Targeted disruption of TORC1 retards young squab growth by inhibiting the synthesis of crop milk protein in breeding pigeon (*Columba livia*)

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ABSTRACT This study was conducted to explore the regulatory role of the target of rapamycin complex 1 (TORC1) signaling pathway in crop milk synthesis in breeding pigeons (*Columba livia*). Three groups of breeding pigeons in the lactation period (n = 30 pairs/group) were respectively injected with rapamycin (RAPA, a specific inhibitor of the target of rapamycin complex) at doses of 0 (vehicle, control), 0.6, or 1.2 mg/kg body weight (BW)/day via the wing vein for 7 days. The average daily feed intake (ADFI) and BW of the breeding pigeons and the BW of young squabs were respectively recorded throughout the experimental period. The breeding pigeons were sacrificed to collect their crop tissues, crop milk, and serum on the eighth day of the experiment. The results showed that neither 0.6 nor 1.2 mg/kg BW RAPA injection affected BW loss or ADFI in breeding pigeons ($P > 0.05$), while crop thickness and crop relative weight were significantly decreased ($P < 0.05$) in the 1.2 mg/kg BW rapamycin-injected group. Simultaneously, RAPA (especially at 1.2 mg/kg BW) decreased the crude protein, α_{s1}-casein, α_{s2}-casein, β-casein, and amino acid contents (Asp, Thr, Ser, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Lys, His, Arg, and Pro) of crop milk ($P < 0.05$) and the concentrations of albumin, total protein, and uric acid in the serum of breeding pigeons ($P < 0.05$). Additionally, the expression of TORC1 pathway-related proteins (TORC1, S6K1, S6, 4EBP1, and eIF4E) was downregulated in the crop tissues of breeding pigeons by 0.6 or 1.2 mg/kg BW/day RAPA injection ($P < 0.05$). Accordingly, the average daily gain (ADG) of young squabs declined, and the mortality rate increased significantly ($P < 0.05$). Together, the results showed that RAPA reduced protein and amino acid levels in the crop milk of breeding pigeons and retarded young squab growth, suggesting a crucial role of TORC1 in crop milk synthesis in breeding pigeons.

Key words: rapamycin, target of rapamycin, breeding pigeon, young squab, crop milk

INTRODUCTION

Unlike other poultry, young squabs cannot eat independently after birth due to their late maturity; specifically, they rely mainly on crop milk (pigeon milk) secreted by the crop of breeding pigeons to obtain nutrients up to 7 days of age (Sales and Janssens, 2003; Pomianowski et al., 2009; Gillespie et al., 2013). Interestingly, several studies have indicated that the pigeon crop undergoes significant changes in morphogenesis during lactation compared with non-lactation conditions and presents a convoluted, highly folded epithelial structure that coalesces as it outgrows the vasculature to form a nutritive cell layer containing lipid-filled vacuoles that are sloughed off to produce the "cheese-like" crop milk (Gillespie et al., 2011, 2013).

Crop milk consists mainly of protein, lipids, and physiologically active substances such as immunoglobulin (Shetty et al., 1992; Shetty and Hegde, 1993). Studies have shown that crop milk contains up to 64.1% crude protein and 29.7% ether extract based on dry weight, and 90% of the protein consists of casein (Hu et al., 2016). Although crop milk is similar to mammalian milk in its function, the synthesis and composition of these products are obviously different. Notably, 13 essential amino acids (**EAA**) that are necessary for poultry are contained in crop milk and account for nearly 50% of the total amino acids present, which suggests that crop milk is beneficial for improving the nitrogen metabolism and growth of young squabs (Vandeputte-Poma, 1980; Bharathi et al., 1997).

Although the synthesis mechanism of crop milk protein is not well characterized, a considerable number of studies have shown a link between pigeon milk and the highly conserved eukaryotic serine-threonine kinase target of rapamycin complex 1 (**TORC1**) signaling pathway, which is a central cell growth regulator connecting

cellular metabolism and growth (Apelo et al., 2014a). In particular, TORC1 phosphorylates the translational regulator eukaryotic translation initiation factor 4E (**eIF4E**) binding protein 1 (**4EBP1**) and is responsible for the S6 kinase (**S6K**)-dependent phosphorylation of the ribosomal protein S6 (Dunlop and Tee, 2009; Suryawan and Davis, 2011; Cargnello et al., 2015). Our previous study showed that the activity of TORC1 in the crop significantly increased during lactation compared with non-breeding conditions (Hu et al., 2016). However, the effect of TORC1 inhibition on crop milk protein synthesis as well as the amino acid composition is uncertain.

