Calcium Overload and Ros Accumulation Induced by Selenium Deficiency Promote Autophagy in Swine Intestine

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Research

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Abstract

Background:

Selenium deficiency can seriously affect the intestinal status of swine, and cause diarrhea in swine. However, the specific mechanism of selenium intestinal injury caused by selenium deficiency is rarely reported.

Methods:

Here, to explore the damage of selenium deficiency on the calcium homeostasis and autophagy mechanism of swine, in vivo and in vitro models of swine intestinal selenium deficiency were established. The intestinal model of swine intestine was established by feeding different selenium concentrations. Besides, selenium-deficient medium and normal medium were used to culture IPEC-J2 cells to establish in vitro models. Morphological observation and cell staining were used to determine the way of intestinal injury, and gene expression was quantitatively detected by qPCR and WB.

Results:

Morphological observations showed that compared with the control group, intestinal cells in the Se-deficiency group were significantly damaged, and autophagosomes increased. MDC staining and cytoplasmic calcium staining results showed that in the Se-deficiency group, autophagy increased and calcium homeostasis was destroyed. Also, according to the ROS test results, the percentage of ROS in the Se-deficiency group is higher than the control group in the in vitro model. Compared with the control group, the protein and mRNA expressions of autophagy and calcium-related genes (Beclin1, LC3-1, LC3-2, ATG5, ATG12, ATG16, mTOR, CAMKK- β , AMPK, SERCA, calpain) in the Se-deficiency group were significantly increased which was consistent in vivo and in vitro. Under the influence of selenium deficiency, the mRNA expression level of selenoproteins decreased significantly, and the steady-state content of some elements was also destroyed.

Conclusion:

Altogether, our results indicated that selenium deficiency could destroy the calcium homeostasis and antioxidant homeostasis of swine intestine to trigger cell autophagy. Moreover, selenium deficiency reduces the overall expression of selenoproteins and affects the content of elements in the intestine.

Introduction

Selenium is an indispensable nutrient element for human and animal organisms, which has physiological effects such as anti-inflammatory and antioxidant[1], anti-mutation[2] in immunity[3]. Selenium also plays an important role in immune function[4]. Studies have shown that selenium deficiency can cause Keshan disease in humans[5], heart failure in swine[6], white muscle disease (WMD) of calves, lambs, ponies, and other animals, and vitamin E/selenium deficiency(VESD) syndrome of swine[7], even affect

maternal thyroid metabolism and oxidative stress, leading to weight loss[8]. These have brought significant economic losses to the swine industry. Selenium has a significant effect on intestinal function in swine, such as diarrhea-induced colitis injury can be alleviated by selenium[9]. Moreover, the intestine is the main organ to absorb selenium[10], in short, the two are closely linked. Besides, swine can be used as a good model to study the potential risks and related mechanisms of human selenium intake[11]. Meanwhile, compared to rodent cell lines, porcine jejunal epithelial cells (IPEC-J2) plays an important role in the study of zoonotic infections, and is often used as an in vitro model for microbial research[12]. Regarding the intestinal tract, past studies have found that selenium deficiency can cause intestinal eosinophilic inflammation[13], but the specific mechanism of selenium deficiency leading to damage is still unclear.

Oxidative stress is due to the imbalance between oxidation and anti-oxidation, which is more prone to oxidation and produces a large number of intermediate products. It is considered to be an important inducing factor leading to aging and disease. Selenium has a good antioxidant function who is considered as scavengers of free radicals and other reactive oxygen species (ROS)[14]. Therefore, selenium deficiency can contribute to oxidative stress and damage to various tissues[15]. As a stimulus point for oxidative stress, ROS is an intracellular chemical capable of triggering various biological responses[16]. Intestinal exposure to adverse environment triggers oxidative stress[17]. Under the condition of selenium deficiency, ROS can trigger the NF- κ B inflammation signaling pathway and the intrinsic apoptosis pathway to cause the apoptosis of duodenal villi cells[18]. Moreover, ROS can mediate autophagy during nutrient deficiency[19]. ROS induces autophagic expression in the nucleus by triggering endoplasmic reticulum stress. Intracytoplasmic ROS may also influence autophagy by modulating ATG4 activity[20]. Our previous experiments have demonstrated that selenium deficiency can activate the ROS mediated MAPK pathways to regulate autophagy[21].

