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The role of nitric oxide and autophagy in liver injuries induced by Selenium deficiency in chickens

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Abstract Selenium (Se) is recognized as a necessary trace mineral in animal diets,including those of birds. Se deficiency induces a number of diseases and injuries in chickens, including liver damage. Nitric oxide (NO) is an essential messenger molecule associated with inflammation and oxidative stress. Autophagy is a cellular pathway that is crucial for development, differentiation, survival, and homeostasis, which maintain the balance of energy and nutrients for basic cell functions in the liver. However, little is known about the role of NO and autophagy in liver injured induced by Se deficiency. The aim of this study was to evaluate the influence of Se deficiency on NO and autophagy in chicken livers. A total of 300 1-day-old sea blue white laying hens were divided into two groups (n=150/group), and each of those groups was randomly divided into groups so that the trials were conducted in triplicate. The Se deficiency group (-Se) was fed a Se-deficient corn-soy basal diet (Se content 0.02 mg/kg), and the Se-adequate group used as a control (+Se) was fed the same basal diet supplemented with Se at 0.2 mg/kg (sodium selenite). The liver tissue was collected and examined for pathological observations, inducible NO synthase (iNOS)-NO activities (including NO content and iNOS activity), and mRNA and protein levels of autophagy genes at 15, 25, 35, 45, 55 and 65 days old. The results showed that numerous autophagosomes, as well as a low density of organelles and glycogen, were observed in the chicken livers from the Se deficiency group. In addition, the NO content and iNOS activity in the Se deficiency group were higher ($p < 0.05$) than in the control group. Transcript expression of autophagy genes (LC3-Ⅰ, LC3-Ⅱ, ATG5, Dynein and Beclin1) increased significantly ($p <0.05$), and TOR gene expression fluctuated (first increased and then decreased) in the Se-deficient group compared with that in the corresponding control group. Meanwhile, the protein expression of autophagy genes (LC3-, LC3-Ⅱ, Dynein and Beclin1) also increased significantly ($p <0.05$) in the

Se-deficient group. This indicated that NO and autophagy are involved in the development of liver injury (pathological processes), which is induced by Se deficiency.

Keywords: Selenium Deficiency, NO, autophagy, Liver, Chicken

Introduction

Selenium (Se) is an essential trace element because it plays a crucial role in antioxidant defence mechanisms [1]. As an important detoxification organ, the liver plays a central role in maintaining nutrient homeostasis by regulating protein, carbohydrate, and fat metabolism [2]. Se deficiency can cause metabolic dysfunction, morphological damage and changes in glutathione peroxidase and the expression of selenoprotein genes in the livers of mice, rabbits, trout, turkeys and chickens [3]. It is also possible that catalytic Se compounds may function in a similar fashion to activate apoptosis [4, 5].

Autophagy or cellular self-digestion is a pathway that is crucial for development, differentiation, survival, and homeostasis [6]. Recent data showed that autophagy is involved in major fields of hepatology. As the best-known function of autophagy concerns nutrient starvation, studies on autophagy and liver diseases have focused on liver ischemia/reperfusion with a demonstrable increase in liver cell autophagy [7]. In addition, autophagy was enhanced during acute liver damage in mice [8]. Donati found that the antilipolytic agent 3, 5'-dimethylpyrazole (DMP) could increase both autophagic proteolysis and expression of the autophagic genes LC3 and beclin1 in mammalian liver cells in vivo [9]. Kim found that autophagy was the primary catabolic process of hepatic proteins and conferred cytoprotection against ischemia/reperfusion liver injury [10]. These studies show that disturbance of autophagy function has a major impact on liver physiology and disease. Although autophagy is primarily a survival mechanism, it can also, under certain conditions, lead to autophagic cell death. However, in most liver diseases, it seems clear that one of the major functions of autophagy is to keep cells alive under stressful ‘‘life-threatening” conditions. In addition, Se in the form of sodium selenite has been reported to exert anti-tumour effect in several cancer cell types by inducing autophagic cell death and

apoptosis mediated by reactive oxygen species (ROS) [11]. In addition, it is of potential clinical importance to better understand the molecular mechanisms regulating the autophagic pathway in selenite-induced apoptosis in NB4 cells [12]. However, the relationship between autophagy and Se deficiency is unclear.

NO has broad biological functions as an active intracellular messenger. Several studies have demonstrated the importance of NOS-mediated signaling in many Se deficiency models. Se deficiency not only induced oxidative damage and upregulated NO and iNOS content in chicken intestinal tracts [13] but also induced oxidative stress with NO by inducing apoptosis in chicken immune cells [14]. In addition, Se deficiency induced pancreatic injury by influencing NO and selenoproteins in the chicken pancreas [15].Thus, it can be seen that NO radicals function efficiently in a number of physiological systems and pathophysiological states. NO impaired autophagy during the early stages of autophagosome formation. Some researchers indicated that NO inhibits autophagosome synthesis via a number of mechanisms [16]. Yang reported that reactive oxygen species (ROS) and NO generation were induced by evodiamine in a time-dependent manner and acted in synergy to trigger mitochondria-dependent apoptosis and autophagy through the induction of mitochondrial membrane permeabilization (MMP) by increasing the Bax/Bcl-2 or Bcl-xL ratio [17].