Therefore, we used rapamycin (**RAPA**), which physically interacts with and suppresses TORC1 activity, to investigate the regulatory role of TORC1 in relation to the crop milk protein composition and the production of breeding pigeons and the growth of young squabs.

MATERIALS AND METHODS

Ethical Statement

All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of South China Agricultural University (Guangzhou, China), and experiments were approved by the Animal Ethics Committee of South China Agricultural University (Guangzhou, China).

Animals

A total of 90 pairs of 3-yr-old breeding pigeons (90 males and 90 females) and 360 1-day-old young squabs were obtained from a commercial pigeon farm (Beilaide Meat Pigeon Industrial Development Co., Ltd., Zhaoqing, China). Each pair of breeding pigeons (1 male and 1 female) was housed in a man-made aviary equipped with 1 nest and 1 perch. Breeding pigeons were fed a mixed-grain diet. The ingredient composition and nutrient levels of the diets are described in Table 1. Grains and water were provided to the pigeons *ad libitum* throughout the experiment.

Experimental Design

A total of 90 pairs of breeding pigeons were randomly assigned to 3 groups. Each pair of breeders fed 4 young squabs ("2+4" feeing pattern). On the first day of the lactation period, the breeding pigeons were randomly subjected to one of the following treatments for 7 days: sham treatment (RAPA vehicle at an equivalent volume, control), low-dose RAPA (Solarbio, Beijing, China; 0.6 mg/kg body weight (BW), dissolved in absolute ethanol and diluted with 0.85% physiological saline, once daily at 09:00 hours, wing vein injection) and high-dose RAPA (1.2 mg/kg BW).

Table 1. Composition and nutrient levels of the basal diets (air-dried basis, %).

Ingredient	Content	Calculated analysis	Content
Corn	42.00	Metabolizable energy (MJ/kg)	12.07
Soybean meal (45% CP)	24.50	Crude protein	18.00
Wheat	24.00	Calcium	0.76
Sorghum	3.00	Total phosphorus	0.56
Soybean oil	1.40	Non-phytate phosphorus	0.32
Dicalcium phosphate	1.10	Methionine	0.48
Shell powder	1.00	Methionine + Cystine	0.75
Salt	0.30	Lysine	1.03
Vitamin and mineral premixb[1]	1.00		
DL-Methionine	0.23		
Lysine-HCl	0.27		
Zeolite powder	1.20		
Total	100.00		

[1]Provided per kilogram of diet: copper, 10 mg; iodine, 0.2 mg; pantothenic acid, 7.5 mg; iron, 35 mg; choline chloride, 200 mg; manganese, 55 mg; biotin, 0.12 mg; vitamin A, 4,000 IU; B$_1$, 3 mg; vitamin B$_2$, 13 mg; vitamin B$_6$, 2 mg; vitamin B$_{12}$, 25 μg; vitamin D$_3$ 1,725 IU; vitamin E, 24 mg; vitamin K$_3$, 1 mg; selenium, 0.2 mg; niacin, 15 mg; folic acid, 0.55 mg; zinc, 35 mg.

The BW and feed intake of the breeding pigeons and the number of live young squabs were recorded throughout the experimental period. The weight loss and average daily feed intake (**ADFI**) of the breeding pigeons and the average daily gain (**ADG**) of young squabs were calculated. All of the birds were euthanized to collect samples by the end of the experiments.

Crop Milk and Crop Tissues

The crops of breeding pigeons were carefully dissected to collect the crop milk and then snap frozen in liquid nitrogen for crude protein and amino acid analysis. After removing the crop milk from the surface of the crop, the thickness and weight of the crop were measured. Samples were collected after phosphate-buffered saline (**PBS**) washing, and one part of the sample was fixed in fresh 4% paraformaldehyde for immunofluorescence analysis, while another was snap frozen in liquid nitrogen for protein analysis.