Autophagy is a cellular process that occurs in eukaryotic cells, degrades cytoplasmic content by lysosomal phagocytosis, and recycles large molecules in the cytoplasm[22]. More studies have shown that differentially expressed genes caused by selenium deficiency can be enriched in the PI3K/AKT/mTOR signaling pathway[23], and the mTOR gene is closely related to the autophagy pathway. Autophagy plays a vital role in cell physiology, including adapting to metabolic stress, clearing dangerous goods, renewing during differentiation and development, and preventing damage to the genome[24]. It has been proved that selenium deficiency induces autophagy in cardiomyocytes[25]. Moreover, the change of calcium(Ca^{2+}) homeostasis is one of the main factors affecting autophagy[26]. The increase of free Ca^{2+} activated by CAMKK- β and AMPK in the cytoplasm can be used as an effective inducer of autophagy and also become an ER target of Bcl-2 against autophagy[27]. In addition, as the Ca^{2+} -ATPase pump, SERCA plays an important role in regulating cellular Ca^{2+} homeostasis, while calpain is activated under the influence of a high concentration of Ca^{2+} [28]. In addition, studies have confirmed the interaction between calpain and SERCA through immunoprecipitation[29]. Experimental results of cancer cell lines show that autophagy even apoptosis is induced by CAMKK- β and AMPK, which will cause [30], and this result has also been verified in carp[31]. Selenium deficiency causes symptoms in the

digestive system of swine and triggers intestinal cell damage. However, it is unclear what role Ca^{2+} homeostasis and autophagy play in selenium-deficient intestinal damage.

Consequently, we established an in vivo experiment of selenium deficiency in swine intestine and an in vitro experiment of selenium deficiency in IPEC-J2 of swine to explore the mechanism of the autophagy-calcium pathway in selenium-deficient intestinal injury.

Material And Method

All procedures used in this study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University (SRM-11).

1. Swine and diets

A total of 24 healthy and similar-weights pure line big white emasculated swine (chosen from 12 nests, 2 heads/nests) were weaned for one week. Then the swine were randomly assigned to 2 groups of 12 (adopting the full sibling pairing test): the Se-deficient group and the control group. The swine were maintained on either a Se-deficient diet (the Se-deficient group) containing 0.007 mg/kg Se or a normal selenite diet (the control group) containing 0.300 mg/kg Se for 16 weeks. The detailed feed ingredients are shown in Table 1. All the swine were housed under the same conditions from the start of the experiment, and the swine were fed in swine pens. The experimental swine were fed three times each day and provided with free access to drinking water. Intestine tissue samples were collected and promptly frozen at the 16th week of the experiment, part of the clean tissue was sliced and immersed in 10% neutral buffered formalin solution and electron microscopy solution at 4 ° C Store and take part of the intestinal tissue frozen at -80°C for future use.

Table 1
Diet formula

Ingredients	Phase 1: Weight < 25 kg	Phase 2: Weight > 25 kg
Corn (%)	66	75
Bean cake (%)	26.4	20
Soybean oil (%)	3.5	1.5
Common salt (%)	0.5	0.3
Stone powder (%)	0.9	0.9
Calcium hydrogen phosphate (%)	1	0.9
Lys (%)	0.7	0.4
Premix (%)	1	1
Digestive energy Kcal / kg	3494.41	3406.7
Crude protein (%)	15.96	14.66
Ca (%)	0.72	0.67
Effective P (%)	0.29	0.27
Lys (%)	1.23	0.98
Met (%)	0.36	0.28
Thr (%)	0.73	0.59
Try (%)	0.2	0.17
Note: Trace elements are provided per kilogram of diet: copper (5mg ^a , 4mg ^b), iodine (0.14mg ^{a,b}), iron (100mg ^a , 60mg ^b), manganese (3mg ^a , 2mg ^b), Zinc (80mg ^a , 60mg ^b); vitamins: VA (1750IU ^a , 1300IU ^b), VD3 (200IU ^a , 150IU ^b), VE (11IU ^{a,b}), VK3 (0.5mg ^{a,b}), biotin (0.05mg ^{a,b}), choline (0.4g ^a , 0.3g ^b), folic acid (0.3mg ^{a,b}), nicotinic acid (30mg ^{a,b}), pantothenic acid (9mg ^a , 8mg ^b), riboflavin (3mg ^a , 2.5mg ^b), thiamine (1mg ^{a,b}), VB6 (3mg ^a , 1mg ^b), VB12 (15µg ^a , 10µg ^b).		

2. Cell culture and treatment

The IPEC-J2 cell line is obtained from the College of animal science, Northeast Agricultural University and cultured using DMEM/High Glucose (GIBCO, NY, USA) medium as a liquid environment which containing 10% FBS (GIBCO, NY, USA), and 1% penicillin-streptomycin (GIBCO, NY, USA). DMEM/High Glucose medium, FBS and penicillin-streptomycin were all sterilized through a 0.22 µm millipore filter to remove any contaminants. Cells were fed once a day and subcultured once every 2–3 days until the cell density reaches 70–80%. When passaging, first inoculated the cells in a culture flask, and incubated for 12 hours to ensure that the cells are attached to the culture flask. Then discarded the original medium and cultured the cells in other mediums. For the Se-deficient group, the cells were cultured in DMEM/High Glucose

medium with 1% FBS, 1% penicillin-streptomycin, 10 µg/ml insulin, and 5 µg/ml transferrin resulting in selenium depletion at least for 5 days [32]. IPEC-J2 cells in the Se-deficient group needs to be changed every day to remove dead cells. But IPEC-J2 cells in the control group were still cultured on the normal medium, passaged, and allowed to grow for 5 days. The cells were cultured at 37 °C and 5% CO₂ and collected for analysis after five days.

