# Effect of Akt activation on apoptosis-related gene expression in the crop tissues of male and female pigeons (*Columba livia*)

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**ABSTRACT** The current study investigated whether the expression of apoptosis genes in the pigeon crops was affected by the Akt signaling pathway during crop milk formation. First, 78 pairs of adult White King pigeons were randomly assigned to 7 groups, and the expression of apoptosis-related genes and Akt signaling pathway-related proteins in the crop tissues during different breeding stages were examined. The results showed that the mRNA levels of Bak, caspase-3, caspase-6, and caspase-9 in female crops all increased and reached their highest levels at d 17 of incubation (**I17**). In male crops, the levels of caspase-3 and caspase-9 gene expression peaked at d 1 of chick rearing (**R1**). The lowest level of Bcl-2 gene expression in females was observed at I17. The expression ratios of p-Akt (Ser473)/Akt and p-Akt (Thr308)/Akt in male crops decreased to their minimum at R1, while it was observed at d 7 of chick rearing (**R7**) in females. Second, 36 pairs of adult pigeons were divided into 3 groups and were subjected to SC79 injections with dosages of 0, 0.02, or 0.04 mg/kg bodyweight. The SC79 injections resulted in a considerable decrease in growth performance of pigeon squabs. In male crops, the expression ratios of p-Akt (Ser473)/Akt and p-Akt (Thr308)/Akt were significantly elevated in the 0.02 mg/kg SC79 group, while in female crops, they were higher in the 0.04 mg/kg SC79 group ($P < 0.05$). The SC79 injection inhibited the gene expression of Bak in female crops, but enhanced the gene expression of Bcl-2 in both male and female crops. In the 0.04 mg/kg SC79 group, a 50.7 to 75.7% decrease was observed in the expression of caspase-3, caspase-6, and caspase-9 in male and female pigeon crops. Expression of the caspase-8 gene and total Akt protein in pigeon crops was not changed in different breeding stages or after SC79 injection. In conclusion, the expression of genes related to mitochondria-dependent apoptosis can be regulated by the Akt signaling pathway, which may play a potential role in pigeon milk formation.

**Key words:** pigeon, crop milk, apoptosis, Akt signaling pathway

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## INTRODUCTION

In addition to the functions of food storage, moistening, and acting as a physical barrier to pathogen, crop sac in pigeon is a highly specialized structure that produce 'milk', so-called 'crop milk' (Kierończyk et al., 2016), as it is in flamingos and male emperor penguins (Prevost and Vilter, 1962; Studer-Thiersch, 1967). The optimal marketing period of meat-type pigeons in China is 28 d. Nutritive cheese-like crop milk mainly consists of protein (60% of dry matter) and fat (30% of dry matter), and greatly lacks carbohydrates (Carr and James, 1931; Xie et al., 2017), which is thought to be an important reason for the much higher growth rate of pigeon squabs than that of other domestic fowls, such as chicks, ducks, and quails (Sales and Janssens, 2002). Until now, most studies have mainly focused on analyzing the nutritive composition (Shetty et al., 1990, 1992, 1994; Shetty and Hegde, 1991) or the physiological mechanism of lipid (Xie et al., 2017; 2020; Wan et al., 2019) and protein formation (Hu et al., 2016; Xie et al., 2019; Chen et al., 2020) in crop milk. The germinal cell layer of the crop is already known to be the cytological basis of milk formation, and these cells rapidly proliferate, which is accompanied by lipid accumulation, in response to prolactin (Wan et al., 2019) and possibly local cell factors (Xie et al., 2018). Therefore, a new question was raised: in what way do lipid-laden

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epithelial cells slough off from the tissue to produce crop milk?

The avian epidermis differs from that of mammals due to the absence of sebaceous glands and the presence of intracellular lipid droplets (Vanhoutteghem et al., 2004). Dramatic upregulation of both alpha and beta keratin genes was found in lactating crops by transcriptome analysis. Therefore, pigeon milk was supposed to be built upon the ability of bird keratinocytes to accumulate lipids at a very high turnover rate during the cornification process, followed by desquamation in the lactating tissue (Gillespie et al., 2011, 2013). Keratinocyte cornification is a mode of programmed cell death that is intimately linked to changes at the cell surface and in the milieu surrounding the cell under the conditions of inflammation, wound healing, or stress hormones (Galand and Vandenhende, 2010; Eckhart, 2018). In mammals, cornification upon the removal of cell organelles finally induces cell desquamation from the epidermis (Eckhart et al., 2013), and the whole process is accompanied with notable cell apoptosis that involves the activity of caspase family members (Demerjian et al., 2008; Erman et al., 2009). The particular hypothesis put forward here was that intense cell apoptosis associated with the changes in related gene expressions probably occurred during the desquamation of the lactating crop epidermis during pigeon milk formation.

Therefore, the present study was designed to first examine the expression profile of apoptosis-related genes and Akt signaling pathway-related proteins in the crops of parental pigeons during incubation and chick-rearing period. Then, whether apoptosis gene expression was affected by the Akt signaling pathway was examined based on SC79 injections. Our results are expected to provide important data for further investigations on pigeon crop milk formation.

## MATERIALS AND METHODS

All procedures used in this study were approved by the Animal Care Advisory Committee of Yangzhou University.