Chemical Analysis

The crude protein content of the crop milk was determined according to Kjeldahl method (Horwitz, 1964; Amamcharla and Metzger, 2010). Briefly, crop milk was freeze-dried for approximately 120 h until all moisture was removed. Then, the crude protein content was determined via the Kjeldahl method using an automatic instrument (Kjeltec-TM 2300, FOSS, Hillerød, Sweden). The content of α_{s1}-casein, α_{s2}-casein, and β-casein were tested by using ELISA kits (Jiangsu Meimian industrial Co. Ltd., Jiangsu, China), and the amino acid content was measured using a Hitachi L-8900 amino acid analyzer (Hitachi, Tokyo, Japan).

Collection of Blood Samples and Measurement of Serum Biochemical Parameters

Blood samples from each breeding pigeon were collected from the jugular vein. After being separated naturally, the serum was centrifuged for 10 min at 4°C. Pure serum samples were aspirated with a pipette and stored in 1.5 mL Eppendorf tubes at –20°C until analyses. The concentrations of albumin, globulin, total protein, and uric acid were measured by using commercial assay kits (Nanjing Jiancheng Biological Product Co. Ltd., Nanjing, China) according to the manufacturers' introductions.

Western Blotting Analysis

Analyses of the TORC1, S6K1, S6, 4EBP1, and eIF4E proteins were performed by western blotting. Total protein was extracted from the crop using radio immunoprecipitation assay (**RIPA**) lysis buffer containing 0.1% phenylmethylsulfonyl fluoride, and the protein concentrations in the homogenates were determined using the BCA protein assay kit (Thermo Fisher Scientific, Rockford, USA). We performed western blotting as previously described (Zhou et al., 2019). Anti-TORC1 (#2972), anti-S6K1 (#9202), anti-S6 (#2708), and anti-4EBP1 (#9644) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA); an anti-eIF4E primary antibody was purchased from Abcam (Cambridge, MA, USA); β-actin (#600,149) antibodies were obtained from Zen BioScience (Chengdu, Sichuan, China). The proteins were visualized using the Beyo ECL Plus chemiluminescence detection kit (Beyotime Institute of Biotechnology, Beyotime, Shanghai, China). Enhanced chemiluminescence (**ECL**) signals were scanned using a FluorChem M apparatus (Protein Simple, Inc., Santa Clara, CA, USA), and band densities were analyzed using image analysis software (Tanon, Shanghai, China).

Immunofluorescence Analysis

Crop tissue samples were fixed with 4% paraformaldehyde overnight and then washed with PBS, dehydrated with alcohol, and embedded in paraffin blocks. Sections of 5 μm were cut for histological analysis. The samples were incubated with the TORC1 antibody overnight at 4°C. Secondary staining was performed at room temperature for 2 h. The nuclei were stained with DAPI for 5 min. Images of tissue sections were obtained using a fluorescence microscope (ECLIPSE-Ti, Nikon, Tokyo, Japan).

Statistical Analysis

The results were analyzed with SAS (Version 9.2; SAS Inst. Inc., Cary, NC) software. All data are represented as the mean ± SEM. Duncan's multiple-range test was used to evaluate the differences in the 3 groups following standard 1-way ANOVA. $P < 0.05$ were considered statistically significant.

RESULTS

Growth Performance of Breeding Pigeons

Overall, the body weight loss and ADFI of the breeding pigeons appeared to be dose dependent, but there was no significant difference between the 3 groups (Figure 1A and B). However, compared with the control group, breeding pigeons subjected to low-dose or high-dose RAPA treatment exhibited a significantly narrower crop thickness ($P < 0.05$; Figure 1C), and the thickness of crop decreased gradually with an increase in RAPA. Moreover, high-dose RAPA treatment significantly reduced the relative crop weight of the breeding pigeons ($P < 0.05$; Figure 1D).

Growth Performance of Young Squabs

As shown in Figure 2A, compared with the control and low-dose RAPA groups, the ADG of the young squabs significantly decreased in the high-dose RAPA group beginning on the third day after RAPA injection ($P < 0.05$), while the ADG was significantly decreased on the fifth day after the injection of low-dose RAPA ($P < 0.05$). The mortality of the young squabs in the high-dose RAPA injection group was also significantly higher than that in the control group ($P < 0.05$; Figure 2B).