3. Morphological examination of swine intestine and IPEC-J2 cells

The technique adopted to observe ultrastructural changes was similar to that of our previous study: The jejunum tissues IPEC-J2 cells were fixed in 2.5% glutaraldehyde phosphate-buffered saline, post-fixed with 1% osmium tetroxide, stained with 4.8% uranyl acetate, and finally dehydrated in a graded ethanol series. The ultra-thin sections were cut, incubated with uranyl acetate and lead citrate. The intestine specimens were visualized using the transmission electron microscopy (GEM-1200ES, Japan).

The treatment method of IPEC-J2 cells electron microscope observation was the same as that of tissue.

4. Cell autophagy detection

Autophagic staining was measured using a cell autophagy detection assay kit (Beijing Solarbio Science & Technology, Beijing, China). The 10 µM MDC staining agent (Dansylcadaverine) was added to the medium containing enterocytes, which incubated in a constant temperature incubator (37 °C) for 25 min. Discarding the medium and washing the cells with PBS (37°C preheat) three times. Finally, cells were collected using a fluorescence microscope at an excitation wavelength of 355 nm and an emission wavelength of 512 nm for observation of fluorescence.

5. ROS activities detection

ROS activities were measured using the ROS assay kit (Nanjing Jiancheng Bioengineering Institute, China). Add 10 µM DCFH-DA (2,7-dichlorofluorescein diacetate) in the culture medium, where there are cell samples to be tested, and incubate in constant temperature incubator (37°C) for 45 min, discard the medium and use PBS (37°C preheat) wash the cells three times, finally, collect the cells for detected the activities of ROS in excitation wavelength 500 ± 15 nm and emission wavelength 530 ± 20 nm. Enterocytes were visualized using fluorescence microscopy.

6. Intracellular Ca²⁺ concentration detection

The Fluo-3 AM assay kit (Beijing Solarbio Science & Technology Co., Ltd) was used to detect the intracellular Ca²⁺ concentration. After 4 days of treatment, the cells cultured in 6-well plates were digested with collagenase- α (0.1 g %), then the cells were resuspended and plated in 12-well plates for 24 h. The Flour-3am mother liquor was diluted with PBS until the concentration reached 1 µM for use. The cells were washed with PBS before they were covered with the diluted working fluid. After incubating at 37 °C for 40 minutes, the cells were cleaned and observed under a fluorescence microscope.

7. Total RNA extraction and determination of the mRNA expression of the autophagy-calcium homeostasis related genes

Total RNA was isolated from intestine tissues and enterocyte using Trizol reagent according to the manufacturer's instructions (Invitrogen, Shanghai China). The dried RNA pellets were resuspended in 50 μ L of diethyl-pyrocyanide-treated water. The concentration and purity of the total RNA were determined by a spectrophotometer. cDNA was synthesized from 5 μ g of the total RNA using oligo dT primers and Superscript II reverse transcriptase according to the manufacturer's instructions (Promega, Beijing, China), and cDNA was stored at -80°C [33].

Specific primers (Beclin1, LC3-1, LC3-2, ATG5, ATG12, ATG16, mTOR, CAMKK, SERCA, calpain, AMPK) for target genes (Table 2) were designed based on known sequences using Primer-BLAST at the National Center for Biotechnology Information (NCBI). Quantitative real-time PCR (qPCR) was performed with a BIOER detection system (China, Hangzhou). Reactions were performed in a 10 μ L reaction mixture containing 5 μ L of $2\times$ SYBR Green I PCR Master Mix (R), 1 μ L of cDNA, 0.3 μ L of each primer (10 μ M), and 3.4 μ L of PCR-grade water. The relative abundance of each mRNA was calculated according to the $2^{-\Delta\Delta\text{Ct}}$ method and normalized to the mean expression of GAPDH.