Although the relationship between Se deficiency and liver damage has been widely studied, the effects of Se deficiency on the NO system and autophagy have not been investigated in the chicken liver. Herein, we first established a model of chicken liver damage induced by Se deficiency. Second, we investigated the pathological changes in liver tissue. Third, we investigated the NO content and iNOS activity in the livers of chickens after induced by Se

88 deficiency. Finally, we investigated the mRNA and protein expression levels of several autophagy
89 genes (LC3-Ⅰ, LC3-Ⅱ, ATG5, TOR, Dynein and Beclin1) in the liver tissue during Se deficiency.
90 The present study provides some compensated data about the effect of Se deficiency on changes in
91 NO and autophagy in chicken liver.
92

Materials and Methods

Birds, Diets and Tissue Collections

All of the procedures used in the present study were approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. A total of 300 1-day-old sea blue white laying hens were divided into control and Se deficiency groups. Each treatment group consisted of 150 chickens, and each of these groups was randomly divided into subgroups so that the trials were conducted in triplicate. The chickens were maintained either on a Se-deficient diet (-Se group, Se-deficient granulated diet including corn, soybean meal and wheat bran from Longjiang County, a typical Se-deficient region of the Heilongjiang Province in China (Weiwei Co. Ltd., Harbin, China)) or a normal Se content diet (Control group, +Se；Weiwei Co. Ltd., Harbin, China) for 65 days. According to the different nutritional needs during the growth stages of sea blue white chickens (0-21 days, 22-42 days and 43-65 days), different types of feed were prepared for the +Se and -Se groups. According to National Research Council (NRC), the nutrient requirements of chicken corresponded to 0.2 mg/kg in the control group (+Se) and 0.02 mg/kg in the Se-deficient group (-Se), which was determined by fluorescence spectrophotometry using GB/T 13,883-2008 (PONY TEST Co., Beijing, China). Throughout the entire experimental period, the chickens were allowed ad libitum consumption of feed and water. Clinical symptoms and mortality were also recorded. The 15 chickens in each group were killed with sodium pentobarbital at 15, 25, 35, 45, 55 and 65 days old. The liver tissues were quickly removed, minced and stored at −80 °C to determine the index of oxidative stress and isolate the RNA and protein. In this study, 15 chickens were killed per group at the six sampling events, and 5 chickens per group were used in the official test (n=5), which was repeated in triplicate. The remaining

115 tissues were used in the preliminary experiment and served as standby tissues.

116 Ultrastructural observations

117 For electron microscopy, liver tissue specimens were fixed with 2.5% glutaraldehyde in 0.1
118 M sodium phosphate buffer (pH 7.2) for 3 h at 4℃, washed in the same buffer for 1 h at 4℃ and
119 postfixed with 1% osmium tetroxide in sodium phosphate buffer for 1 h at 4℃. The tissues were
120 then dehydrated in a graded series of ethanol starting at 50% for 10 min after two changes in
121 propylene oxide. The tissue specimens were embedded in araldite. Ultrathin sections were stained
122 with Mg-uranyl acetate and lead citrate for transmission electron microscope evaluation.

123 Determination of NO Content and iNOS Activity in Liver

124 Chicken livers were homogenized on ice in physiological saline and centrifuged at 700×g for
125 15 min at 4℃ before supernatant collection. Here, we detected NO and iNOS as indices of
126 oxidative damage. The NO content and iNOS activity were determined using NO and iNOS
127 activity assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The method used
128 in the present study was according to the procedure previously published by our lab [18] with an
129 ELX800 Microplate reader (BioTek Instruments, USA) to detect the OD at 550 and 530 nm,
130 respectively.

131 Quantification of autophagy gene mRNA expression

132 The method of quantification used was same as in our previous research [19]. After quantification,
133 the expression levels of autophagy genes were determined by quantitative reverse transcription
134 PCR using SYBR Premix ExTaq TM (Takara, China) and an ABI PRISM 7500 real-time PCR
135 system (Applied Biosystems). The PCR primers (Table 1) were designed using Oligo Primer
136 Analysis software (version6.0) and synthesized by Invitrogen (Shanghai, China).