Crop Milk Component Analysis

The results showed that the crude protein contents of the crop milk were 43.0, 38.6, and 31.6% in the control, low-dose RAPA, and high-dose RAPA groups, respectively, suggesting that RAPA reduces the crude protein level, especially high doses of RAPA injection ($P < 0.05$; Figure 3A). The tendency of α_{s1}-casein, α_{s2}-casein, and β-casein level was consistent with the crude protein after RAPA treatment ($P < 0.05$; Figure 3B–D). Moreover, the analysis of amino acids in crop milk showed that the levels of 16 amino acids (Asp, Thr, Ser, Glu, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Lys, His, Arg and Pro) were also significantly reduced in a RAPA dose-dependent manner ($P < 0.05$; Table 2).

Serum Biochemical Parameters of Breeding Pigeons

Assessment of the serum biochemical parameters of the breeding pigeons revealed significant reduction in albumin, total protein, and uric acid levels in the high-dose RAPA group compared with the control group ($P < 0.05$, Table 3). There was no significant change in serum globulins among the groups ($P > 0.05$, Table 3).
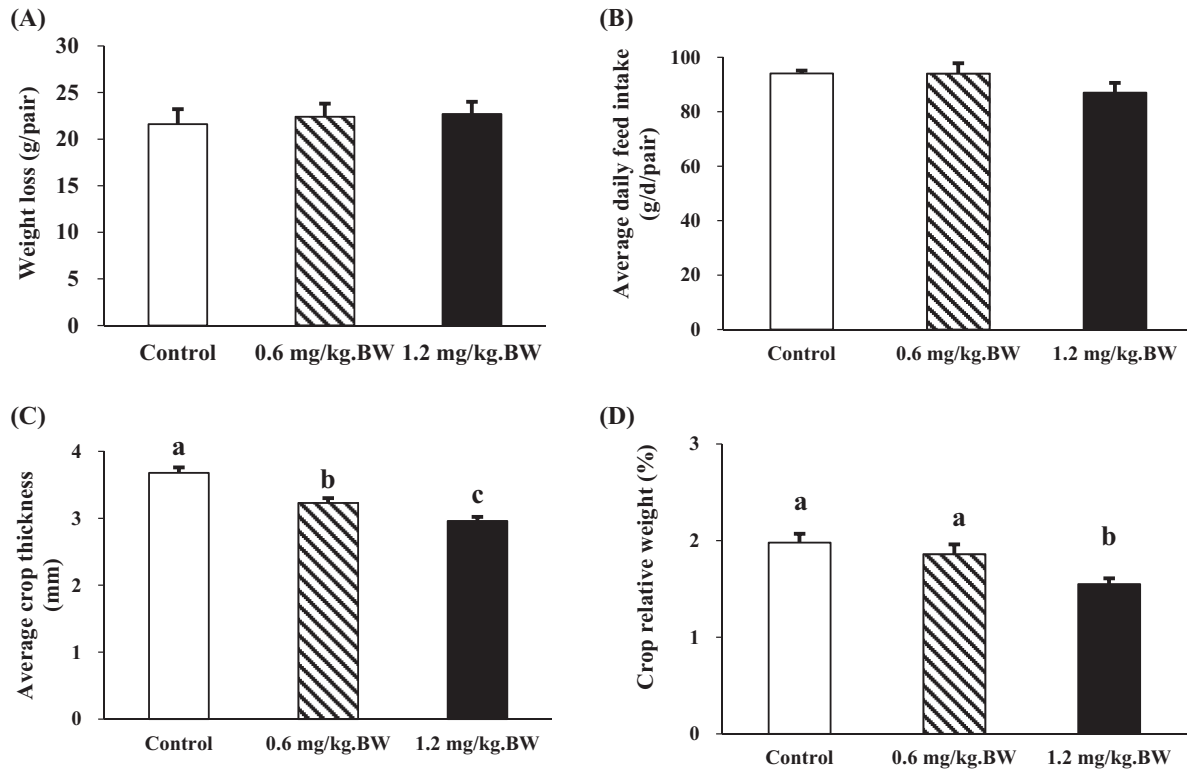
Figure 1. Inhibition of the production performance of breeding pigeons by the injection of rapamycin. (A) Weight loss of breeding pigeons was determined at the end of the experiment (g/pair). The values are the mean ± S.E.M. with n = 30. (B) The average daily feed intake of the breeding pigeons was determined (g/d/pair). The values are the mean ± S.E.M. with n = 10. (C, D) Statistical analysis of crop thickness (C) and crop relative weight (D) in breeding pigeons. The values are the mean ± S.E.M. with n = 10. a–c, no identical letters on the column indicate significant differences ($P < 0.05$).
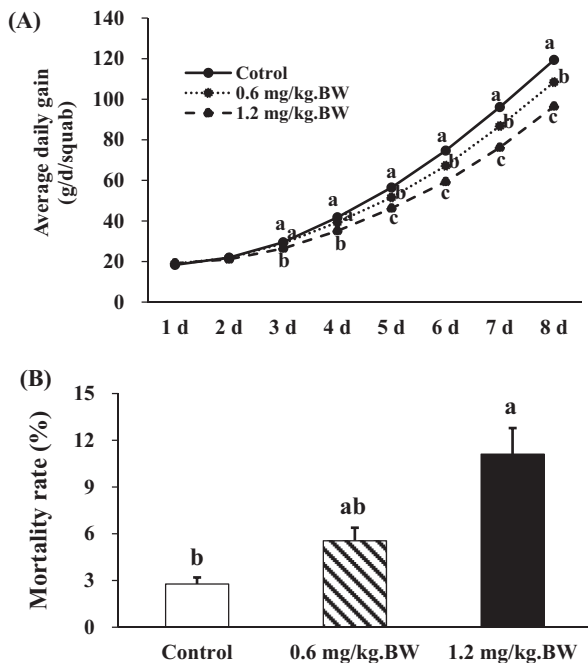