Table 2
Gene-specific primers used in the real-time quantitative reverse-transcription PCR

Gene	Primer sequence (5'→3')
GAPDH	Forward: GTGAACGGGTGAGTTAGGGG
	Reverse: CGATGCGGCCAAATCTTGAG
Beclin1	Forward: GCAGGTGAGCTTCGTGTGTCAG
	Reverse: CCTGGGCTGTGGCAAGTAATGG
LC3-1	Forward: CCAGTCCTGGACAAGACCAAGTTC
	Reverse: GGTTCACCAGCAGGAAGAAGGC
LC3-2	Forward: TTCCTGGTGCCTGATCATGTCAAC
	Reverse: ACTCACCATGCTATGTCCGTTAC
ATG5	Forward: AGAAACCTAGAGAGGGCCACA
	Reverse: TCTTCCTAGTCAAACAACGTCA
ATG12	Forward: CAACTGCTGCTGAGGGCGATG
	Reverse: CACCGGCAGGTTCTTCTGTTCC
ATG16	Forward: TCGCAGAAGCAGCAAAGGAACC
	Reverse: TGATGGCTCGCACGGGAGAG
DIO1	Forward: GCTGAAGGTCCGATGGCAACG
	Reverse: AGATGTGGCACCTCTGTCCTGAC
DIO2	Forward: TGGTGGAGGAGTTCTCATCAGTGG
	Reverse: GCACATCGGTCTTCCTGGTTCTG
DIO3	Forward: CAACAGTGATGGCGACGAGGTG
	Reverse: CGAGGATGTGCTGGTTCTGGAAG
TXNRD1	Forward: GCTCAAGTGCGGACTGACCAAG
	Reverse: AGCAACCGGCCTGGAGGATG
TXNRD2	Forward: ACGGTCTTCACGCCACTGGAG
	Reverse: CGCATCTCGTTCAGGCACTGTG
TXNRD3	Forward: ATGTCACTCAGAAGGGCTGC
	Reverse: TCCAGGGACGATGGATGAGT

Gene	Primer sequence (5'→3')
GPX1	Forward: GCGTCGCTCTGAGGCACAAC
	Reverse: GGTCGGACGTACTTGAGGCAATTC
GPX2	Forward: CTCGCTCTGAGGCACAACCAC
	Reverse: GCGGACGTACTTGAGGCTGTTC
GPX3	Forward: AGAACGCCTGTCCTCCTACTTCG
	Reverse: GTTGATGGTGGTGCGGTGGTAC
GPX4	Forward: GCCTGTTCCGCCTGCTGAAG
	Reverse: GGTGACGATGCACACGTAGCC
GPX7	Forward: GGCAAGTTGGTGTCGCTGGAG
	Reverse: GCGGTAGTGCTGGTCTGTGAAG
SELT	Forward: CGATGCCTCGGCCAACATGG
	Reverse: CTCTCCTTCAATGCGGATGTCTGG
SELS(SELENOS)	Forward: GGCTGGTACATCGTCTTCTGCTG
	Reverse: TCCAGGTGCCTCTGCCTCAAG
SELM	Forward: GCTCGGCTTCTATCGCAAGGC
	Reverse: TACAGGTCAGCGCGGTCAGG
SELO	Forward: GCCGACTTCACCAACACCTTCTAC
	Reverse: AGCCAGGAACTCAGCCAGGTC
SELH	Forward: GCTTCGAGGTGACGCTGATGC
	Reverse: CAGCTCCTCCACCACCTCTTGAG
SELI	Forward: TTCAGCACCAGGTCACAAGCATG
	Reverse: TCCACACCATCCAGAGTGTAGGC
SELK	Forward: GCGTTGGACAGCAGGAGTCAG
	Reverse: GGAATCAGATGAGCCTCCATAGCC
SELW	Forward: GCCGTTGAGTCGTCTATTGTGG
	Reverse: GGAGTGAACCAACTTCCTGCTACC
SELP	Forward: CATCACCATCGCCATCACCATCAC
	Reverse: GGCTGCTCAGGAGCGTCTGG

Gene	Primer sequence (5'→3')
SEPX1	Forward: ACCGAGACCATCCACGCTGAC
	Reverse: GTTGCCACATCTGCCACAGGAC
SPS2	Forward: CTCCTCAGCGTCAGCCAGAATATG
	Reverse: CCACTCCGCCGATGATAATCCAAG
AMPK	Forward: CACCAGGACCCTTTGGCAGTTG
	Reverse: TCTTTCAGGATGGGGCCGAGTC
CAMKK	Forward: GAGTCGCACCATGTCTCCATCAC
	Reverse: GCCAACTTGACCACGCCATAGG
calpain	Forward: AACACAAAGACCTGCGGACCAAG
	Reverse: CCAGCTTCCCGTTGCCATCAC
SERCA	Forward: CCTTACCAGTCATCGGGCTC
	Reverse: GACACGGTTCAAAGAGGTGGA

8. Detection of selenoproteins

Detection of selenoproteins content in intestine tissue and IPEC-J2 cells was performed by qRT-PCR, whose method was the same as the detection of autophagy-calcium target genes, and the specific primers for selenoproteins genes (Table 2) were designed based on known sequences using Primer-BLAST at the NCBI.