### Birds and Housing

A total of 78 pairs of adult White King pigeons (78 males and 78 females, 60 wk of age) with an average body weight of 580 ± 20 g were obtained from a commercial pigeon farm (Kunpeng Pigeon Co., Ltd., Xuzhou, China). All the pigeons were chosen from a large flock (approximately 2,000 pairs) and had the same oviposition interval. Each pair was housed in an artificial aviary equipped with a nest and perch, and subjected to a 50-d study, which included a 7-d acclimation period and a 43-d experimental period (18-d incubation and 25-d chick rearing). To maintain the broodiness of parental birds, plastic eggs were added to cages only after the second egg was laid, as described previously (Xie et al., 2018). Baby squabs hatched from the incubator were reared by parents after 18 d of incubation. The birds were fed a pellet compound diet based on corn, soybean meal, and wheat (16.67% crude protein, 12.00 MJ/kg of metabolizable energy, 1.13% calcium, 0.34% available P, 0.89% lysine, and 0.31% methionine). The nutrient data can be found in our previous study (Xie et al., 2016). The birds received feed, sand and water ad libitum. Light was provided for 16 h daily throughout the experiment.

Forty-two parent pigeons were randomly assigned to 7 groups based on different breeding stages, which included d 4 (**I4**), 10 (**I10**), and 17 (**I17**) of the incubation period and d 1 (**R1**), 7 (**R7**), 15 (**R15**), and 25 (**R25**) of the chick-rearing period. After 12-h fast, all the pigeons were euthanized by cervical dislocation. Crop tissues were quickly frozen in liquid nitrogen and stored at −80°C for subsequent examination. Eggs and baby squabs were transferred to the pigeon farm to be cared for by other pigeons.

### SC79 Injection

SC79 (Selleck, Shanghai, China) was freshly prepared in 2% dimethyl sulfoxide (**DMSO**) and corn oil, and administered by wing vein injection. Thirty-six pairs of parent pigeons were randomly assigned to the following 3 groups: 1) the control group (SC79 vehicle at an equivalent volume); 2) the low-dose SC79 (0.02 mg/kg bodyweight); 3) the high-dose SC79 (0.04 mg/kg bodyweight). Previous studies showed that pigeon crops had a rapid development in morphology, and lipid synthesis in epithelial cells increased significantly at 14 d of incubation (Hu et al., 2016; Xie et al., 2017). Therefore, the birds were injected every other day at 7:00 before feeding from 14 d of incubation to 7 d of chick rearing. Six pigeon squabs from each group were weighed at 1, 2, 3, 4, 5, 6, and 7 days of age. At 1 d of chick rearing, 6 pairs of randomly chosen adult pigeons were killed, and the crops were frozen in liquid nitrogen and stored at −80°C for future gene and protein expression determination. Pigeon milk cannot be stored by the crop organ, so it was often collected from squabs soon after being fed by parents (Bharathi et al., 1997). At 7 d of chick rearing, 6 pigeon squabs were used to sample crop milk according to the method described previously (Xie et al., 2017). Briefly, slits on the crop of squabs were made carefully by a surgical blade. Crop milk was collected and incision was closed by double sutures. Samples were also transferred into −80°C low temperature refrigerator for the future examination. After sampling, squabs were put into nests at the farm to be cared for by other pigeons.

### Western Blot

Tissue samples were thawed and homogenized in lysis buffer containing phenylmethanesulfonyl fluoride (Beyotime Institute of Biotechnology, China) and PhosSTOP phosphatase inhibitor (Roche, Germany) on ice. The protein concentration was determined using the Bradford assay. A total of 25 $\mu$g of proteins were separated

using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% fat-free milk or bovine serum albumin at room temperature for 1 h and incubated with primary antibodies, including antibodies against Akt (Cell Signaling, dilution 1:1000), phosphorylated (Ser473)-Akt (Cell Signaling, dilution 1:2000), phosphorylated (Thr308)-Akt (Cell Signaling, dilution 1:1000), and $\beta$-actin (Abcam, dilution 1:5000), overnight at 4°C. The membranes were then probed with horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies (Cell Signaling, dilution 1:2000) at room temperature for 1 h after 3 washes in Tris-buffered saline containing 0.2% Tween 20 (**TBST**). Finally, staining was visualized by enhanced chemiluminescence (**ECL**) detection reagents and imaged under a Bio-Rad scanning densitometer. The intensity of the individual bands was calculated using Quantity One software (Bio-Rad, Hercules, CA).

### Crop Milk Analysis

Dry matter (**DM**) of crop milk was determined according to the method of 925.09 of AOAC (1990). To determine crude protein (**CP**), a 0.3 g sample of crop milk on a DM basis was digested with sulfuric acid, and then analyzed by an automatic Kjeldahl nitrogen analyzer (UDK 159, VELP Scientifica, Italy). Crude fat of crop milk was determined according to a modified method as descibed before (Folch et al., 1957). Briefly, 1 g of sample on a DM basis was homogenized with a 20 mL of chloroform:methanol solution (2:1 vol/vol). The mixture was vortexed, and allowed to be agitated for 30 min. Four mL Distilled water was added. Following overnight incubation at 4°C, the organic layer was transferred to glass tube of known weight and oven-dried at 105°C to constant weight.

### RNA Isolation and Real-Time Quantitative PCR

Total RNA extraction from crop tissues for target gene expression analysis was performed using the TRIzol reagent method. Briefly, frozen samples were ground with liquid nitrogen, and tissue powder was immediately transferred into TRIzol reagent, deproteinized by chloroform, precipitated with isopropanol and washed in 75% ethanol. The RNA was resuspended in RNase-free water, and its quality was confirmed by the determination of the absorbance ratio 260 nm/280 nm. cDNA was synthesized by M-MLV reverse transcriptase at 42°C for 60 min with an oligo dT-adaptor primer.