Reactions consisted of the following: 10 μl of 2× SYBR Green I PCR Master Mix (TaKaRa, China), 0.4 μl of 50× ROX reference Dye II, 0.4 μl of each primer (10 μM), 2 μl of diluted cDNA, and 6.8 μl of PCR-grade water. The PCR program for amplification of Hsp genes and GAPDH consisted of 95°C for 30 s followed by 40 cycles at 95°C for 15 s and 60°C for 30 s. The results (fold changes) were expressed as $2^{-\Delta\Delta Ct}$ in which

$\Delta\Delta Ct = (Ct_{LC3\text{-}1} - Ct_{GAPDH})\ t - (Ct_{LC3\text{-}1} - Ct_{GAPDH})\ c$, where $Ct_{LC3\text{-}1}$ and Ct_{GAPDH} are the cycle thresholds for chicken LC3-1 and GAPDH genes in the different treated groups, respectively, t is the Se-deficient group, and c is the control group (Se-adequate group).

Western blot analysis

Protein extracts were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions on 12% gels. Separated proteins were then transferred to nitrocellulose membranes using a tank transfer for 2 h at 200 mA in Trisglycine buffer containing 20% methanol. Membranes were blocked with 5% skim milk for 16-24 h and incubated overnight with diluted primary chicken antibodies against Dynein (1:1,400), Beclin1 (1:500), LC3-Ⅰ and LC3-Ⅱ (1:500; Dynein, Beclin1, LC3-Ⅰ and LC3-Ⅱ polyclonal antibodies were produced by our lab) followed by a horseradish peroxidase (HRP)-conjugated secondary antibody against rabbit IgG (1:1,500, Santa Cruz, CA, USA). To verify the equal loading of samples, the membrane was incubated with a monoclonal β-actin antibody (1:1,000, Santa Cruz, CA, USA) followed by a HRP-conjugated goat antimouse IgG (1:1,000). The signal was detected by X-ray films (Trans Gen Biotech Co., China). The optical density (OD) of each band was determined using the Image VCD gel imaging system, and Dynein, Beclin1, LC3-Ⅰ and LC3-Ⅱ expression were detected as the OD ratio between Dynein, Beclin1, LC3-Ⅰ and LC3-Ⅱ and that of β-actin respectively.

159 Statistical analyses

160 Data analysis was performed using SPSS for Windows system (SPSS, Chicago, IL, USA).
161 Data were expressed as the mean ± standard deviation. The K-S test was used to verify normal
162 distribution in the data and the homogeneity of variances. The data that meet the normal
163 distribution and showed no significant difference (>5% significance level) was used for further
164 analyzing. The differences between means were assessed using a two-tailed paired Student's T-test.
165 $P < 0.05$ was considered statistically significant."

166

Results

Ultrastructural observations

The Se deficiency effects on the histopathology of the liver electron microscopy are shown in (Fig. 1). No obvious ultrastructural changes were observed in the control group (Fig. 1A). Widened intercellular space between hepatocytes was observed in the Se-deficient group. In the Se-deficient group, hepatocytes showed numerous autophagosomes as well as a low density of organelles and of glycogen. Moreover, some hepatocytes presented morphological characteristics of autophagic cell death (also called type II cell death) (Fig. 1B and 1C).

Changes in NO Content and iNOS Activity

The effects of Se deficiency on NO content and iNOS activity in liver are shown in (**Table. 2and Fig. 2**). As shown in Fig. 2A, the NO content in the Se-deficient group was significantly increased ($p < 0.05$) compared to that of the each corresponding control groups in a time-dependent manner (from day 15 to day 65). The NO content at day 65 in the Se-deficient group was increased 4.45-fold in the liver compared with the control group. During the trial period, the NO content peaked at day 65 in the Se-deficient group. The NO content increased 4.45-fold in liver compared to the control group.

As shown in Fig. 2B, iNOS activity in the Se-deficient group was also significantly increased ($p < 0.05$) compared to that of the each corresponding control group in a time-dependent manner (from day 15 to day 65). iNOS activity at day 65 in the Se-deficient group was increased 2.29-fold in liver compared with that of the control group.

Effects of Se deficiency on the mRNA expression levels of LC3-Ⅰ, LC3-Ⅱ, ATG5, TOR, Dynein and Beclin1 in chicken livers

189 To determine the effects of Se deficiency on the expression of autophagy genes in livers of
190 chickens at different time intervals, the mRNA expression levels were examined by quantitative
191 reverse transcription PCR (**Table. 3**). Compared with the corresponding control groups, the
192 mRNA levels of the LC3-Ⅰ, LC3-Ⅱ, ATG5 and Dynein gene in the livers of Se-deficient
193 chickens in the 35-, 45-, 55- and 65-day treatment groups were significantly increased ($p < 0.05$)
194 (**Figs. 3A, 3B, 3C** and **3E**). Compared with the corresponding control groups, the mRNA levels of
195 the Beclin1 gene in livers of Se-deficient chickens in the 25-, 35-, 45-, 55- and 65-day treatment
196 groups all significantly increased ($p < 0.05$) (**Fig. 3F**). However, TOR gene expression fluctuated,
197 first increasing ($p < 0.05$) and then decreasing ($p > 0.05$) (**Fig. 3D**).