Figure 2. Inhibition of the growth performance of young squabs by injection of rapamycin. (A) The average daily gain of young squabs at 1, 2, 3, 4, 5, 6, 7, and 8 D was monitored (g/d/squab). The values are the mean ± S.E.M. with n = 30. (B) The mortality rate (%) of each young squab was determined. The values are the mean ± S.E.M. with n = 10. a–c, no identical letters on the column indicate significant differences ($P < 0.05$).

Expression of TORC1 Signaling Pathway-Related Proteins

To elucidate the regulatory role of TORC1 in crop milk protein synthesis, we analyzed TORC1 signaling pathway-related proteins and found that the protein levels of TORC1, S6K1, S6, 4EBP1, and eIF4E were decreased in the low- or high-dose RAPA injection group ($P < 0.05$, Figure 4A and B). The results of immunofluorescence staining with the TOR antibody in the crop tissues of the breeding pigeons were identical to the results of western blotting (Figure 4C).

DISCUSSION

TORC1 regulates body growth mainly by promoting anabolic processes and by suppressing catabolic processes; in particular, TORC1 has been demonstrated to be the key spatiotemporal regulation hub in the coordination of nutrient status (Apelo et al., 2014b; Gordon et al., 2015; Miniaci et al., 2015). As expected, the injection of 1.2 mg/kg BW RAPA significantly reduced the level of total protein (including albumin and globulin) in serum, indicating that RAPA inhibits protein synthesis in breeding pigeons. Likewise, the lower uric acid concentration after treatment with RAPA suggested that nitrogen metabolism in the body was reduced. Curiously, the difference in total protein between the
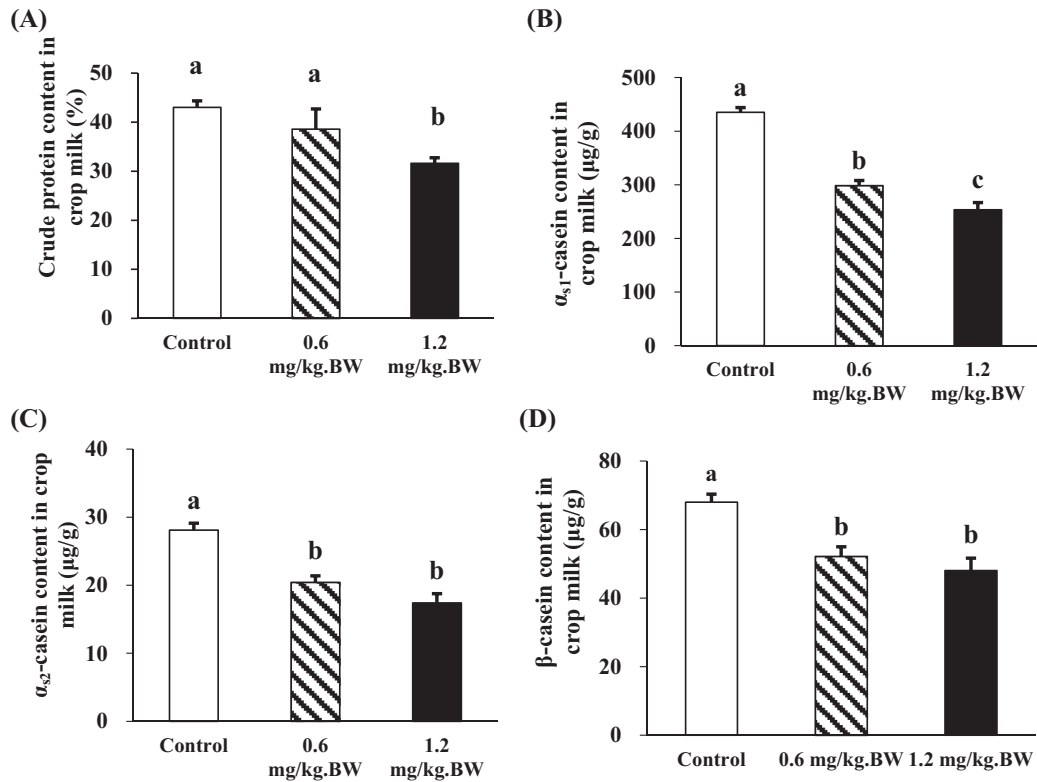
