Dynamic Expression of TRPV6, Cal Bind in D28k and PMCA 1B in the Egg During the Oviposition Cycle in Laying Hens

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Abstracts

The aim of this study is to investigate the effect of active Ca^{2+} transcellular transport on the eggshell calcification. Forty ISA laying hens at the peak stage (220 days old) were assigned to five reproductive stages for sampling. The samples of eggshell gland (ESG) were obtained by the chickens were sacrificed at 0, 2, 4.5, 8 and 16 h after post-oviposition, respectively. Then, quantization's of mRNA level and protein concentration of TRPV6, CaBP-D28K and PMCA 1b at different stages in the ESG were carried out by real-time PCR and western-blot analysis, respectively. The results are as follows: the expression levels of TRPV6, CaBP-D_{28K} and PMCA 1b mRNA in the ESG were retained very low until the egg movement into the shell gland (0~4.5 h after ovulation), then significantly increased at 16 h during eggshell calcification. In addition, TRPV6, and CaBP-D_{28K} indicated significant statistical difference ($P < 0.01$), respectively. Furthermore, western blotting showed that the expression of TRPV6 and PMCA 1b reached the maximum at 16 h after ovulation, but the statistical difference was not significant. The change of CaBP-D_{28K} expression was very similar to that of TRPV6, but the concentration was significantly increased at 16 h than that at 0 h after ovulation ($P < 0.05$). In conclusion, the expression of TRPV6, CaBP-D_{28K} and PMCA 1b in the ESG was regulated by the oviposition cycle, suggesting that active Ca^{2+} transcellular transport exerted significant effects in calcium delivering in the ESG.

Keywords—*ESG; TRPV6; CaBP-D_{28K}; PMCA 1b; the oviposition cycle*

I. INTRODUCTION

The shell gland, that is, the uterus, is the important reproductive organs of the laying hens. During the peak period of the egg laying, every day, a large amount of calcium ions are transported to the shell cavity to deposit the eggshells. At present, the existing data are insufficient to explain the eggshell gland calcium ion transport, but studies have pointed out that there may be three mechanisms as follow: First, the calcium ion trans cell transport pathway [1,2]; Second, The positive potential difference between the plasma membrane and glandular cavity of egg shell can overcome the high concentration of calcium ion in the lumen of the shell.[3-5];Third, the high concentration of HCO^{3-} in the gland cavity can rapidly combine with Ca^{2+} to form calcium carbonate deposits [6]. However, it is not yet reported that in eggshell glandular cavity is a way to complete calcium ion transport or several ways to complete with, therefore, to be further explored.

Studies have shown that calcium ion Trans cell transport can promote the transfer of calcium ions in the eggshell gland. At the last 15 h of eggshell formation, calcium moves through the hen's eggshell glandular at the rate of 100~150 mg · h^{-1}, and the calcium concentration in the shell gland is 2~4 times that in the plasma. Therefore, the presence of calcium ions in the plasma into the shell gland must overcome this concentration gradient, indicating that this transport may be active. At the same time, when the egg shell gland activity increased oxygen consumption, due to lack of oxygen and metabolic poison, the operation of calcium and potential gradient are inhibited, indicating the formation of eggs when the metabolism of energy consumption [7]. In addition, CaBP-D_{28K} and PMCA 1b were confirmed to be distributed in the eggshell gland, the CaBP-D_{28K} mRNA level was significantly increased during the calcification of the eggshell, and the activity and expression of PMCA 1b were increased Eggshell calcification. At present, TRPV6 research reports less, in the previous study, I study confirmed TRPV6 expression in the eggshell gland [11], it is speculated that calcium transporter transport may play an important role in the process of calcification of eggshells.

To prove the above guess, this experiment is to start from the normal laying eggs cycle of the laying hens, and study the different stages of the

completion of a cycle, that is 0 h, 2 h, 4.5 h, 8 h, 16 h after laying, (follicle at different positions of the fallopian tube: ovary, magnum, isthmus, shell gland I(ion and water is formed, the shell gland (II) eggshell calcification)), egg shell gland calcium cross-cell transport related proteins: TRPV6, CaBP-D28K, PMCA 1b mRNA and protein levels, and provide a theoretical basis for further revealing the important role of Ca^{2+} cross-cell transport pathway in the transfer of bone calcium in eggshells.

II. MATERIALS AND METHODS

A. The Main Reagents

SYBR Prime Script RT-PCR Kit、SYBR Premix Ex Taq（TaKaRa， Dalian Baosheng Bioengineering Co., Ltd.); Trizol (Tianan, Nanjing Arnold Biology Co., Ltd.); TRPV6 rabbit anti-human polyclonal antibody (ACC-036, Alamone Labs Ca^{2+}-ATPase murine monoclonal antibody (BM0203, Wuhan Bude Biological Engineering Co., Ltd.); GAPDH murine cloning antibody (KC-5G4, Shanghai, China); CaAP-D28K mouse monoclonal antibody (Wuhan Bude Biological Engineering Co., Ltd.) (GGHL-15P, Immunology Consultants Laboratory, Inc., USA); horseradish peroxidase-labeled goat anti-mouse IgG (BS50350, & lt; RTI ID = 0.0 & gt; Bioworld Technology Co., Ltd., USA); ECL Chemiluminescence Kit (Tiangen, Nanjing Arne Biological Co., Ltd.); BCA Protein Concentration Detection Kit (Wuhan Bude Biological Engineering Co., Ltd.); Other reagents are of analytical grade.