The mRNA abundances of B-cell lymphoma antagonist/killer (**Bak**), B-cell lymphoma-2 (**Bcl-2**), caspase-3, caspase-6, caspase-8, caspase-9, and $\beta$-actin were detected by real-time quantitative PCR (**qRT-PCR**). The primers designed by Primer Premier 5.0 software are shown in Table 1. qRT-PCR was performed using SYBR Premix Ex Taq (TaKaRa, Dalian, China) in a C1000 Touch Thermal Cycler equipped with a CFX96 Real-Time PCR Detection System (Bio-Rad) and evaluated with CFX Manager 3.1 software (Bio-Rad). The reaction conditions were as follows: 95°C for 30 s followed by 39 cycles of 95°C for 5 s and 60°C for 30 s. Each sample was analyzed in triplicate. Melting curve analysis was used to verify amplification specificity. The relative expression of target genes was calculated based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with $\beta$-actin as the internal reference.

### Statistical Analysis

All the data were presented as the mean ± SE. The data were statistically evaluated using SPSS 17.0 (SPSS Inc., Chicago, IL). Differences among breeding stages were estimated by Duncan's post-hoc test. All statements of significance were based on $P < 0.05$.

## RESULTS

### mRNA Levels of Apoptosis-Related Genes During Breeding Stages

The mRNA expression of Bak in male and female crops increased to a peak level at I17 (Figure 1A).

**Table 1.** Primers used in the present study.

| Target gene | Nucleotide sequence (5'→3')[1] | Acession No. | Size (bp) |
|---|---|---|---|
| Bak | F: GCTGCACTCAATCTGGACAA<br>R: AGATTTGAGAGCGAGCTTGG | XM_005513713 | 154 |
| Bcl-2 | F: GATGACTGAGTACCTGAACCG<br>R: TGCCATACAACTCCACGAAG | XM_005509733 | 83 |
| caspase-3 | F: GCTTTGTTTGCGTGTTGCTGAG<br>R: TCTGCTTCTACGGGTATTTTTTGAC | XM_005500178 | 235 |
| caspase-6 | F: AGCCTCTAAGGACGACCACAGC<br>R: GGGCATCAAACGCATAAACG | XM_021283084 | 92 |
| caspase-8 | F: GAAGGAACACGGAAAGATGGAGA<br>R: ATGGGACAAAATGAAGCAAACG | XM_021299505 | 171 |
| caspase-9 | F: CCCATTGCCCGAGTTTGAGA<br>R: GGCTGGTCCACATTACCTGCT | XM_021295214 | 178 |
| $\beta$-actin | F: TCAGGGTGTGATGGTTGGTAT<br>R: TCATTGTAGAAAGTGTGGTGCC | XM_005504502 | 159 |

[1]Abbreviations: F, forward; R, reverse.

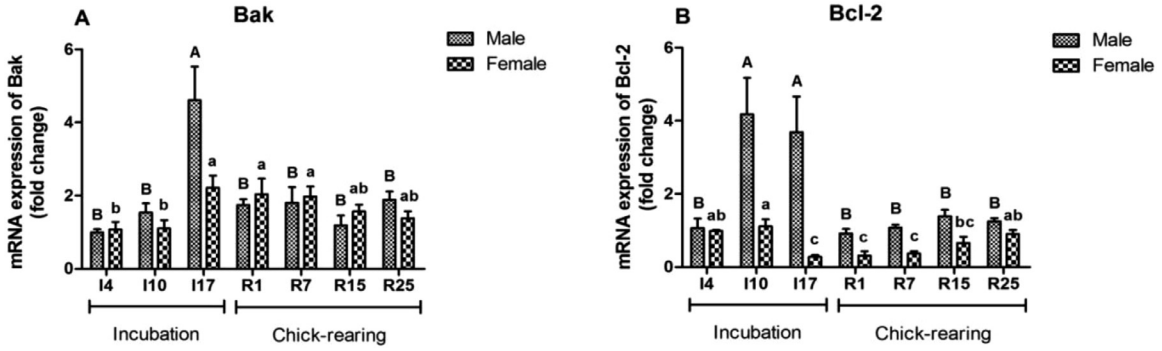


**Figure 1.** The mRNA expressions of Bak (A) and Bcl-2 (B) in crop tissues of male and female pigeons during incubation and chick-rearing periods. The stages included incubation period: I4, I10, and I17; chick-rearing period: R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A-B) or lowercase letters (a-c) are significantly different ($P < 0.05$).

Bcl-2 gene expression in males increased significantly at I10 and I17, but in females, lower levels were observed from I17 to R15 ($P < 0.05$; Figure 1B). The expression levels of caspase-3, caspase-6, and caspase-9 in female crops all reached to their maximum levels at I17 (Figure 2A−2C), whereas in male crops, the expression of caspase-3 and caspase-9 peaked at R1 (Figure 2A and 2C). These proteins all showed similar changing patterns during the whole breeding cycle. However, no significant changes were found in the gene expression of caspase-8 in either male or female pigeons from I4 to R25 ($P > 0.05$; Figure 2B).