198 **Western bolt analysis of autophagy levels**

199 The protein expression levels of LC3-, LC3-Ⅱ, Dynein and Beclin1 were examined by
200 western blots. The results (**Table. 4** and **Fig. 4**) revealed that protein expression of the four
201 autophagy genes in the Se-deficient group were gradually increased in the livers of chickens
202 compared with the control group. What is more, the western blot results of LC3-Ⅰ, LC3-Ⅱ,
203 Dynein and Beclin1 were consistent with LC3-Ⅰ, LC3-Ⅱ, Dynein and Beclin1 mRNA responses
204 to Se deficiency.

205

206

Discussion

Se is an essential micronutrient and plays an important role in biological functions in humans and many other species [20-22]. Se-deficient diets not only increase the AST and ALT values (clinical indices of liver damage) and [23] caused morphological damage in rat livers [24]; but also caused mouse liver cytosolic oxidative stress [25] and down-regulation of GPx1 mRNA. The loss of GPx1 activity causes cellular damage in rabbit livers [26]. Some research has also shown that apoptosis or a synchronized process of apoptosis and necrosis can be induced by Se deficiency [27, 28]. Abundant studies have indicated that macroautophagy in the liver is important for the balance of energy and nutrients for basic cell functions, the removal of misfolded proteins and the turnover of major subcellular organelles, such as the endoplasmic reticulum, mitochondria, and peroxisomes, under both normal and pathophysiological conditions [29]. Therefore, the aim of the present study was to examine autophagy during liver damage induced by Se deficiency. In the present study, the data of histopathological changes indicated that autophagy occurred in the chicken liver tissues in which damage was induced by Se deficiency (Fig. 1B, C).

NO is a small signalling molecule with important regulatory effects in many tissues. Some reports showed that Se deficiency up-regulated NO concentrations and led to lesions in the small intestines of humans [30]; Se deficiency can induce iNOS activity and NO overproduction in chicken immune tissues [14]. Consistent with the studies described above, we found that Se deficiency induced high levels of iNOS and NO in chicken livers and that the NO content and iNOS activity were significantly increased in the Se-deficient chicken livers throughout the experimental period (from day 15 to 65). These results indicated that under conditions of Se deficiency, the antioxidant defence system was undermined in vivo, which led to the accumulation of ROS and the subsequent release of a large number of inflammatory mediators while stimulating an increase in

iNOS expression and the excessive release of NO [13].

NO, provided by the donor S-nitrosocysteine (SNOC), induced cell death and autophagy with autophagosomes engulfing injured mitochondria [31]. However, little is known about the effect of NO on autophagy induced by Se deficiency. In the present study, Se deficiency induced a statistically significant increase in the levels of the NO system and hepatic tissue autophagy in chickens. Some data show that NO inhibits autophagic flux in mammalian cells. NO S-nitrosylates JNK1 and IKKβ affect autophagy, as NO decreases JNK1 activity and Bcl-2 phosphorylation and activates mTORC1 in an IKKβ- and TSC2-dependent manner. Overexpression of NOS isoforms also impairs autophagic flux. Conversely, the inhibition of NO synthesis induces autophagy and protects against neurodegeneration in models of Huntington's disease [16]. Our data indicate the role of NO in regulating autophagy, which may have underlying implications for its myriad of cellular functions. A previous study showed that ROS/NO contributed to the activation of autophagy over a short incubation period and acted to prevent autophagy during a long incubation period. Such reports have demonstrated that ROS can exert an inductive effect on autophagy, the mechanism of which has been suggested to be related to its specific regulation of the activity of Atg4 [32]. This may be because ROS can regulate autophagy under amino acid and serum starvation conditions, whereas superoxide has been suggested to be the major ROS species involved in ROS-mediated autophagy [33,34].

The autophagy-related genes play essential roles at different stages of the autophagic process, including induction, vesicle formation, autophagosome formation and autolysosome formation, and were first identified and characterized in yeast [35]. LC3 is a specific marker of autophagosome formation and the Atg proteins are crucial for autophagosome assembly. It has been reported that

LC3 and ATG5 take part in the formation of the autophagosome [36.] In the present study, the mRNA expression of LC3-I, LC3-II and ATG5 significantly increased in the chicken livers in which damage was induced by Se deficiency. The protein kinase target of rapamycin (TOR) is inactivated in response to an exogenous stimulus [37]. In the current study, the mRNA levels of TOR fluctuated (first increased and then exhibited the largest decrease observed on the 65 day) in the livers exposed to Se deficiency, which reflects the development of a compensatory mechanism in response to increased oxidative stress. Dyneins are large multi-component microtubule-based molecular motors involved in many fundamental cellular processes, including vesicular transport, mitosis and ciliary/flagellar beating [38]. In our study, the mRNA and protein levels of Dynein were increased in chicken livers exposed to Se deficiency. Thus, Dynein may be a transporter that plays an important role in the combination of autophagosomes and lysosomes in the liver after exposure to Se deficiency. Beclin1 participates in the early stages of autophagosome formation, promoting autophagosome nucleation [39]. Protein expression of Beclin 1 was increased in PC-12 cells exposed to cadmium [40]. Consistent with the prior study, the mRNA and protein levels of Beclin 1 were increased in chicken livers exposed to Se deficiency.