9. Total protein extraction and determination of the protein expression of autophagy-calcium homeostasis related genes

Total protein was extracted from intestine tissues and enterocytes by lysis buffer for Western blotting with phenylmethanesulfonylfluoride (PMSF) (100 mM). These extracts were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Separated proteins were transferred to nitrocellulose membranes in Tris-glycine buffer containing 20% methanol at 4 °C. The membranes were blocked with 5% skim milk for 2 h and incubated overnight with diluted primary antibodies against Beclin1(1:500, Wanleibio, China), LC3(1:500, ABclonal, China), mTOR(1:500, Wanleibio, China), AMPK(1:800, the polyclonal antibody produced by our lab), CAMKK-β(1:500, Proteintech, China), SERCA(1:500, the polyclonal antibody produced by our lab) and β-actin (1:10000, ABclonal, China) followed by goat anti-rabbit IgG (H + L) (1:10000, Immuno Way, China). The signal was detected using an enhanced chemiluminescence system [34].

10. Ions detection of swine intestine tissue

ICP-MS method was used to detect the levels of 23 ions in the intestine tissue of the control group and the Se-deficiency group. Start and measure under optimized instrument working conditions.

11. Statistical analysis

Each group consisted of 6 single observation replications ($n = 6$) and two parallel experiments were performed to ensure the accuracy of the experimental data. The data are expressed as the mean $\pm$ standard deviation (mean $\pm$ SD), and GraphPad Prism v8.0 software was used for all the statistical analyses and the Multiple t-tests showed that the data were normally distributed. The data were compared using a t-test analysis of variance to determine the difference between the control group and the Se-deficient group. * Significant difference from the corresponding control ($P < 0.05$).

Results

1. Selenium deficiency induced swine intestinal autophagy

1.1 Ultrastructural observation of autophagosomes in swine intestine

The ultrastructure of the swine intestine tissue (**Fig. 1A**) of the control group and the Se-deficient group was observed with a transmission electron microscope (TEM). In the control group, normal mitochondria and a few lysosomes were observed. However, a large number of autophagic vesicles with a double-layer membrane structure and seldom lysosomes appeared in the Se-deficient group.

1.2 Ultrastructural observation of autophagosomes in IPEC-J2 cells

Observing the ultrastructure of the control group and Se-deficient group of IPEC-J2 cells (**Fig. 1B**) by the transmission electron microscope. In the control group of IPEC-J2 cells, a large number of normal mitochondria could be observed. There were a small number of autophagy lysosomal vesicles characterized by incomplete boundary membrane, intact intima, and amorphous substances. There were also a small number of autophagic vesicles that do not contain substances. In the Se-deficient group, the cytoplasm was mainly autophagic lysosome vesicles, which contained mitochondria. There were also a large number of uncovered secondary lysosomes and autophagic vesicles, as well as a small number of mitochondria with normal morphology.

2. Effects of selenium deficiency on the selenium content of swine intestine tissue and IPEC-J2 cells

In intestine tissue and IPEC-J2 cells, the expression of 22 selenoproteins in the control group was higher than that in the Se-deficiency group. It should be noticed that the expression of GPX2 in the tissue selenium deficiency group was slightly higher than that of the control group.

3. Effects of selenium deficiency on ROS viability in IPEC-J2 cells

Affected by the lack of selenium nutrition, the ROS activity of the Se-deficient group had a significant increase compared to the control group ($p < 0.01$). The activities of ROS increased significantly ($p < 0.01$) in the Se-deficient group.

4. Effects of selenium deficiency on autophagy in IPEC-J2 cells

Dansylcadaverine-MDC is a fluorescent pigment that stains normal cells into a uniform yellowish-green, and autophagy becomes bright green. The IPEC-J2 cells in the control group were yellowish-green with few green highlights. Compared with the control group, the bright green spot of the Se-deficiency group was dense, which meant that the Se-deficient group had a more autophagosome accumulation ($p < 0.01$).

5. Effects of selenium deficiency on the concentration of Ca^{2+} in IPEC-J2 cells

The lack of selenium nutrition caused an increase in the cytoplasmic Ca^{2+} concentration of the Se-deficiency group. Compared with the control group, the Se-deficiency group had more bright green spots, and calcium overload occurred significantly ($p < 0.05$).

6. Protein and mRNA expression of autophagy- Ca^{2+} related genes in swine intestine tissues

Affected by selenium deficiency, protein and mRNA expression abundance of autophagy-related genes in intestine tissues are shown in **Fig. 4**.

qPCR results revealed that mRNA expression of autophagy-related genes (Beclin1, LC3-1, LC3-2, ATG5, ATG12, ATG16, mTOR) significantly increased ($p < 0.05$) in the Se-deficient group, compared with the control group. Similarly, the mRNA expression of Ca^{2+} pathway-related genes (CAMKK, SERCA, and calpain) in the Se-deficiency group also increased significantly compared to the control group. But the mRNA expression of LC3-1 and AMPK increased slightly, did not show significant differences ($p > 0.05$). Meanwhile, the protein expression of LC3-1, LC3-2, AMPK, CAMKK- β significantly increased ($p < 0.05$) in the Se-deficient group compared with the control group respectively. Compared with the control group, the protein expression of Beclin1 in the Se-deficiency group increased slightly, and there was no significant difference. The protein expression of SERCA and mTOR in the Se-deficiency group was significantly lower than that in the control group ($p < 0.01$). The expression results of the above genes indicated that selenium deficiency caused Ca^{2+} overload and autophagy accumulation in the swine intestine.

