BIOLOGICAL SCIENCES

Effect of dietary NiCl$_2$ on the cell cycle of cecal tonsil in the chicken broiler

LU HU, BAOLIN SONG, HENGMIN CUI & BANGYUAN WU

Abstract: Although the effects of nickel chloride (NiCl$_2$) on the immune system have long been recognized, little is known about the effects of nickel (II) on the cell cycle and related signaling events in immune organs, such as cecal tonsil, a key immune organ of chicken. In the present study, we investigated the effect of NiCl$_2$ on the cell cycle of cecal tonsil. The cell cycle was detected by the methods of flow cytometry (FCM), quantitative real-time PCR (qRT-PCR) and immunohistochemistry (IHC). The results showed that dietary NiCl$_2$ in excess of 300 mg/kg caused the G2/M cell cycle arrest and the reduction of cell proportion at S phase of the cecal tonsil. The G2/M cell cycle arrest was accompanied by the up-regulation of p53, p21 protein expression and mRNA expression, and down-regulation of cyclinB and proliferating cell nuclear antigen (PCNA) protein expression and mRNA expression. The data suggested that the cells' (mainly the T lymphocytes) proliferation in the cecal tonsil was inhibited by the high dietary NiCl$_2$.

Key words: NiCl$_2$, cell cycle, cecal tonsil, chicken.

INTRODUCTION

Nickel (Ni) is a metal widely distributed in the environment, and is considered to be a transition metal with mutagenic and carcinogenic activities as well as immune regulatory functions. High nickel exposure has the potential to produce a variety of pathological effects, such as immunological effects. Previous studies have reported that dietary nickel chloride (NiCl$_2$) could cause the splenocyte apoptosis (Huang et al. 2013), has inhibitive effects on thymocytes (Tang et al. 2015), has toxic effect on development and induce the histopathological lesions in the broiler's bursa of Fabricius (Yin et al. 2016a, 2016b), as well as significantly declines the cytokine mRNA expression and protein levels in intestinal mucosal immunity of broilers (Wu et al. 2015). Decreased number and activity of immune cells or the immune factors (such as cytokines) can induce immune dysfunction and impair a wide range of tissue responses. In addition, it has been reported that nickel nanowires could induce cell cycle arrest and apoptosis by generation of reactive oxygen species in HeLa cells (Ma et al. 2014). Other studies also reported that nickel acetate can induce the increase of the cell percentages in G2/M phase (Shiao et al. 1998), and NiCl$_2$ can induce cell cycle arrest at the phases of G0/G1 in thymus (Tang et al. 2014).

Cell cycle, as an important cellular mechanisms in life, which plays central roles in maintaining the homeostasis in the multicellular organisms. The process of the cell cycle includes DNA replication (S phage), nuclear division and cell division (M phage), the cell-cycle gap phase between M phase and S phase (G1 phage) and the cell-cycle gap phase between S phase and M phase (G2 phase) (Hartwell & Kastan 1994). The proteins regulate the cell's progression through the stages of the cell cycle and are in turn regulated by numerous proteins, including p53,

p21, cyclin-cdk complexes, including proliferating cell nuclear antigen(PCNA) and cyclin A and B1 and so on (Schafer 1998). The imbalances in cell proliferation and cell death will be caused when cell cycle control is disturbed (Mechali & Lutzmann 2008). However, little research has been aimed at cell cycle arrest of the cecal tonsil which induced by NiCl$_2$. Therefore, the objective of this study was to investigate the effect of NiCl$_2$ on cell cycle in chicken broilers. In this study, we detected the cell cycle by flow cytometry (FCM), and the protein expression and the mRNA expression levels of p53, p21, cyclin B and PCNA involved in the cell cycle arrest.

MATERIALS AND METHODS

Chickens and diets

One hundred and twenty one-day-old healthy Cobb broilers were randomly allotted by body weight to 2 groups with 6 replicates each and 6 broiler chickens in each replicate. Broilers were housed in cages with electrically heated units and provided with water as well as undermentioned experimental diets *ad libitum* for 42 days.