Figure 3. Effects of intravenous injection of rapamycin on the protein content of crop milk. (A) Crude protein content in crop milk. (B) α_{s1}-casein content in crop milk. (C) α_{s2}-casein content in crop milk. (D) β-casein content in crop milk. The values are the mean $\pm$ S.E.M. with n = 30. a–c, no identical letters on the column indicate significant differences ($P < 0.05$).

Table 2. Effect of the injection of rapamycin on amino acid contents of crop milk (%) of breeding pigeons.

Amino acid	Control	0.6 mg/kg.BW	1.2 mg/kg.BW
Asp	6.5 ± 0.55[a]	5.8 ± 0.33[a,b]	5.0 ± 0.19[b]
Thr	3.3 ± 0.23[a]	3.1 ± 0.26[a,b]	2.4 ± 0.16[b]
Ser	3.4 ± 0.31[a]	3.1 ± 0.27[a,b]	2.4 ± 0.10[b]
Glu	11.4 ± 0.55[a]	10.2 ± 0.47[a,b]	9.3 ± 0.18[b]
Gly	3.4 ± 0.29[a]	2.7 ± 0.19[a,b]	2.2 ± 0.10[b]
Ala	3.7 ± 0.24[a]	3.1 ± 0.13[a,b]	2.6 ± 0.10[b]
Cys	0.7 ± 0.01[a]	0.6 ± 0.02[a]	0.5 ± 0.04[b]
Val	4.1 ± 0.28[a]	3.4 ± 0.18[b]	2.8 ± 0.06[b]
Met	1.2 ± 0.09[a]	1.0 ± 0.05[a]	0.8 ± 0.05[b]
Ile	3.5 ± 0.27[a]	3.0 ± 0.15[a,b]	2.5 ± 0.01[b]
Leu	7.1 ± 0.53[a]	6.3 ± 0.33[a,b]	5.3 ± 0.09[b]
Tyr	3.1 ± 0.28[a]	2.4 ± 0.24[a,b]	2.0 ± 0.14[b]
Phe	4.0 ± 0.27	3.5 ± 0.22	3.3 ± 0.19
Lys	5.1 ± 0.43[a]	4.1 ± 0.32[a,b]	3.2 ± 0.30[b]
NH3	1.1 ± 0.06	1.1 ± 0.11	1.1 ± 0.05
His	1.7 ± 0.09[a]	1.4 ± 0.11[a,b]	1.3 ± 0.08[b]
Arg	5.2 ± 0.43[a]	4.5 ± 0.26[a]	3.4 ± 0.06[b]
Pro	3.0 ± 0.07[a]	2.8 ± 0.08[a,b]	2.9 ± 0.02[b]

The values are the mean $\pm$ S.E.M. with n = 30. a–c, no identical letters in the same row indicate significant differences ($P < 0.05$).

high-dose RAPA group and the control group was mainly reflected in the albumin level, rather than the globulin level. Albumin, similar to total protein, exhibits important physiological functions such as maintaining plasma colloid osmotic pressure and transporting various metabolites (Fanali et al., 2012; Li et al., 2017). Accordingly, we conjecture that RAPA reduces serum albumin levels by inhibiting protein synthesis,

which might hinder the transport of substances to various tissues such as the crop.