7. Protein and mRNA expression of autophagy- Ca^{2+} related genes in IPEC-J2 cells

Affected by selenium deficiency, protein and mRNA expression abundance of autophagy-related genes in IPEC-J2 cells are shown in **Fig. 5**.

Similar to the expression in intestine tissue, compared with the control group, autophagy-related genes (LC3-1, LC3-2, ATG5, ATG12, ATG16, mTOR) were significantly increased in the mRNA expression of Se-deficient group IPEC-J2 (**Fig. 5A**). The mRNA expression of Beclin1 was not significantly different in the two groups. The mRNA expression of Ca^{2+} related genes (CAMKK, SERCA, calpain, AMPK) had the same trend as that of the main autophagy-related genes, and the expression in the Se-deficiency group was significantly higher than that in the control group ($p < 0.01$).

The change in protein expressions of autophagy- Ca^{2+} related genes was shown in **Fig. 5B**. As detected, compared with the control group, the protein expressions of Beclin1, LC3-1, LC3-2, and ATG16 were significantly increased. However, the protein expression of SERCA in the Se-deficiency group was significantly lower than that in the control group. The results of gene expression in IPEC-J2 cells are the same as in vivo, showing that selenium deficiency can lead to Ca^{2+} overload and autophagy accumulation in cells.

8. Results of detection and analysis of multiple elements in intestine tissue

The detection results of 23 elements in swine intestine tissue are shown in **Fig. 6**. It can be roughly divided into three groups of main elements, majorelements (Na, Mg, K, Ca), essential trace elements (Fe, Zn, B, Cu, Mn, Ni, Ba, Sb, Se, Ti, V), and toxic trace elements (Al, Li, As, Cd, Pd, Sn, Sr). The results showed that selenium deficiency caused an increase in the major element Ca ($p < 0.05$), while the levels of other major elements were basically not affected ($p > 0.05$). The content of essential trace elements B, Cu, Ni, V was significantly reduced ($p < 0.05$), and other essential trace elements were not affected ($p > 0.05$). At the same time, selenium deficiency led to a decrease in the content of toxic trace element Al ($p < 0.05$). The results showed that the lack of selenium caused the imbalance of the ion level in the swine intestine tissue, the compensation of Ca increased, and the loss of B, Cu, Ni, V, and Al.

Discussion

Selenium is an indispensable trace element in the body and affects the body's health. Selenium protein is involved in the regulation of cellular redox homeostasis, protection of oxidative stress[35]. Selenium deficiency can cause swine mulberry hearts and has a particularly significant effect on the digestive system of swine[36], which can lead to enteritis[37] and even death. Although different forms of selenium supplements are a convenient method to treat these diseases, such as selenium can be supplemented to the host by adding a daily diet. The fact has also proved that this method can indeed effectively reduce the incidence and mortality of mouse models and human colon cancer[38]. However, there are still many factors that affect its effectiveness, such as the chemical form of selenium additives and the health of animals[39]. Moreover, some studies have shown that dietary selenium affects the host's intestinal flora

balance and gastrointestinal colonization, thereby affecting the host's selenium status and the expression of selenoprotein[40]. Besides, the study has shown that selenium deficiency can change the distribution and steady-state of other minerals [32], which has been confirmed again in our experiment. In this study, we established the selenium deficiency model in swine and the selenium deficiency model in IPEC-J2 cell in vitro, in which results showed that selenium deficiency caused a downward trend in the overall selenoprotein expression and disrupted the balance of intestinal trace elements. Selenium deficiency induced the occurrence of oxidative stress, the imbalance of Ca^{2+} homeostasis in the intestine and in vitro, and ultimately promotes autophagy.