## *Expression of Akt Signaling Pathway-Related Proteins During Breeding Stages*

To investigate the involvement of the Akt signaling pathway during crop milk formation, the protein expression levels of total Akt, phosphorylated (Ser473)-Akt, and phosphorylated (Thr308)-Akt were detected by western blot analysis. As shown in Figure 3, the expression of total Akt in both male and female crop tissues remained stable during the incubation and chick-rearing periods ($P < 0.05$). However, the expression ratios of p-Akt (Ser473)/Akt and p-Akt (Thr308)/Akt in male

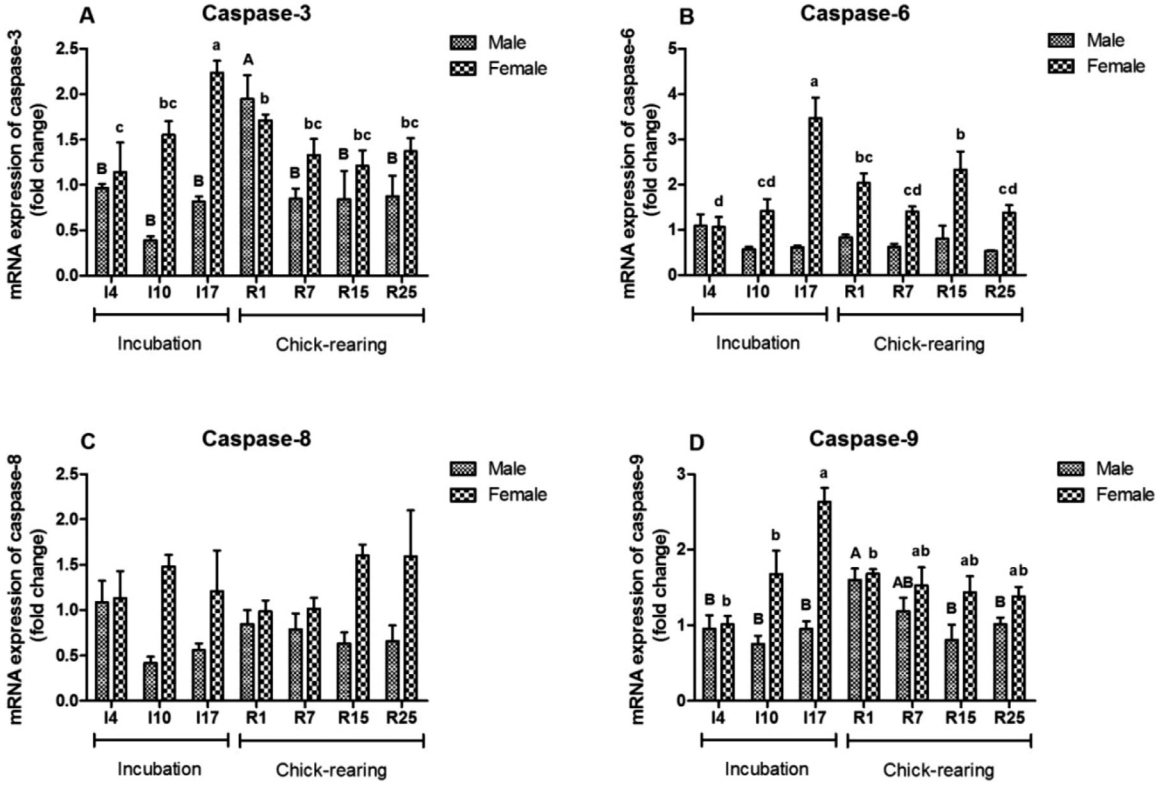


**Figure 2.** The mRNA expressions of caspase-3 (A), caspase-6 (B), caspase-8 (C), and caspase-9 (D) in crop tissues of male and female pigeons during incubation and chick-rearing periods. The stages included incubation period: I4, I10, and I17; chick-rearing period: R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A-B) or lowercase letters (a-d) are significantly different ($P < 0.05$).