In conclusion, the present study clearly showed that Se deficiency increased the NO production and the activity of iNOS and eventually induced the autophagy of liver tissue. These results strongly indicate that NO and autophagy are involved in the development of liver injury and play a major role in the liver damage induced by Se deficiency.

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351 **Table. 1 Primers used for quantitative real-time PCR**

Target gene	Primer Sequence(5'-3')
LC3-Ⅰ	Forward 5'-TTACACCCATATCAGATTCTTG-3'
	Reverse 5'-ATTCCAACCTGTCCCTCA-3'
LC3-Ⅱ	Forward 5'-AGTGAAGTGTAGCAGGATGA-3'
	Reverse 5'-AAGCCTTGTGAACGAGAT-3'
ATG5	Forward 5'-GGCACCGACCGATTTAGT-3'
	Reverse 5'-GCTGATGGGTTTGCTTTT-3'
TOR	Forward 5'-GGACTCTTCCCTGCTGGCTAA-3'
	Reverse 5'-TACGGGTGCCCTGGTTCTG-3'
Dynein	Forward 5'-CGGCTTGACCTATGGAATCT-3'
	Reverse 5'-CATCACTGCGAGGAACTGC-3'
Beclin1	Forward 5'- CGACTGGAGCAGGAAGAAG-3'
	Reverse 5'- TCTGAGCATAACGCATCTGG-3'
GAPDH	Forward5'-AGAACATCATCCCAGCGT-3'
	Reverse5'-AGCCTTCACTACCCTCTTG-3'

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354 **Table. 2** Effects of dietary Se deficiency on activity of NO and iNOS in chicks' liver

	group	Relative level and value P					
		15day	25day	35day	45day	55day	65day
	+Se	11.982±0.877	11.993±0.869	12.061±0.622*	12.018±0.207*	12.148±1.270*	12.329±0.374*
	-Se	17.219±0.356	26.920±1.703	31.192±1.024	42.098±1.043	58.591±3.441	67.288±4.334
NO	P	0.0001	0.0002	0.0001	0.0001	0.0001	0.0001
	+Se	1041.826±33.417	1095.995±169.673	1131.809±148.519*	1164.417±79.787*	1215.533±68.573*	1226.174±61.165*
	-Se	1571.171±31.659	1964.438±236.664	2289.594±167.672	2386.171±83.249	3258.926±249.659	4034.070±244.579
iNOS	P	0.0001	0.0014	0.0002	0.0001	0.0001	0.0001

355

356

357 **Table. 3** Effects of dietary Se deficiency on liver relative mRNA abundance of autophagy genes in
358 chicks

gene	group	Relative mRNA level and value P					
		15day	25day	35day	45day	55day	65day
	+Se	1.000±0.000	1.052±0.020	1.091±0.139*	1.108±0.086*	1.085±0.031*	1.086±0.086*
	-Se	1.134±0.112	1.264±0.111	1.573±0.125	1.772±0.160	2.723±0.150	3.290±0.316
LC3-1	P	0.344	0.255	0.045	0.036	0.047	0.001
	+Se	1.000±0.000	1.033±0.091	1.079±0.106*	1.159±0.101*	1.208±0.115*	1.235±0.104*
	-Se	1.260±0.101	1.519±0.126	2.130±0.126	2.387±0.197	2.451±0.231	3.475±0.238
LC3-2	P	0.159	0.205	0.032	0.012	0.018	0.014
	+Se	1.000±0.000	1.010±0.100	1.040±0.103*	1.075±0.104*	1.084±0.043*	1.103±0.106*
	-Se	1.036±0.090	1.955±0.123	2.415±0.224	2.416±0.180	2.967±0.274	4.514±0.347
ATG5	P	0.743	0.054	0.031	0.001	0.020	0.043
	+Se	1.000±0.000*	1.042±0.100*	1.093±0.108	1.099±0.104	1.121±0.079	1.292±0.118*
	-Se	3.399±0.298	3.048±0.268	1.942±0.142	1.668±0.110	1.180±0.114	0.279±0.022
TOR	P	0.010	0.007	0.053	0.365	0.693	0.041
	+Se	1.000±0.000	1.023±0.097	1.053±0.084*	1.023±0.101*	1.059±0.102*	1.146±0.111*
	-Se	1.050±0.083	2.070±0.139	2.224±0.160	2.669±0.231	3.450±0.252	4.717±0.254
Dynein	P	0.688	0.199	0.003	0.039	0.001	0.014
	+Se	1.000±0.000	1.025±0.101*	1.145±0.100*	1.115±0.109*	1.108±0.110*	1.153±0.087*
	-Se	1.081±0.094	1.804±0.156	2.897±0.189	3.294±0.285	4.451±0.315	4.628±0.358
Beclin1	P	0.490	0.001	0.029	0.003	0.001	0.015