A corn–soybean basal diet formulated by the National Research Council (NRC 1994) was the control diet. NiCl$_2$·6H$_2$O (CAS.No.13931-83-4, Chengdu Kelong Chemical Reagent Company, Chengdu, China) was mixed into the corn–soybean basal diet to produce experimental diets with 300 mg/kg, 600 mg/kg and 900 mg/kg of NiCl$_2$, respectively. The environment and facilities of the laboratory animal are in line with the national standard (Chinese, GB4925-2010). The research compliesied with China West Normal University Animal Care Committee guidelines.

Determination of the cell cycle of the cecal tonsils cell cycle analysis by flow cytometry (FCM)

The preparation of cecal tonsil mono nuclear cells was performed as described by Wu et al. (2014). Briefly, the cecal tonsils were immediately removed and ground to form a cell suspension with mechanical method, and then was filtered through a 300-mesh nylon screen to get, and then the mononuclear cells were washed twice with cold PBS (phosphate buffer solution, pH 7.2-7.4) and suspended in PBS at a concentration of 1×10^6 cells/mL. A total of 500 µL of the cell suspension was transferred to a 5-mL culture tube. After centrifugation (600 rpm, 5 min), the supernatant was decanted, the cells were incubated for 30 min at room temperature in the dark with 5 µL 0.25% Triton X-100 and 5 µL Propidium Iodide (PI) (Cat.No.51-66211E). Finally, 500 µL of PBS were added to each tube, and cells were analyzed by flow cytometry (BD FACS Calibur) within 45 min of preparation. The results were analyzed using the Mod Fit LT for Mac V3.0 computer program.

Determination of the cell cycle regulatory molecule mRNA expression by quantitative real-time PCR (QRT-PCR)

The cecal tonsil from six chickens in each group were taken at 14, 28, and 42 days of age and stored in liquid nitrogen, then homogenized in liquid nitrogen using a mortar and pestle. Total RNA was extracted, using RNAiso Plus (9108/9109, Takara, Japan), and the cDNA was synthesized using a Prim- Script™ RT reagent Kit (RR047A, Takara, Japan) according to the manufacture's protocol. The cDNA product was used as a template for qRT-PCR analysis. Sequences for target genes were obtained from the NCBI database. Oligonucleotide primers were designed using Primer 5 software and synthesized at Takara (Dalian, China, Table I).

Table I. Sequence of primers used in qRT-PCR.

Gene symbol	Accession number	Primer	Primer sequence(5'-3')	Product size	Tm (°C)
p53	NM205264.1	Forward Reverse	ACCTGCACTTACTCCCCGGT TCTTATAGACGGCCACGGCG	127bp	59
p21	AF513031.1	Forward Reverse	TCCCTGCCCTGTACTGTCTAA GCGTGGGCTCTTCCTATACAT	123bp	60
cyclinB	NM205239.2	Forward Reverse	ATCACCAACGCTCACAAGAAC AGGCTCCACAGGAACATCTG	171bp	59
PCNA	AB053163.1	Forward Reverse	GATGTTCCTCTCGTTGTGGAG CAGTGCAGTTAAGAGCCTTCC	104bp	60
β-actin	L08165	Forward Reverse	TGCTGTGTTCCCATCTATCG TTGGTGACAATACCGTGTTCA	178bp	62

For qRT-PCR reactions, 25 µL mixtures were made by using SYBR® Premix Ex TaqTM II (DRR820A, Takara, Japan), containing 12.5 µL Tli RNaseH Plus, 1.0 µL of forward and 1.0 µL of reverse primer, 8.5µL RNAase-free water and 2 µL cDNA. Reaction conditions were set to 3 min at 95 °C (first segment, one cycle), 10 s at 95 °C and 30 s at Tm of a specific primer pair (second segment, 44 cycles) followed by 10 s at 95 °C, and 72 °C for 10 s (dissociation curve segment) using Thermal Cycler (C1000, BIO RAD, USA). mRNA expression was analyzed for 4 genes (Table I), and β-actin expression was used as an internal reference housekeeping gene. All qRT-PCR were performed using the SYBR® Premix Ex TaqTMII system (DRR820A, Takara, Japan) using on a Model C1000 Thermal Cycler (Bio Rad, USA). Gene expression values from control group subsamples at 14, 28, and 42 days of age were used to calibrate gene expression in subsamples from corresponding experimental subsamples. All data output from the qRT-PCR experiments were analyzed using the 2-ΔΔCT method (Livak & Schmittgen 2001).