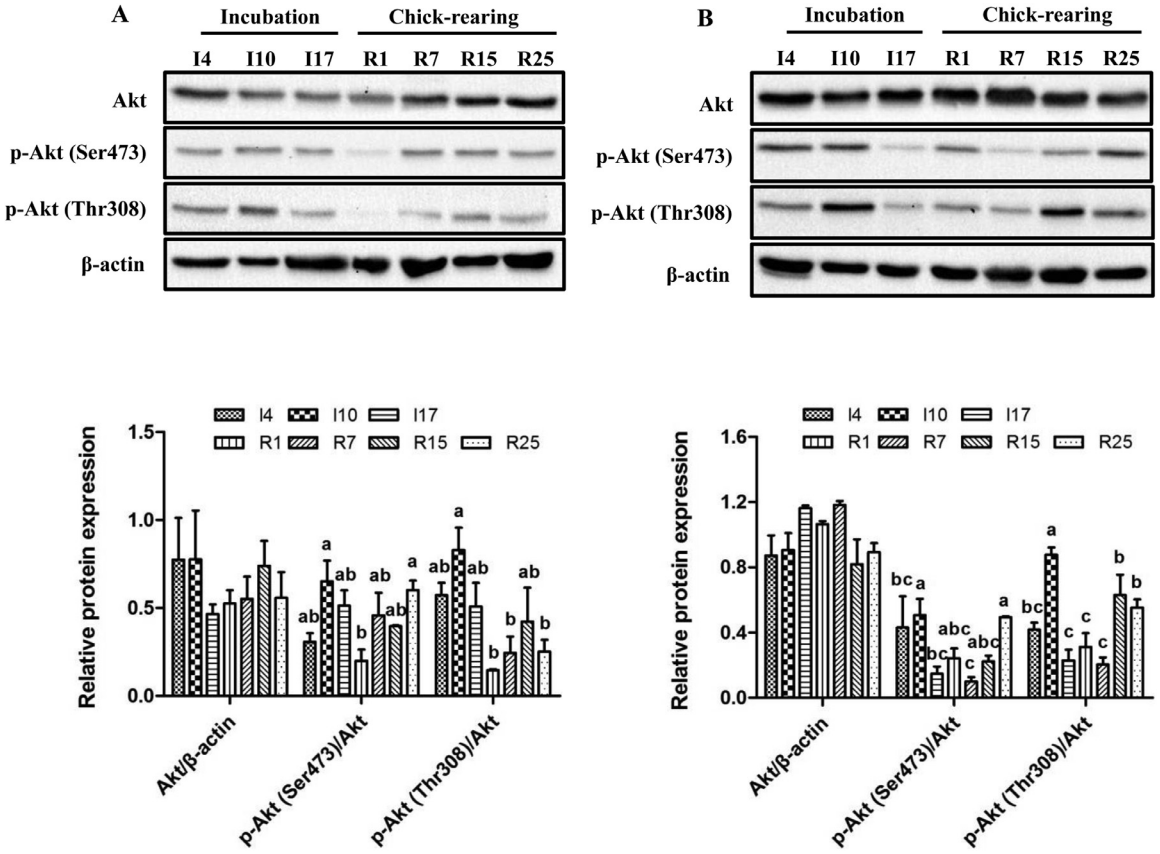


**Figure 3.** Representative western blot and densitometric analysis of Akt signaling pathway-related proteins in crop tissues of male (A) and female (B) pigeons during incubation and chick-rearing periods. Expression levels of Akt protein are normalized to $\beta$-actin levels. Expression levels of phosphorylated Akt (Ser473 and Thr308) are normalized to Akt levels. The stages included incubation period: I4, I10, and I17; chick-rearing period: R1, R7, R15, and R25. Values are means ± SEM (n = 6 males and females). Data points with the different lowercase letters (a-c) are significantly different ($P < 0.05$).

crops decreased to the lowest level at R1 ($P < 0.05$). Compared to the levels at the beginning of incubation and late chick-rearing, Akt phosphorylation at Ser473 and Thr308 in females was inhibited from I17 to R7, and decreased to the lowest value at R7 ($P < 0.05$).

## Growth Performance of Pigeon Squabs

Compared to the control vehicle group, SC79 injection resulted in considerably decreased growth performance of pigeon squabs (Figure 4). The body weight of the squabs in the 0.04 mg/kg SC79 group was lower than that of the squabs in the control group from 5-day-old to 7-day-old, while it was lower in the squabs in the 0.02 mg/kg SC79 group from 6-day-old to 7-day-old. A dose-dependent effect was not observed in the present experiment.

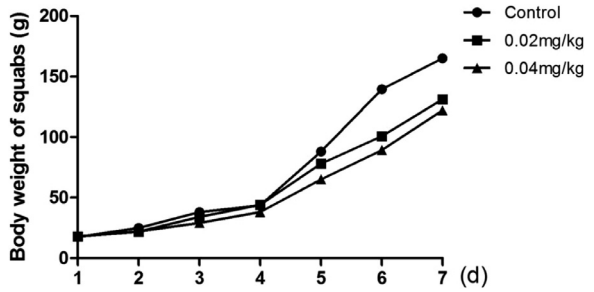


**Figure 4.** The effect of SC79 injection on the growth performance of pigeon squabs (n = 12). Body weight of squabs at 1, 2, 3, 4, 5, 6, and 7 d was recorded.

## Nutrient Composition of Crop Milk

As shown in Table 2, the content of crude protein in pigeon crop milk was more than double that of crude fat at 7 day of chick-rearing period. However, SC79 injection had no significant effect on the content of DM, CP, and CF in crop milk ($P < 0.05$).

**Table 2.** Effects of SC79 injection on the nutrient composition of crop milk at d 7 of incubation period[1].

| | SC79 injection (mg/kg) | | |
|---|---|---|---|
| Item[2] | 0 | 0.02 | 0.04 |
| DM (%) | 29.93 ± 0.46 | 28.38 ± 1.40 | 29.20 ± 0.81 |
| CP (%) | 25.56 ± 1.01 | 26.90 ± 1.01 | 25.91 ± 1.09 |
| CF (%) | 10.51 ± 0.84 | 9.12 ± 0.30 | 10.32 ± 0.92 |

[1]Data are shown as means ± SEM; n = 6.
[2]Abbreviations: CF, crude fat; CP, crude protein; DM, dry matter.