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361 **Table. 4** Effects of dietary Se deficiency on liver on liver protein expression levels of autophagy
362 genes in chicks

gene	group	Relative protein level and value P					
		15day	25day	35day	45day	55day	65day
	+Se	1.000±0.000	1.061±0.040	1.100±0.137*	1.017±0.105*	1.094±0.051*	1.094±0.105*
	-Se	1.110±0.121	1.227±0.120	1.506±0.134	1.685±0.168	1.821±0.158	1.951±0.322
LC3-1	P	0.196	0.103	0.023	0.031	0.022	0.016
	+Se	1.000±0.000	1.043±0.110	1.088±0.125*	1.167±0.120*	1.115±0.134*	1.142±0.122*
	-Se	1.224±0.110	1.457±0.134	1.507±0.135	2.018±0.205	2.296±0.238	2.217±0.245
LC3-2	P	0.072	0.077	0.019	0.005	0.002	0.003
	+Se	1.000±0.000	1.033±0.116	1.062±0.103*	1.032±0.120*	1.068±0.121*	1.054±0.130*
	-Se	1.035±0.092	1.353±0.148	1.459±0.168	1.792±0.239	2.195±0.259	2.335±0.261
Dynein	P	0.605	0.109	0.013	0.005	0.0001	0.0001
	+Se	1.000±0.000	1.135±0.119*	1.153±0.119*	1.123±0.128*	1.117±0.129*	1.162±0.106*
	-Se	1.063±0.102	1.314±0.165	1.698±0.197	2.054±0.292	2.295±0.322	2.551±0.364
Beclin1	P	0.161	0.016	0.011	0.004	0.0006	0.004

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Figure legends:

Fig. 1 The effects of Se deficiency on liver tissue changes, as observed by histopathology under an electron microscopy. A is in control group. B and C are in Se-deficient group.

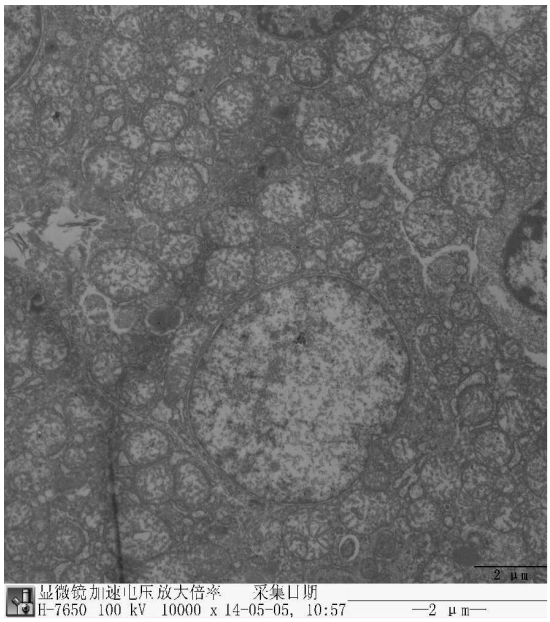
Fig. 2 The effect of redox activities on the liver induced by selenium deficiency in chickens. A, shows the changes in NO activity. B, shows the changes in iNOS activity. *indicates that there were significant differences ($p<0.05$) between the control group and the Se-deficient group at the same time point. Each value represents the mean ± SD of five individuals.

Fig. 3 Effects of Se deficiency on mRNA expression in chicken livers. A–F The mRNA expression of LC3-Ⅰ, LC3-Ⅱ, ATG5, TOR, Dynein and Beclin1 genes, respectively. In the experiment, the relative mRNA levels of the autophagy genes were detected by qPCR; the relative mRNA levels from the 15-, 25-, 35-, 45-, 55- and 65-day control group were used as the reference values in A–F. Each value represents the mean ± SD of five individuals. *, significant differences ($p<0.05$) between the control group and the Se-deficient group at the same time point.

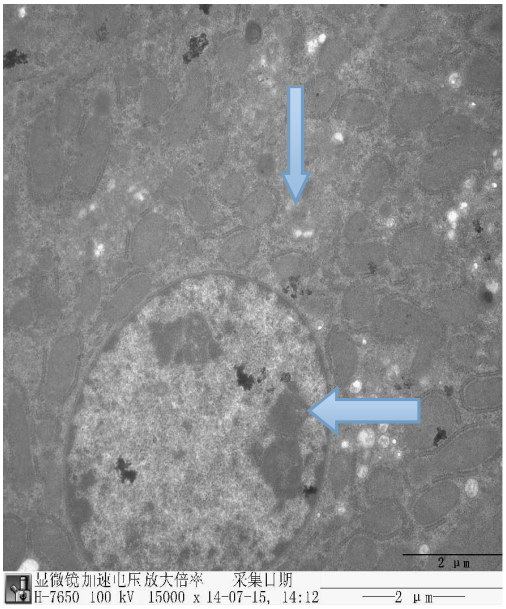
Fig. 4 Effects of Se deficiency on LC3-, LC3-Ⅱ, Dynein and Beclin1 protein expression in chicken livers. C indicates the control group, and L indicates the Se-deficient group. The experiment time points are 15-, 25-, 35-, 45-, 55- and 65-days.