Selenium is involved in regulating ROS levels and redox balance in all tissues[41], a large number of studies have shown that ROS is an important target for tissue damage caused by selenium deficiency. Gao et al. found that selenium deficiency could stimulate ROS-induced inflammation[42]. Selenium can be used as a ROS scavenger to exert additive effects on the proliferation and paracrine of human amniotic fluid-derived mesenchymal stem cells with bFGF (Basic Fiber Growth Factor)[43]. Due to the combined effect of selenium deficiency and low protein intake, the levels of ROS in the serum and myocardial tissue defects of rats are significantly increased. Eventually, it causes myocardial oxidative stress and induces apoptosis through mitochondrial-mediated pathways[44]. The imbalance between ROS production and the elimination of protective mechanisms may lead to chronic inflammation[45]. Besides, our previous research also showed that selenium deficiency did cause inflammation of swine intestinal tissue and IPEC-J2 cells[46]. In our present experiment, it was observed that ROS production in IPEC-J2 cells increased due to selenium deficiency stimulation. Similar to the specific expansion of myeloid cells that causes excessive ROS and promotes intestinal pathology[47], we believe that the swine intestinal damage caused by selenium deficiency is also closely related to the accumulation of ROS. Also, studies have shown that the addition of trace element chelating agents can improve the antioxidant capacity and immune function[48]. But excessive cadmium can promote oxidative stress [49][50]. Therefore, we speculate that the destruction of the homeostasis of trace elements in our experiment may also be one of the reasons for the accumulation of ROS.

Moreover, a large number of reports indicate that ROS is an early inducer of autophagy during nutritional deficiencies[51]. NOX2 production of ROS is the key to phagocytic cells to kill microorganisms, and LC3 needs to recruit to phagosomes[52]. ROS can induce autophagy by activating the MCOLN1-lysosome Ca^{2+} -TFEB pathway to eliminate damaged mitochondria and excess ROS[53]. Our previous research found that selenium deficiency could inhibit the expression of TNNT2, thereby activating the channel for Ca^{2+} to flow outside the cell, and inhibiting the channel for Ca^{2+} to flow inward[54]. We note that AMPK is an important signaling molecule for Ca^{2+} -dependent autophagy activation[55]. AgNP induces ER stress and autophagy occurs through the Ca^{2+} /CAMKK-beta/AMPK/mTOR pathway. The specific mechanism is that CAMKK- β and AMPK are activated by the increase of Ca^{2+} levels in the cytoplasm after being stimulated by AgNP, which leads to the downregulation of mTOR and the upregulation of Beclin1, eventually activating autophagy [56]. In this experiment, selenium deficiency also caused the accumulation of autophagy in swine intestine by increasing the expression of CAMKK- β and AMPK in the

Ca²⁺/CAMKK-β/AMPK/mTOR pathway, and decreasing the expression of mTOR. However, it is worth noting that the mRNA expression of Beclin1 in our experimental results decreased slightly in vitro. We believe that there may be other pathway genes that are stimulated by selenium deficiency at the mRNA level and inhibit the expression of Beclin1. For example, the anti-apoptotic protein, Bcl-2, which interacts with Beclin1 to regulate autophagy, keeps cells alive rather than dead[57].

Autophagy can prevent cell damage and promote cell survival in the absence of nutrition, and can also respond to cytotoxic damage[58]. Therefore, autophagy is sensitive to the lack of dietary selenium. Selenium deficiency can cause increased expression of autophagy in the chicken spleen, bursa, and thymus and cause damage to chicken immune organs[59]. Our previous studies on selenium nutrition have shown that selenium deficiency can cause autophagy in cardiomyocytes[60]. Autophagy can antagonize apoptosis mechanisms in cardiomyocytes knocked out of GPX3 gene, a selenoprotein[61]. Autophagy plays a key role in regulating the interaction between the gut microbiota and innate and adaptive immunity and the host's defense against intestinal pathogens, maintaining intestinal homeostasis[62]. In this experiment, the key genes of autophagy (LC3, Beclin1) were up-regulated in swine intestinal tissues and IPEC-J2 cells treated with selenium deficiency, and the ultrastructure also showed autophagic vacuoles and encapsulated mitochondria. The above results suggest that due to the stimulation of selenium deficiency, homeostasis of Ca²⁺ and ROS are destroyed and overload occurs, leading to increased autophagy. To maintain normal cell function and protect the intestine from selenium deficiency, autophagy plays a role in resisting damage.

However, it is too early to say that autophagy effectively protects swine intestine injury caused by selenium deficiency. Because we observed the apoptotic bodies such as the formation of apoptotic bodies and the shrinkage of the nucleus while observing the Se-deficient group autophagic vesicles, we suspect that the lack of selenium nutrition may also trigger apoptosis of intestinal. It may be due to excessive autophagy or damage that has exceeded cell death caused by autophagic protection. Studies have shown that inducing autophagy in tumor cells lacking nutrition can maintain cell metabolism and vitality during periods of nutritional deficiencies, but long-term lack of nutrition can lead to cell death[63]. Moreover, oxidative stress is also a key factor in triggering apoptosis[64][65].

Attention should also be paid to the destruction of element balance. In this experiment, the contents of B, V and Cu were reduced by selenium deficiency, which could increase the risk of dementia, autism and depression[66]. Decreased levels of Ni and Al affect fasting blood glucose [67]. Increased Ca content can exacerbate the risk of diabetes, and even accumulation of Ca²⁺ in mitochondria will promote apoptosis [68]. Calcium accumulation is often regarded as one of the signs of injury[69]. Our results show that selenium deficiency disrupts the balance of elements in the intestines of swine and increases the risk of suffering from various diseases.