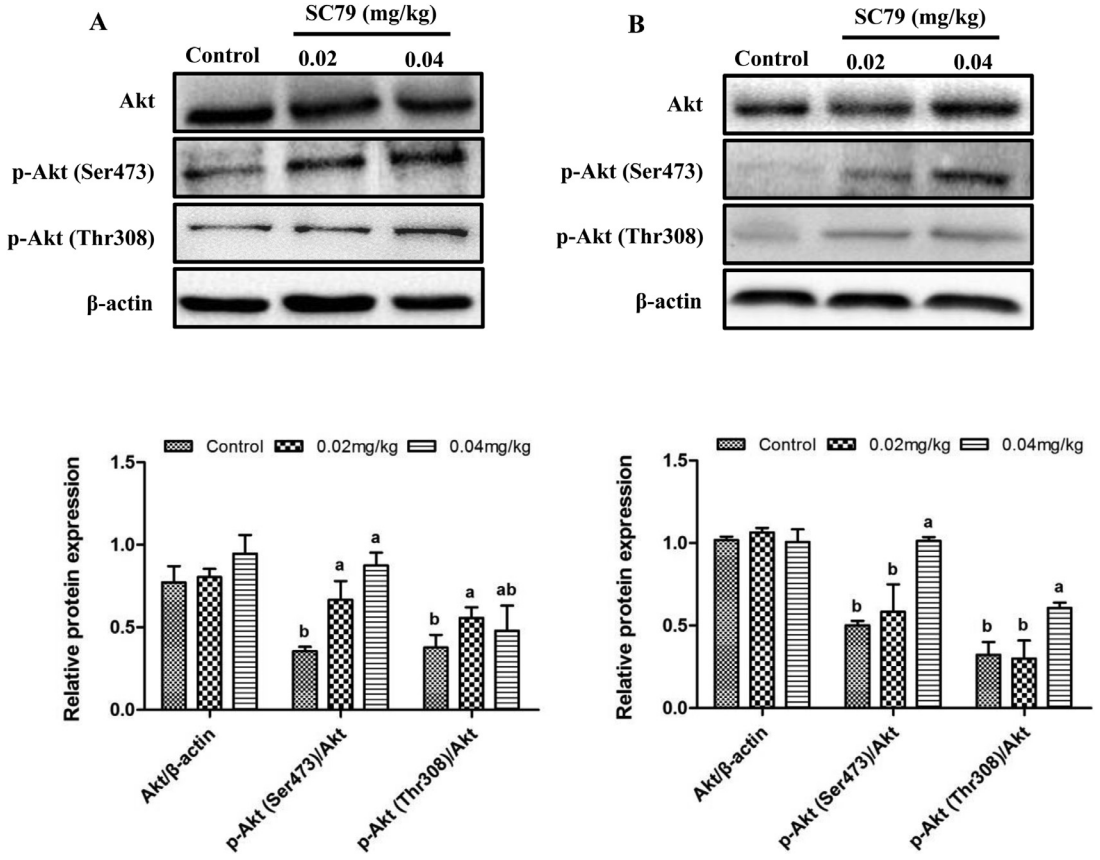


**Figure 5.** Representative western blot and densitometric analysis of Akt signaling pathway-related proteins in crop tissues of male (A) and female (B) pigeons injected with SC79. Expression levels of Akt protein are normalized to $\beta$-actin levels. Expression levels of phosphorylated Akt (Ser473 and Thr308) are normalized to Akt levels. Values are means ± SEM (n = 6 males and females). Bars with the different lowercase letters (a-b) are significantly different ($P < 0.05$).

## *Expression of Akt Signaling Pathway-Related Proteins Under SC79 Treatment*

Total Akt protein expression in both male and female pigeon crops was not affected by SC79 treatment ($P > 0.05$; Figure 5A and 5B). However, Akt phosphorylation at Ser473 and Thr308 was enhanced when subjected to SC79 injection. In male crops, the expression ratios of p-Akt (Ser473)/Akt and p-Akt (Thr308)/Akt were significantly elevated in the 0.02 mg/kg SC79 group ($P < 0.05$; Figure 5A), while in female crops, they were higher in the 0.04 mg/kg SC79 group ($P < 0.05$; Figure 5B).

## *mRNA Levels of Apoptosis-Related Genes Under SC79 Treatment*

As shown in Figure 6, the gene expression of Bak in female crops was remarkably inhibited in response to SC79 treatment ($P < 0.05$), but it was not affected in male crops ($P > 0.05$). SC79 injection of 0.04 mg/kg significantly increased the mRNA level of Bcl-2 in male crops, and a 3.60- to 5.59-fold increase was detected in female crops in the 0.02 and 0.04 mg/kg SC79 groups ($P < 0.05$). SC79 injection induced a significant decrease in the gene expression of caspase-3, caspase-6, and caspase-

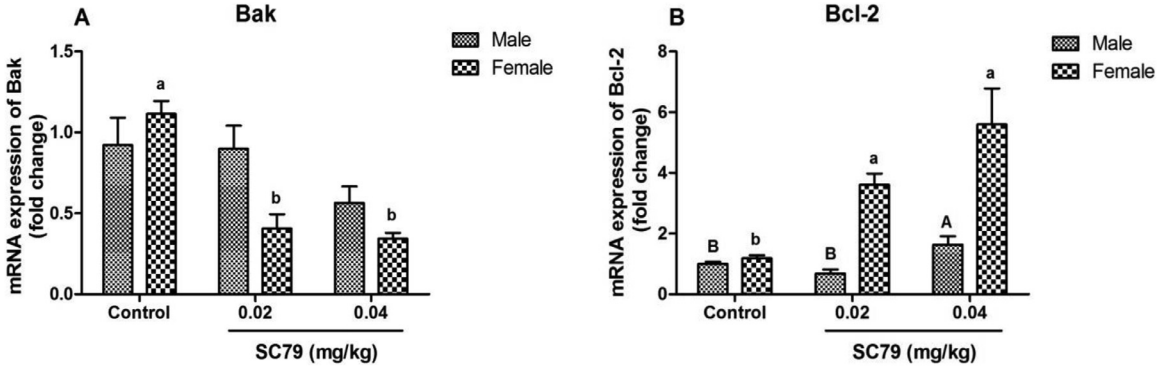


**Figure 6.** Effects of SC79 injection on the mRNA expressions of Bak (A) and Bcl-2 (B) in crop tissues of male and female pigeons during incubation and chick-rearing periods. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A-B) or lowercase letters (a-c) are significantly different ($P < 0.05$).