Determination of the cell cycle regulatory molecule protein expression by immunohistochemistry (IHC)

Six chickens in each group were humanely sacrificed and then the cecal tonsils were collected and fixed in 4% paraformaldehyde, dehydrated in ethanol and embedded in paraffin.

The method was described as followings: Cecal tonsils slices were dewaxed in xylene, rehydrated through a graded series of ethanol solutions, washed in distilled water and PBS and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 15 min. The sections were subjected to antigen retrieval procedure by microwaving in 0.01 M sodium citrate buffer pH 6.0. Additional washing in PBS was performed before 30 min of incubation at 37 °C in 10% normal goat serum (Cat.No.AR0009, Boster, Wuhan, China). The slices were incubated overnight at 4 °C with the primary antibodies (Table II). After washing in PBS, the slices were exposed to 1% biotinylated goat anti-rabbit IgG secondary antibody (BM2004, Boster, Wuhan, China) for 1 h at 37 °C, and then incubated with strept avidin-biotin complex (SABC, Cat.No.

SA1022, Boster, Wuhan, China) for 30 min at 37 °C. To visualize the immunoreaction, sections were immersed in diaminobenzidine hydrochloride (DAB, Cat.No.AR1000, Boster, Wuhan, China). The slices were monitored microscopically and stopped by immersion in distilled water, as soon as brown staining was visible. Slices were lightly counterstained with hematoxylin, dehydrated in ethanol, cleared in xylene and mounted.

The cell cycle checkpoint protein expression was counted using a computer-supported imaging system connected to a light microscope (OlympusAX70) with an objective magnification of ×400. The mean intensity of staining for each protein was quantified using Image-pro Plus 5.1 (USA) as described previously. Each group was measured five sections and each section was measured five visions and averaged.

Statistical analysis

The significance of difference among four groups was analyzed by variance analysis, and results presented as mean ± standard deviation (X ± SD). The variation was measured by one-way analysis of variance (ANOVA) test of SPSS 16.0 for windows. Statistical significance was considered at $P<0.05$, $P<0.01$ was considered highly significant.

RESULTS

Cell cycle changes in the cecal tonsil

Figure 1 shows that the cell percentages in G0/G1 phase were significantly lower ($P<0.05$ or $P<0.01$) in the 600 and 900 mg/kg groups at 28 or 42 days of age when compared with those in the control group. The cell percentages in G2/M phase were significantly increased ($P<0.05$ or $P<0.01$) in the 600 and/or 900 mg/kg groups from 14 to 42 days of age and in the 300 mg/kg group at 28 and 42 days of age in comparison with those in the control group. The cell percentages in S phase were significantly decreased ($P<0.05$ or $P<0.01$) in the 600 and 900 mg/kg groups at 28 and 42 days of age than those in the control group. The results show a dose and time dependent increase in G2M phase cells and a corresponding decrease in cells at G2M and S stages of the cell cycle.

Changes of the cell cycle regulatory molecule protein expression in the cecal tonsil

The p53 protein expression was higher ($P<0.05$) in the 900 mg/kg group at 14 days of age and significantly higher ($P<0.05$ or $P<0.01$) in the 600 and 900 mg/kg groups at 28 days of age and in the 300, 600 and 900 mg/kg groups at 42 days of age when compared with those in the control group. The p21 protein expression was increased ($P<0.05$) in the 900 mg/kg group at 14

Table II. Antibodies used in immunohistochemistry (IHC).