The rapid growth of young squabs requires adequate nutrition; thus, in preparation for lactation, the surface area of the breeding pigeon crop is extended through an increase in rete pegs. This hyperplasia followed by desquamation results in large numbers of epithelial cells accumulating in the crop lumen, which provides nourishment for the young squab (Gillespie et al., 2011, 2012). Our results showed that crop thickness in the low- or high-dose RAPA group was significantly narrower than that in the control group, which was partly due to the inhibition of cell proliferation and differentiation caused by an insufficient supply of nutrients. Consequently, it was understandable that the crude protein and α_{s1}-casein, α_{s2}-casein, and β-casein contents of crop milk showed the same trend as the crop. Furthermore, RAPA treatment resulted in a significant decrease in 16 amino acids, including 11 EAA (Met, Lys, Thr, Val, Leu, Ile, Arg, His, Gly, Cys, and Tyr), in crop milk. Because crop milk is the main source of nutrients for young squabs, low-quality pigeon milk, especially a lack of EAA, would inevitably lead to growth retardation and high mortality of young squabs (Vandeputte-Poma, 1980; Gillespie et al., 2013). Liu et al. (2016) also found that treatment with 0.4 and 1.0 mg/kg BW RAPA induced 10% and 50% mortality of broiler chicks, respectively. Surprisingly, even 1.2 mg/kg BW RAPA had no significant effect on the BW loss

Table 3. Effect of the injection of rapamycin on serum biochemical parameters of breeding pigeons.

Group	Albumin (mmol/L)	Globulin (mmol/L)	Total protein (mmol/L)	Uric acid (mmol/L)
Control	12.8 ± 0.45[a]	16.7 ± 0.81	29.5 ± 1.16[a]	445.8 ± 47.07[a]
0.6 mg/kg.BW	10.8 ± 0.20[b]	16.9 ± 0.71	27.7 ± 0.69[a]	391.8 ± 30.80[a]
1.2 mg/kg.BW	9.9 ± 0.58[b]	14.6 ± 1.34	24.5 ± 1.20[b]	219.5 ± 24.20[b]