Figures:

389 Fig. 1



A



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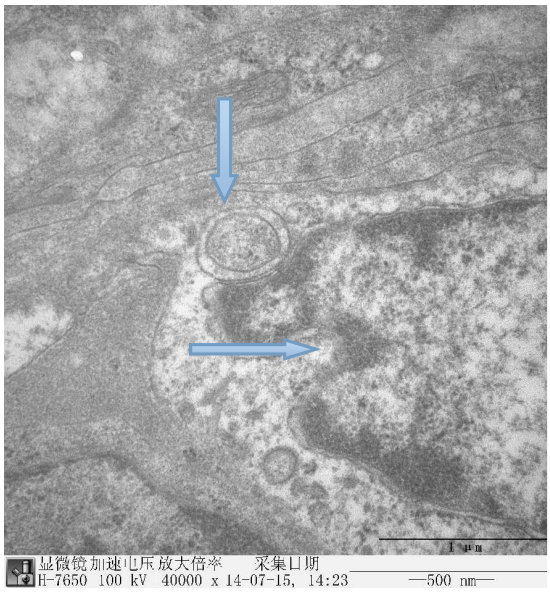

C

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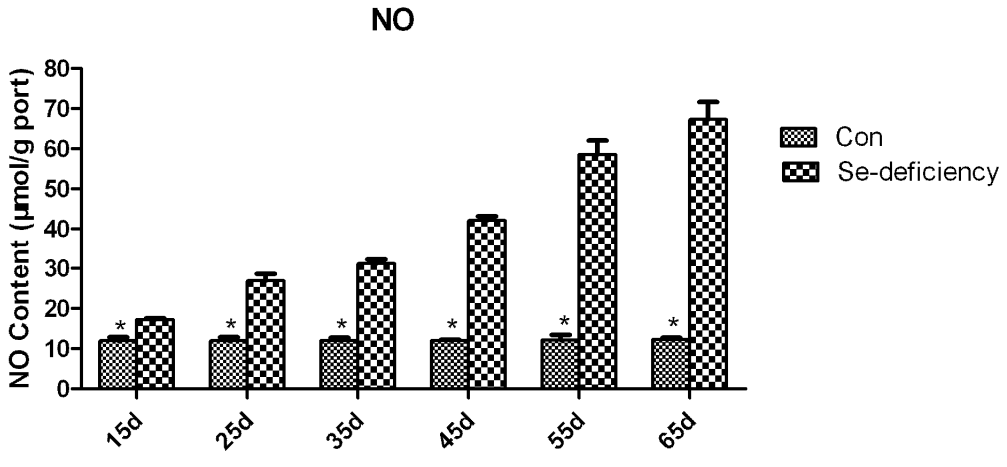
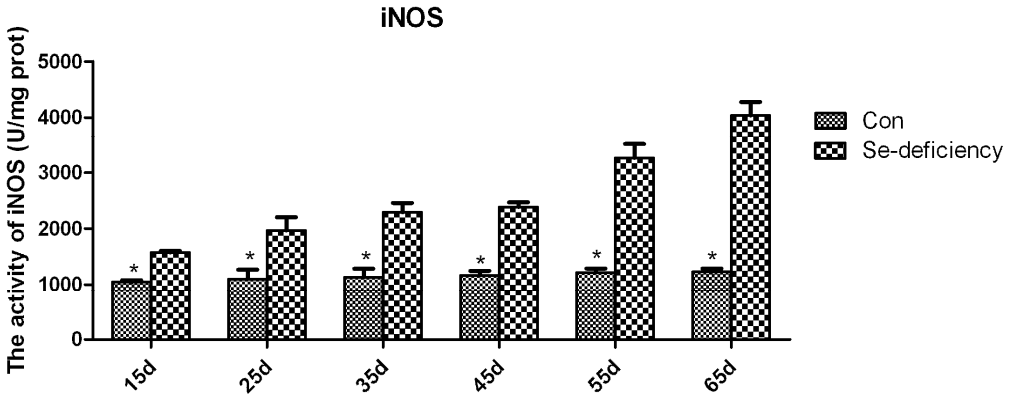
391 Fig. 2