name	Company	Cat.No	Dilution
p53	Boster, China	BM0101	1:100
p21	Boster, China	BA0227	1:100
cyclinB1	Bioss, China	BS-0572R	1:100
PCNA	Boster, China	BM0104	1:100
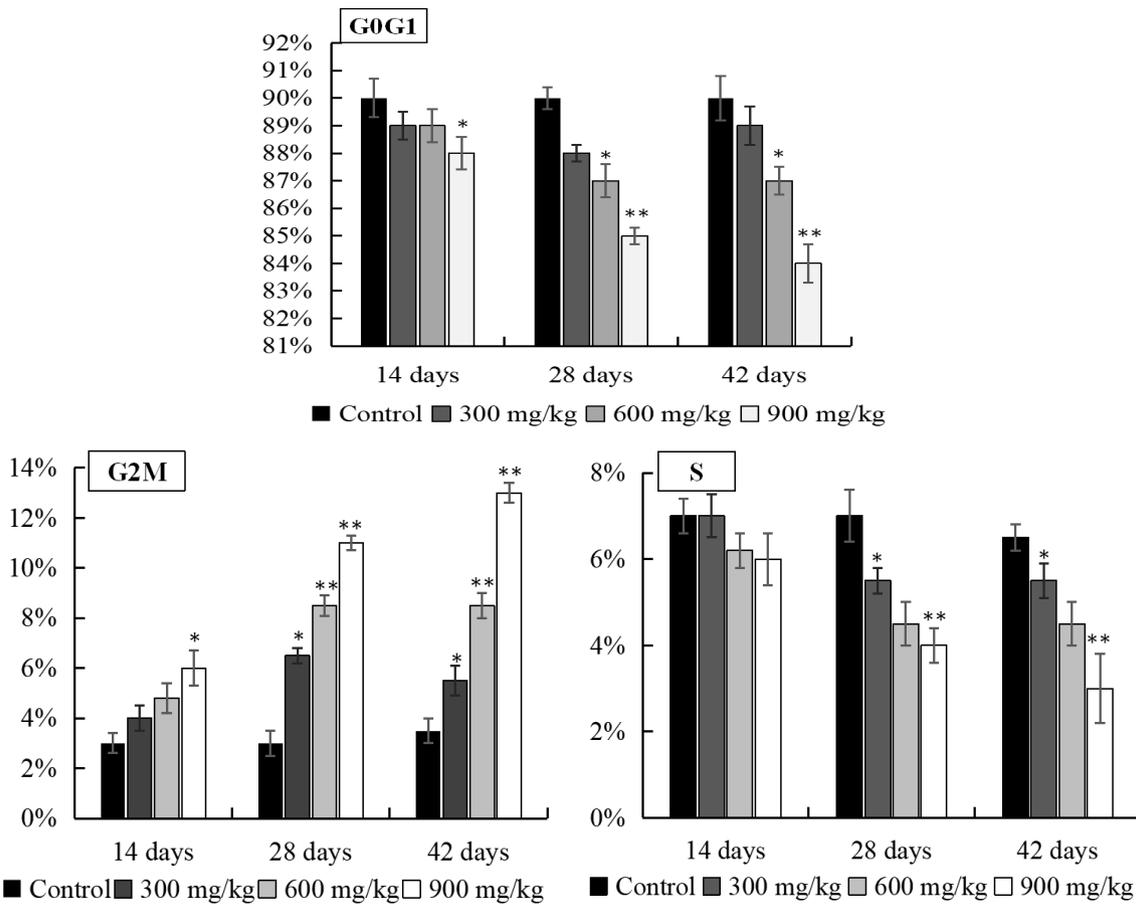
Figure 1. Changes of cell cycle phase distribution (%) in the cecal tonsil. Data are presented with the mean ± standard deviation (n=6) *P<0.05, compared with the control group **P<0.01, compared with the control group.

days of age, was significantly increased (P<0.05 or P<0.01) in the 600 and 900 mg/kg groups at 28 and 42 days of age in comparison with those in the control group. The cyclin B1 protein expression was significantly higher (P<0.05 or P<0.01) in the 600 and 900 mg/kg groups than those in the control group from 14 to 42 days of age. The PCNA protein expression was decreased (P<0.05) in the 900 mg/kg group at 14 days of age, and was significantly decreased (P<0.05 or P<0.01) in the 600 and 900 mg/kg groups at 28 days of age, and in the 300, 600 and 900 mg/kg groups at 42 days of age, as showed in Figures 2 and 3.

