ECL Western Blotting Substrate (Solarbio, Beijing, China). The images of the protein bands were obtained with a Bio-Rad ChemiDocTM XRS+ (Bio-Rad, Hercules, CA, USA).

Immunohistochemistry: Immunohistochemistry was performed as previously described.[2] Intestinal tissue specimens were embedded in paraffin and sectioned at 5 μm for processing by the immunohistochemistry method using a commercial kit (Bioss Biotechnology, Beijingi, China), using DAB peroxidase substrate (Beyotime Biotechnology, Shanghai, China) and counterstained with hematoxylin. Primary antibodies and secondary antibodies were listed in Tab. S4. Specimens were evaluated by Motic digital miccroscope.

Statistical analysis: Each sample was assessed in triplicate and the experimental data were expressed as the mean ± SD. Significance values were calculated by one-way analysis of variance (ANOVA) using SPSS version 19.0 software (SPSS, IBM, Armonk, NY, USA). The differences were considered significant at p-values of < 0.05 and highly significant at p-values of < 0.01.

Reference:

1. Miksa I R, Buckley C L, Carpenter N P, et al. Comparison of selenium determination in liver samples by atomic absorption spectroscopy and inductively coupled plasma-mass spectrometry. Journal of Veterinary Diagnostic Investigation, 2005, 17(4):331.
2. Duan W R, Garner D S, Williams S D, et al. Comparison of immunohistochemistry for activated caspase-3 and cleaved cytokeratin 18 with the TUNEL method for quantification of apoptosis in histological sections of PC-3 subcutaneous xenografts. Journal of Pathology, 2003, 199:221-228.