A



B

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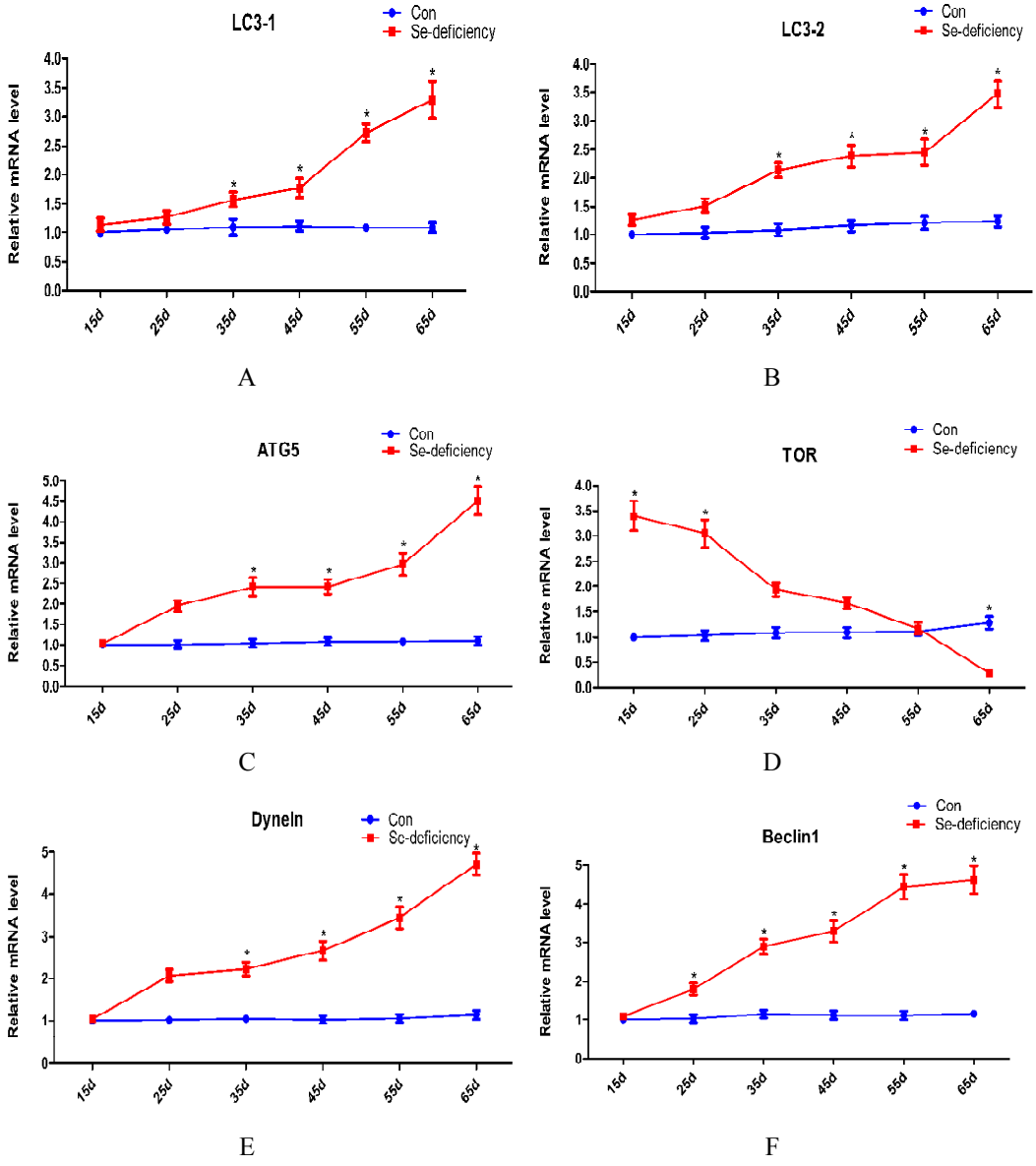
393 Fig. 3



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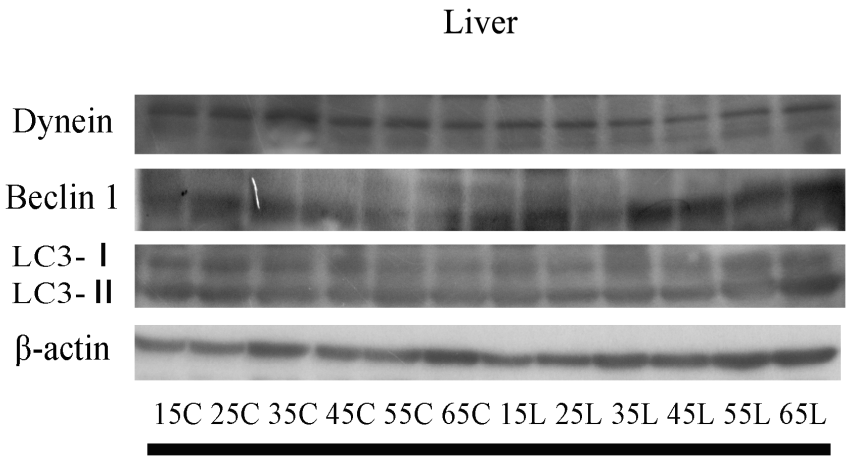
395 Fig. 4



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