Changes of cell cycle regulatory molecule mRNA expression in the cecal tonsil

As showed in Figure 4, the mRNA expression of p53 was increased (P<0.05) in the 600 mg/kg and 900 mg/ kg groups at 28 days of age and was significantly increased (P<0.05 or P<0.01) in the 300, 600 mg/kg and 900 mg/kg groups at 42 days of age than that in control group. The mRNA expression of p21 was significantly higher (P<0.01) in the 900 mg/kg group at 14 days of age, was significantly higher (P<0.05 or P<0.01) in the 600 and 900 mg/kg groups at 28 days of age, and was significantly higher (P<0.05 or P<0.01) in the 300, 600 and 900 mg/kg groups at 42 days of age. The mRNA expression of cyclin B was decreased (P<0.05) in the 900 mg/kg group
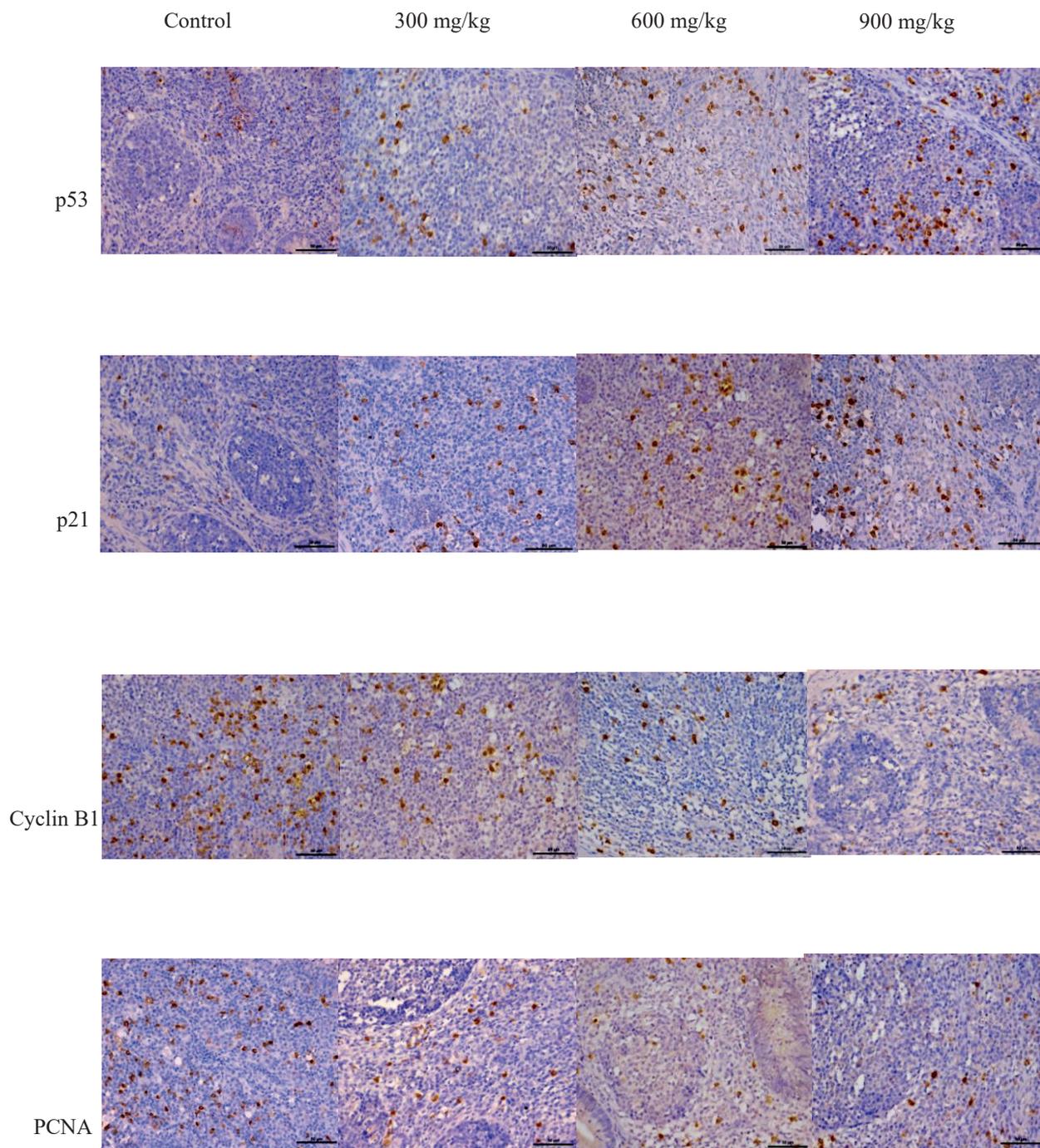
Figure 2. Changes of p53, p21, Cyclin B1 and PCNA protein expression levels in the cecal tonsil at 42 days of age. (Immunohistochemistry, ×400).