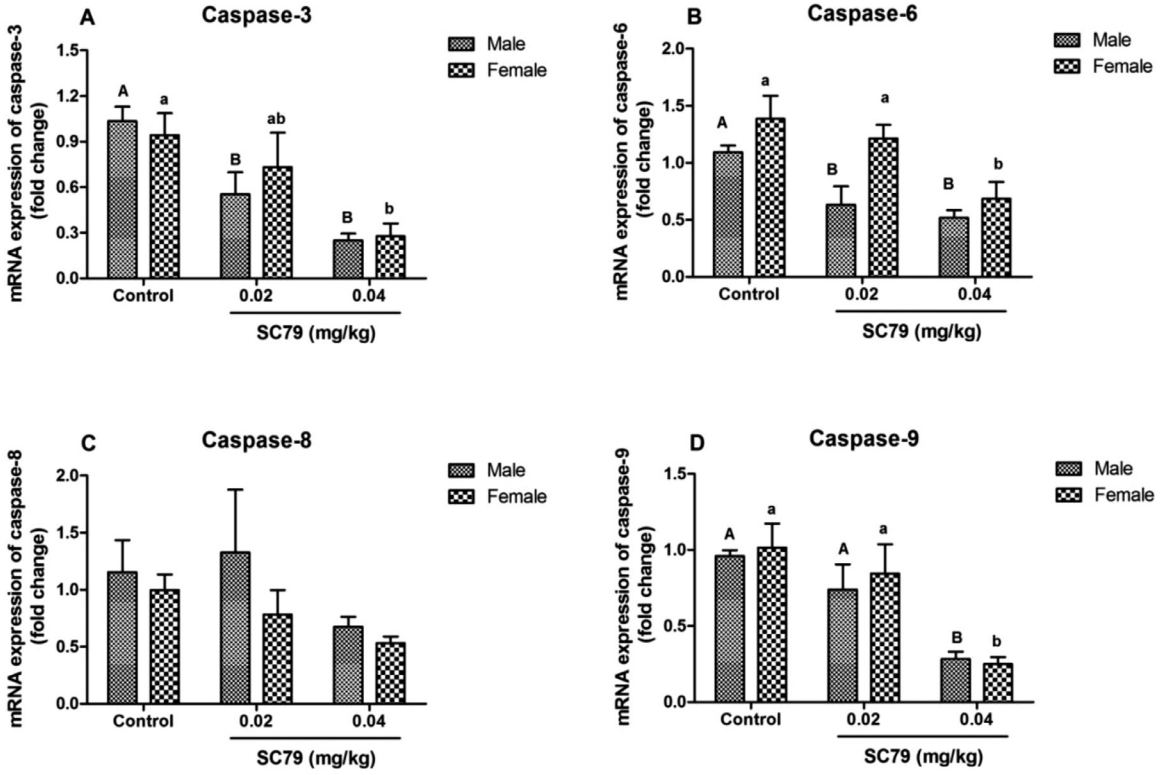


**Figure 7.** Effects of SC79 injection on the mRNA expressions of caspase-3 (A), caspase-6 (B), caspase-8 (C) and caspase-9 (D) in crop tissues of male and female pigeons during incubation and chick-rearing periods. Values are means ± SEM (n = 6 males and females). Bars with the different capital letters (A-B) or lowercase letters (a-c) are significantly different ($P < 0.05$).

9 in male and female pigeon crops (Figure 7; $P < 0.05$). Especially in the 0.04 mg/kg SC79 group, a 50.7 to 75.7% decrease was observed in the expression of the three genes, but caspase-8 gene expression showed no changes under SC79 treatment (Figure 7C; $P > 0.05$).

## DISCUSSION

Cell desquamation and apoptosis are 2 major ways of cell loss for maintaining homeostasis. Both in a model of ischemia reperfusion injury or the development of stratified epithelia, cell overproduction was observed to be followed by intensive apoptosis and desquamation (Shimizu and Yamanaka, 1993; Saathof et al., 2004). The strong proapoptotic activity that was observed during the regression of tissue hyperplasia was thought to be due to programmed removal of excess cells (Columbano et al., 1985; Bhathal and Gall, 1985). Epithelial desquamation from crop sacs has already been observed in pigeons when they regurgitate to squabs (Mirarchi et al., 1986). Therefore, confirming whether cell apoptosis occurs in the tissue would provide a better understanding for crop milk formation in pigeons.

Cell apoptosis has been extensively reported in embryogenesis, metamorphosis, the control of normal tissue turnover (Ishizuya-Oka and Ueda, 1996; Eijnde et al., 2015; Lynch et al., 2015), and pathological tissue (Guevara et al., 2001). Biological events can be initiated by the intrinsic pathway or the extrinsic pathway (Budihardjo et al., 1999). The intrinsic pathway of mitochondria-dependent apoptosis hinges on the balance of activity between pro- and antiapoptotic signals of the Bcl-2 family. The release of cytochrome c was regulated by Bcl-2 family members by changing the membrane permeability of mitochondria, and the activation of initiator caspases (caspase-2, -8, -9, and -10) and effector caspases (caspase-3, -6, and -7) were triggered sequentially. In the extrinsic pathway of death receptor-dependent apoptosis, the expression of caspase-8 gene was enhanced by Fas, or TNF-receptor, which directly activate caspase-3 followed by apoptosis.

From transmission electron microscopy (**TEM**) analysis (Dumont, 1965), mitochondira with typical cristae were relatively small but numerous in cells of the proliferative stratum in non-brooding pigeons. However, mitochondrial swelling and cristae reduction were observed in the epithelial cells of lactating crops. It was suggested that this organelle experienced some stresses during crop milk formation. In the present study, the proapoptotic gene Bak was upregulated in pigeon crops at the end of incubation and at the beginning of chick rearing. However, Bcl-2 gene expression showed different changing patterns in males and females from I10 to R7. Bcl-2 inhibits apoptosis by interacting and forming inactivating heterodimers with Bax/Bak (Diaz et al., 1997). Much lower Bcl-2 gene expression together with higher expression of Bak in females indicated that intense mitochondria-dependent cell apoptosis possibly contributed to the desquamation to form pigeon milk, but its

potential role in crop epithelial cell apoptosis may also be different in the 2 sexes.