The values are the mean ± S.E.M. with n = 30. a, b, no identical letters in the same column indicate significant differences ($P < 0.05$).
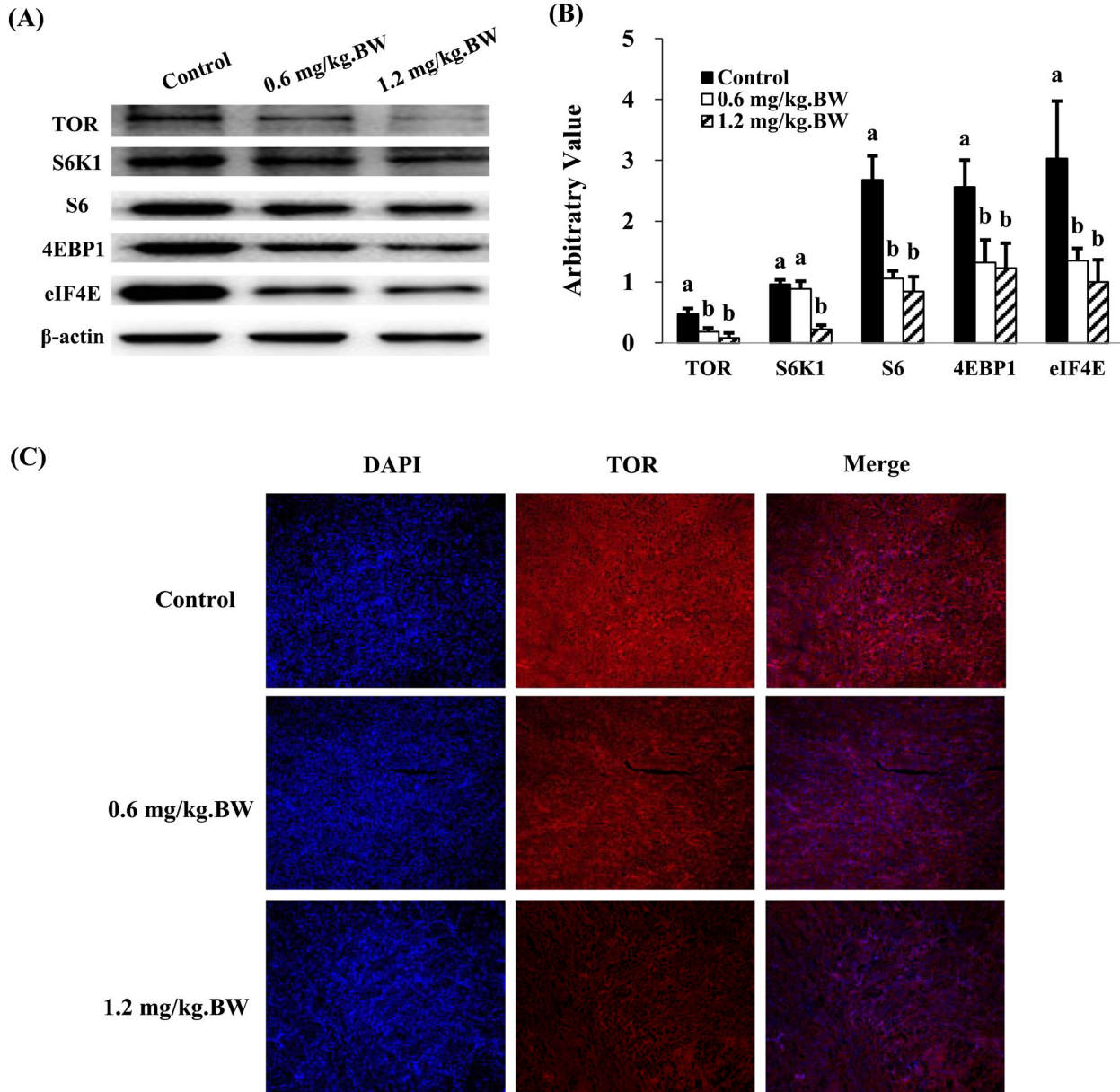

Figure 4. The expression of TORC1 pathway-related proteins in the crop of breeding pigeons injected with rapamycin during lactation periods. (A, B) The levels of proteins related to the TORC1 signaling pathway were measured by western blotting. The results are shown in the form of the total protein level relative to β-actin. The values are the mean ± S.E.M. with n = 6. a,b, no identical letters on the column indicate significant differences ($P < 0.05$). (C) Representative images of immunofluorescence (IF) staining with the TOR antibody in the crop of the breeding pigeons are shown (200×; n = 3).

and ADFI of breeding pigeons in the present study, while 0.4 mg/kg BW RAPA could reduce the body weight of broilers. These differences may be related to the sensitivity to RAPA between different poultry species.

Previous studies have revealed that TORC1 is a bona fide target that is inhibited by RAPA and regulates its direct targets S6 and 4EBP1 to initiate mRNA translation (Mahoney et al., 2009; Liu et al., 2016; Miao et al., 2017). Inhibition of the TORC1 signaling pathway

reduces protein synthesis and cell proliferation (Albert and Hall, 2015; Yang et al., 2015; Zhao et al., 2015). In recent years, TORC1 signaling has been found to regulate mammalian milk protein synthesis (Apelo et al., 2014a; Gordon et al., 2015). Interestingly, during the feeding period when breeding pigeons secrete crop milk vigorously, the activity of TORC1 in the crop significantly increases (Hu et al., 2016). In this experiment, RAPA-induced TORC1 disruption might have directly inhibited the synthesis of proteins (the main components of crop milk) and the proliferation of epithelial cells in the crops of breeding pigeons.

Taken together, the results showed that RAPA reduces the contents of protein and amino acids in crop milk by inhibiting the activity of TORC1 in the crop, eventually leading to growth retardation of young squabs.

FUNDING

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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