at 14 days of age and was significantly decreased (P<0.05 or P<0.01) in the 300, 600 and 900 mg/ kg groups at 28 and 42 days of age than that in the control group, except for 300 mg/kg group at 28 days of age. The PCNA mRNA expression was lower (P<0.05) in the 900 mg/kg group, and was significantly lower (P<0.05 or P<0.01) in the 600 and 900 mg/kg groups at 28 and 42 days of age when compared with that in the control group.
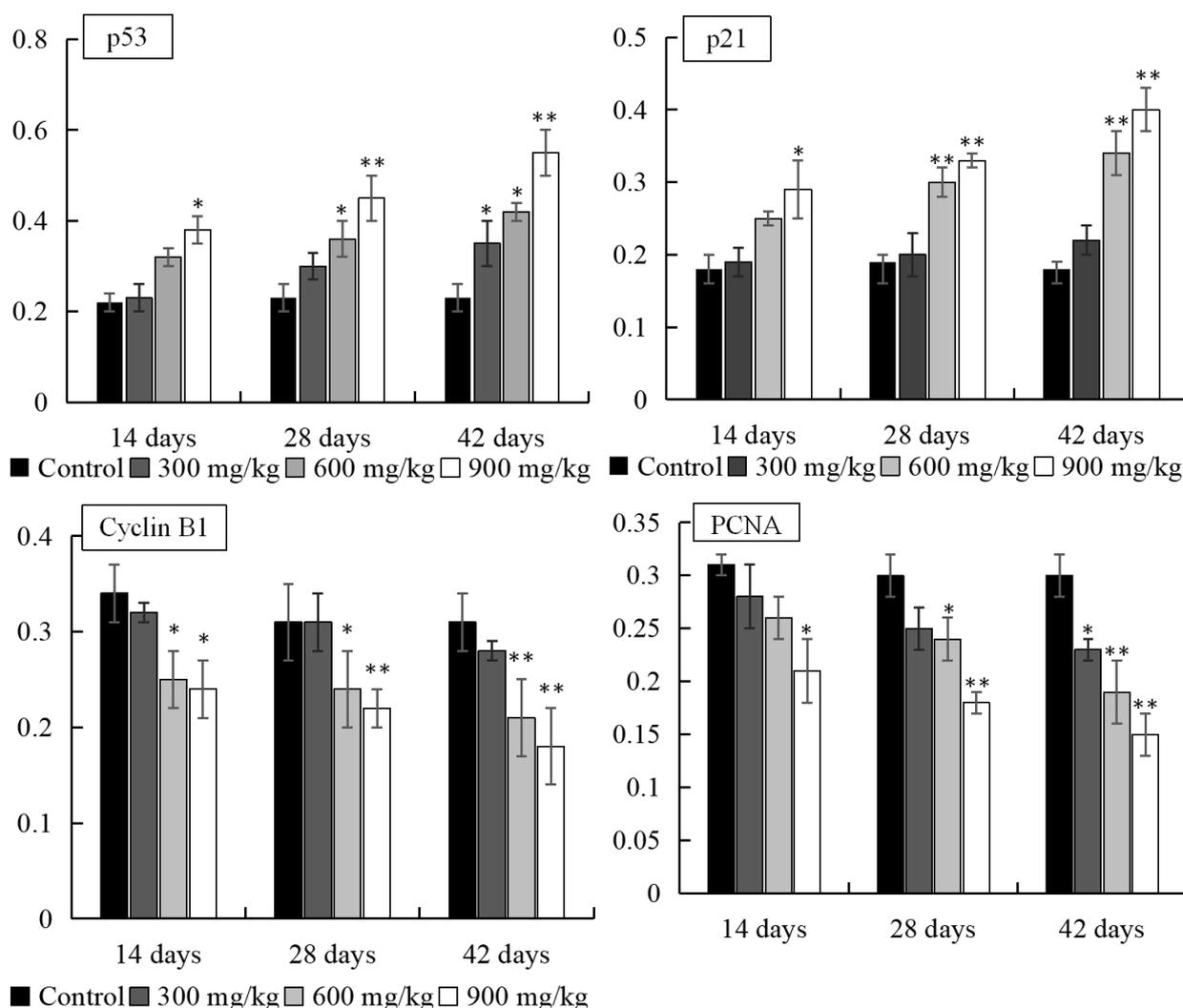
Figure 3. Changes of the mean density of p53, p21, Cyclin B1 and PCNA protein expression in the cecal tonsil. Data are presented with the mean ± standard deviation (n=6×6) *P<0.05, compared with the control group **P<0.01, compared with the control group.