Conclusions

In conclusion, our research showed that the lack of selenium nutrition could destroy the Ca^{2+} homeostasis of the swine intestine and trigger the overload of ROS, which ultimately leads to the accumulation of autophagy. The key mechanism for promoting autophagy is the Ca^{2+} -CAMKK- β -AMPK-mTOR pathway. Selenium deficiency also destroys the balance of other elements, increasing the risk of physical and even psychological diseases. However, the specific relationship and mechanism of crosstalk between autophagy and apoptosis will be explored in the next experiments. While many questions remain, these insights open a new line of investigation concerning how selenium deficiency modulates intestinal autophagy.

Abbreviations

Se	Selenium
LC3	GABA(A) receptor-associated protein
ATG5	autophagy-related protein 5
ATG12	autophagy-related protein 12
ATG16	autophagy-related protein 16
mTOR	serine/threonine-protein kinase
CAMKK-β	calmodulin-dependent protein kinase kinase β
AMPK	adenosine 5'-monophosphate-activated protein kinase
SERCA	sarco(endo)plasmic reticulum Ca ²⁺ -ATPase
calpain	calcium-activated neutral protease
ROS	reactive oxygen species
ER	endoplasmic reticulum
ATG4	autophagy-related protein 4
Ca ²⁺	calcium
GPX3	Glutathione Peroxidase 3
NF-κB	the Nuclear receptor-κB
MAPK	mitogen-activated protein kinase
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
AKT	RAC serine/threonine-protein kinase
FBS	fetal bovine serum
PBS	phosphate buffer
ICP-MS	inductively coupled plasma mass spectroscopy
NOX2	NADPH oxidase 2

Declarations

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author upon request.

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Contributions

Ziwei Zhang conceived and designed the experiments, and critically reviewed the manuscript. Yingying Zheng performed the experiments, analyzed the data and wrote the article. Haoyue Guan, Jie Yang, Jingzeng Cai, and Qi Liu assisted in analyzing the data and reviewing the manuscript.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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Figures

Figure 1

Ultrastructural observation of swine intestine tissues and IPEC-J2 cells. A (swine intestine tissues) and B (IPEC-J2 cells) were intestine tissues observed using TEM.

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A

B

Figure 2

The mRNA expressions of 22 selenoproteins in IPEC-J2 cells and swine intestine tissues. (A) Selenoprotein expressions in intestine tissues on mRNA level, (B) Selenoprotein expressions in IPEC-J2 cells on mRNA level. * indicate that there were significant differences ($P < 0.05$) between the two groups. Results were expressed as mean $\pm$ SD ($n=6$).

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Figure 3

Cell staining results. (A) ROS fluorescence of IPEC-J2 cells was detected by fluorescence microscope, (B) the ROS fluorescence intensity quantitative analysis by Image J. (C) Autophagy fluorescence of IPEC-J2 cells were detected by fluorescence microscope. (D) the autophagy fluorescence intensity quantitative analysis by Image J. (E) The fluorescence of cytoplasm Ca^{2+} in IPEC-J2 cells was detected by the fluorescence microscope in WB, BF, and merged in 4 times mirror. Green fluorescence is cytoplasmic calcium. (F) the Ca^{2+} fluorescence intensity quantitative analysis by Image J. * indicate that there were significant differences ($P < 0.05$) between the two groups and the data are presented as the mean $\pm$ SD ($n=6$).

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Figure 4

Detection of autophagy-Ca²⁺ homeostasis-related genes in intestine tissue. The expression of autophagy-Ca²⁺ homeostasis related genes at mRNA (A) and protein levels (B) in intestine tissue control and selenium-deficient groups, respectively. * indicate that there were significant differences ($P < 0.05$) between the two groups and the data are presented as the mean $\pm$ SD (n=6).

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Figure 5

Detection of autophagy-Ca²⁺ homeostasis-related genes in IPEC-J2 cell. The expression of autophagy-Ca²⁺ homeostasis related genes at mRNA (A) and protein levels (B) in IPEC-J2 cell control and selenium-deficient groups, respectively. * indicate that there were significant differences ($P < 0.05$) between the two groups and the data are presented as the mean $\pm$ SD ($n=6$).

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Figure 6

Using ICP-MS technology detected 23 kinds of trace element ion levels, roughly divided into three groups of main elements, macroelements (Na, Mg, K, Ca), essential trace elements (Fe, Zn, B, Cu, Mn, Ni, Ba, Sb, Se, Ti, V), and toxic trace elements (Al, Li, As, Cd, Pb, Sn, Sr). * indicate that there were significant differences ($P < 0.05$) between the two groups and the data are presented as the mean $\pm$ SD ($n=6$).

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