Overall, the gene expression levels of caspase-3 and caspase-9 showed similar changing tendencies, although they peaked at different time points in males and females. The cytochrome-c-independent activation of caspase-9 induces the activation of downstream caspase-3. It results in the induction of caspase-activated DNAse (**CAD**), cleavage of poly-ADP-ribose polymerase (**PARP**) and degradation of fordin, which ultimately leads to the demise of the cell (Wang et al., 2001; Yeh et al., 2007). Caspase-6 can directly cleave several cytoskeleton-associated proteins (Jin et al., 2008). In our study, female caspase-6 expression increased to a peak level at I17, while it remained stable during the whole breeding cycle, and a sex effect once again possibly existed in mitochondria-dependent cell apoptosis in pigeon crops. Furthermore, there were no changes in caspase-8 gene expression in males or females during the incubation and chick-rearing periods, which showed that the extrinsic pathway of death receptor-dependent apoptosis was probably not involved in cell apoptosis during crop milk formation.

Although both parent pigeons can produce milk, our previous data comparing pigeon milk between males and females, such as its lipid, amino acid, and growth factor contents (Xie et al.,2013, 2016, 2018, 2020), showed that male pigeon milk seemed to have higher nutritive value than that of female in the first 10 days of milk regurgitation. Despite the much greater time allocation of female birds devoted to egg hatching (Lehrman, 1964), male pigeons were thought to be more important for promoting squab growth and development through their higher nutritive milk by a different physiological mechanism of milk formation.

The Akt signaling pathway is a crucial network of proteins, that play a role in a variety of cellular physiologies, such as survival, growth, proliferation, and differentiation (Bhaskar and Hay, 2007). Although total Akt protein expression was unchanged in the present study, its phosphorylation at both Ser473 and Thr308 was significantly inhibited at the beginning of chick rearing. Due to the obvious lipid accumulation in crop epithelial cells (Gillespie et al., 2013), oxidative stress is inevitable, and the activation of Akt has been shown to be regulated by reactive oxygen species (**ROS**) (Cao et al., 2009). Therefore, it is reasonable that Akt activity in crop cells was attenuated by a dramatic increase in oxidative stress resulting from lipid burden during the peak of crop milk formation.

SC79 is a highly efficient and selective Akt activator that is cell and blood-brain barrier permeable (Jo et al., 2012). SC79 physically binds to the Akt PH domain, and promotes its activation by phosphorylating Akt at both Ser473 and Thr308 in the cytosol (Jo et al., 2012; Zhu et al., 2019). Our results showed that SC79 injections induced relatively higher expression ratios of p-Akt (Ser473)/Akt and p-Akt (Thr308)/Akt in both male and female pigeon crops. This finding proved that SC79 also acts on birds. Interestingly, parent pigeons were subjected to SC79 injection, and the growth performance of their baby squabs was depressed. The body weight of squabs is known to be closely related to the quality and quantity of crop milk produced by their parents (Sales and Jassens, 2002). Previous studies reported that crop organ cannot store pigeon milk, and a turnover of cornified epithelium was just over a 4-h period (Bharathi et al., 1997; Gillespie et al., 2013), so it was hardly to quantify the milk production. In the present study, SC79 showed no effects on DM, CP, and CF in pigeon crop milk at 7 day of chick-rearing period, and the nutrient composition seemed to be unchanged. It was suggested that Akt signaling pathway may be involved in crop milk production quantity.

Downregulation of Akt signaling is an important event in drug-induced cell apoptosis (Knobloch et al., 2008; Kang et al., 2018), while activation of Akt protects cells in tissue injury by inhibiting apoptosis (Takatani et al., 2004; Li et al., 2017). In addition to the changes in Akt phosphorylation, SC79 injection in the present study also induced a significant fluctuation in apoptotic gene expression in male and female pigeon crops. SC79 has been demonstrated to prevent cell death due to nutrition deprivation, drug toxicity, or physical damage in mammals (Gong et al., 2016; Li et al., 2016; Zheng et al., 2017). Our data provide the first evidence that apoptotic gene expression in pigeon crop cells is regulated by Akt phosphorylation.

Akt signaling has been demonstrated to counteract both the intrinsic pathway and the extrinsic pathway of apoptosis. In mammals, Akt inhibits mitochondria-mediated cell death through phosphorylation of BAD (a member of proapoptotic Bcl-2 family) and caspase-9 (Datta et al., 1997; Cardone et al., 1998), and induces the activation of the NF-$\kappa$B transcription factor, which promotes the transcription of Bcl-2 and Bcl-x-L (Seo et al., 2012). This could be the explanation for the changes in Bax, Bcl-2, caspase-3, caspase-6, and caspase-9 in the crop tissue subjected to SC79 injection in the current study. On the other hand, decreased Akt phosphorylation leads to the translocation of Forkhead transcription factors, which increase FasL expression and caspase-8 (Li et al., 2006). In our study, caspase-8 expression in crops was not changed under the treatment of SC79, which further indicated that extrinsic apoptosis signaling pathways may not be involved in crop milk formation.

## CONCLUSIONS

The expression of apoptosis-related genes and Akt signaling pathway-related proteins in male and female pigeon crops varied significantly in a sex-dependent manner during the incubation and chick-rearing periods. In general, proapoptotic gene expression was enhanced at the end of incubation or at the beginning of chick rearing, whereas Akt phosphorylation was significantly inhibited. SC79 injections increased Akt phosphorylation and inhibited apoptotic gene expression. Together

with the depressed growth performance of squabs under SC79 treatments, our results indicated that the expression of genes related to mitochondria-dependent cell apoptosis can be regulated by the Akt signaling pathway, and the biological event probably contributed to the desquamation to form pigeon milk.

## DISCLOSURES

No conflict of interest exits in the submission of this manuscript (Effect of Akt activation on apoptosis-related gene expression in the crop tissues of male and female pigeons (*Columba livia*)). We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the present work submitted and manuscript is approved by all authors for publication.

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