DISCUSSION

The present study shows the effects of dietary NiCl₂-induced the cell cycle arrest in the cecal tonsil of broiler chickens. The results suggested that dietary NiCl₂ induce cell cycle arrest, mainly at the G2/M phase and S phase (Figure 1), which may finally result in cell-growth arrest or even apoptosis (Lupertz et al. 2010). Similar results were reported by Shiao et al. (1998) and Guo et al. (2015), they found that nickel acetate or nickel chloride could increase the cell proportion in

G2/M phase and decreases the cell proportion in S phase in the Chinese hamster ovary cells or renal cells.

The G1 and G2 phases as checkpoints in the cell cycle which play key roles in the regulation of cells proceeding to S and M phases (O'connor 1996). However, if the normal proceeding was affected by some factors, the balances of the cell cycle would be broken and the generation or expression of the cell cycle regulatory molecules would also be promoted or inhibited. The p53 protein is a transcription factor that enhances
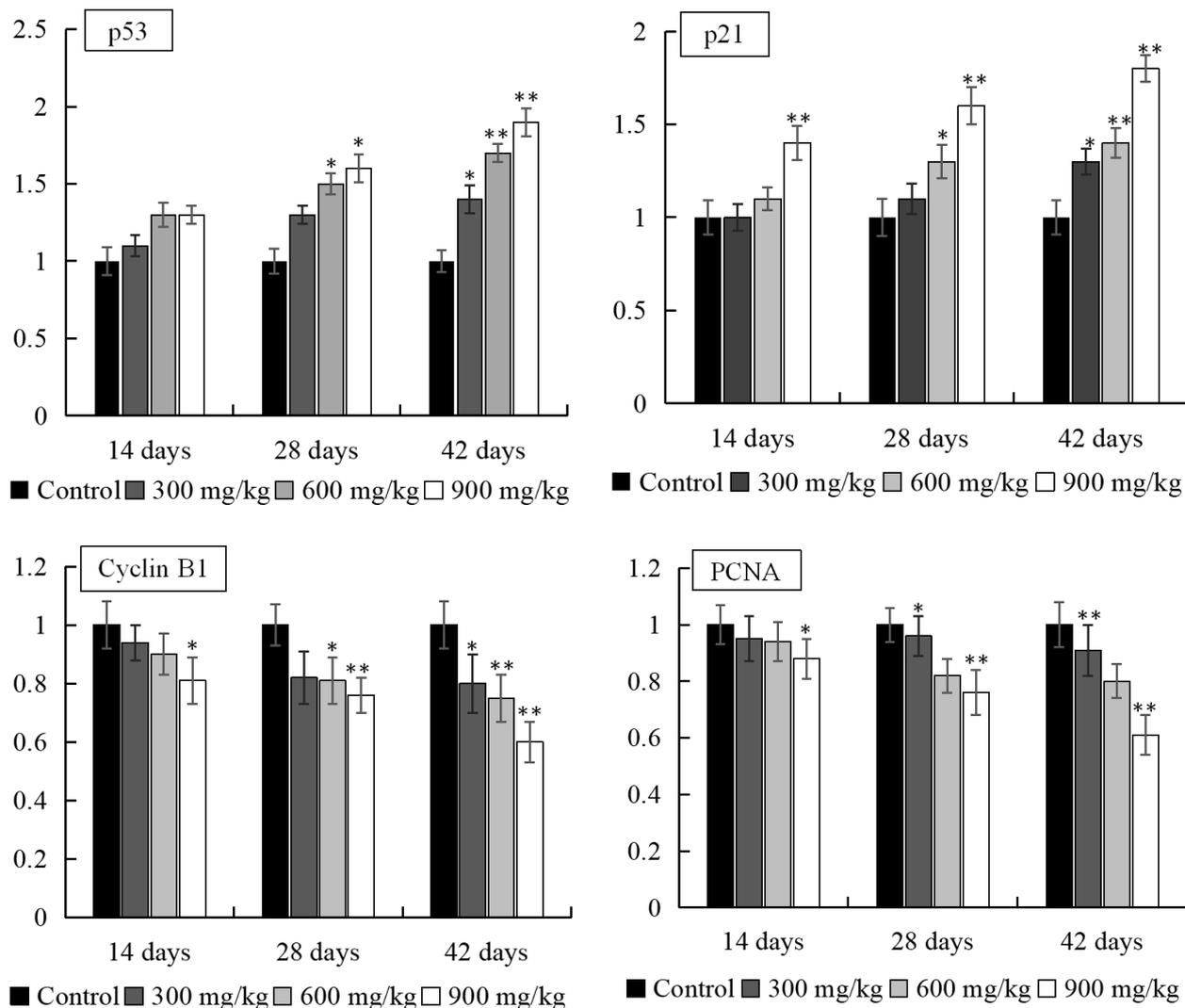
Figure 4. Changes of p53, p21, cyclin B and PCNA mRNA expression levels in the cecal tonsil. Data are presented with the mean ± standard deviation (n=6) *P<0.05, compared with the control group **P<0.01, compared with the control group.

the rate of transcription of six or seven known genes (p21 or WAF1 or Cip1, Cyclin G, Bax, MDM2, GADD45, IGF-BP3), at least in part, the p53-dependent functions in a cell (Levine 1997). P53 regulate the G1-S phase restriction point, which is related to a DNA damage checkpoint mediated by p53, and the choice by p53 whether to initiate a G1 arrest (via p21) or apoptosis (Levine 1997). P53-dependent G2/M cell cycle arrest is an important component of the cellular response to stress (Taylor & Stark 2001), and the transcription of the p21 gene can be activated by p53-dependent and -independent mechanisms, which cause cells to arrest in G2/M phase (Waga et al. 1994). In this study, we found that the protein and mRNA expression of p53 and p21 are increased, which showed that the G2/M cell cycle arrest is activated by NiCl$_2$. The results are consistent with a study that high-dose estrogen and clinical selective estrogen receptor modulators can induce cell cycle arrest through p53-dependent p21 activation in primate ovarian surface epithelial cells (Wright et al. 2005). Progression through the cell cycle is

also regulated by cyclins and cyclin-dependent kinases (Cdks). The cyclin kinase inhibitor p21 (also known as WAF1, CIP1, SDI1, and MDA-6) can induce G1 arrest and block entry into S phase by inactivating Cdks or by inhibiting activity of proliferating cell nuclear antigen (PCNA) (Gartel et al. 1996). So in the study, we also detect the PCNA and cyclin B1. PCNA is a key factor in DNA replication and cell cycle regulation (Strzalka & Ziemienowicz 2010, Bravo et al. 1987), cyclin-dependent protein kinases are key regulators of the eukaryotic cell cycle, and cyclin B1 plays a key role in regulating G2/M phase transition (Re et al. 1995). In the present study, the cyclinB1 and PCNA protein and mRNA expression are decreased induced by dietary high level of NiCl$_2$. Our results are consistent with the studies that performed on terminally differentiated cardiomyocytes showed that cell cycle arrest was dependent on maintaining a high concentration of p21, which in turn reduced the level of PCNA (Engel et al. 2003).

In conclusion, our study shows that dietary high level NiCl$_2$ increases p53 and p21 protein and mRNA expression, and decreases cyclin B, PCNA protein and mRNA expression. At the same time, p53 (via p21) inhibits DNA replication and maintains G2/M cell cycle arrest through the reduction of cyclin B1 and PCNA.

Acknowledgments

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