B. Test animals and test designs

Nanjing Qinglong mountaina breeding plant 220-day-old high-yield layer house (about 7,000, laying rate more than 90%), then select 40 good physical condition, the laying rate consistent,similar physquel ISA layer, single cage reared in the same care ladder egg chicken in the cage number, according to the conventional method of feeding and management of intensive chicken farms chicken, the use of natural light and artificial light supplement in the house, 16:8 (L/D). Test chicken free drinking water. According to the production schedule of the chicken farm, every morning at 7:30, one-time feed their own formulated feed, composition: corn 63%, bran 2%, 24% soybean meal, stone powder 8% and additives 3%.

After one week, the laying time, the number of eggs and the laying rules of each chicken were recorded in detail, and then divided into 5 groups according to the concentration degree of laying time.

C. Sample Collection and Processing

The trial lasted two weeks; Samples were collected at 0 h, 2 h, 4.5 h, 8 h and 16 h after laying respectively. 8 rats in each group were sacrificed, the surgical collection of the shell gland (uterus) tissue was about 1 g. preparation PBS (pH7.4) buffer use of 4 ℃ pre-cold DEPC and rinse 3 times, absorbent paper dry, divided into two and place into freezing tube, liquid nitrogen frozen, then transferred to 80 ℃ refrigerator for analysis of gene and protein changes.

D. Changes in the Expression of Calcium Ion Tran's Cell Transport Related Proteins During Egg Laying Cycle

1) Tissue total RNA extraction

The total RNA of eggshell gland tissue was extracted by Trizol one-step method. The total RNA levels and purity (A260 / A280, 1.82.0) were determined by a nucleic acid protein detector. RNA integrity was detected by 1% denaturing gel electrophoresis.

2) Preparation of sample cDNA

Useing SYBR PrimeScript RT-PCR Kit kit, 10 μL reverse transcription (RT) system, 500 μl total RNA, 25 pmol Oligo (dT) 18, 50 pmol Random 6 mers, 2 μL 5 × RT Buffer, ， 0.5 μL RT Enazyme Mix I， supplemented with DEPC (diethyl pyrocarbonate) water treatment to 10 L; reacting at 37 DEG C for 15 min, 85 C inactivated 5 s. CDNA samples are stored at 20 DEG C for reserve.

3) Primer design

According to the gene order of GenBank chicken TRPV6 and PMCA 1b, using Primer 5 software to design primers, reference gene beta -actin and CaBP-D28K primer sequences according to the literature, the primer sequences are shown in Table 1, synthesized by Shanghai Invitrogen biotechnology company.

4) Real time quantitative fluorescence PCR

Used SYBR Green I fluorescence quantitative PCR. According to the real-time fluorescence quantitative kit (SYBR Premix Ex Taq, TaKaRa) operating instructions ice preparation amplification system 20 μL: SYBR Premix Ex Taq（2×）10 μL，ROX Reference Dye（50×）0.4 μL，Forward Primer（10 pM）0.8 μL，Reverse Primer（10pM） 0.8 μL， cDNA template 2.0 μL, supplemented with Nuclease-Free water to 20 μL. The amplification conditions of the target gene are shown in Table 1. Melting curve conditions: 95 ℃, 15 s; 60 ℃, 1 min; 95 ℃, 15 s, 60 ℃, 15 s. Each sample was repeated twice, while a blank control was

set.YBR Green I fluorescence quantitative PCR was used.

Table 1 Primer Sequence of Target Gene for Real-Time PCR

Target genes	GenBank accession	Primer sequences	PCR products (bp)	PCR conditions
TRPV6	XM_416530	F:5'-tggaacggactaagtcagaagttg-3' R: 5'-cgttatggctgggatgttgtt -3'	141	94 ℃,15 s, 60 ℃, 10 s, (40)
CaBP-D28K	EU_404189	F:5'-ttaaatctgcgttgcttccataca -3' R:5'-ggcccatcctgcactccataac-3'[12]	297	94 ℃, 15 s, 64 ℃, 10 s, (40)
PMCA 1b	XM_416133	F:5'-ttcaggtactcatgtgatggaagg -3' R:5'-cagccccaagcaaggtaaag -3'	98	94 ℃, 15 s, 62 ℃, 10 s, (40)
β-actin	NM_205518	F:5′- tgcgtgacatcaaggagaag -3′ R:5′- tgccagggtacattgtggta -3′[13]	300	94℃,15 s, 60-62 ℃, 10 s, (40)

5) ***Determination of TRPV6, CaBP-D28K and PMCA Amount of Gene Expression in Tissues***

By TRPV6, CaBP-D28K, PMCA and β-actin genes experienced the cycle number Ct value when the fluorescence signal reached the threshold, the data of validity were analyzed by 2-ΔΔCT method, and the relative expression of TRPV6, CaBP-D28K and PMCA genes was calculated by using the formula of β-actin as the internal standard gene.

E. Changes of Calcium Ion Trans Cell Transfer Related Protein Expression in Eggshell Gland During Egg Laying Cycle

*1)****Sample Preparation***

Take tissue 100 mg in 1 μL lysis solution on the ice homogenizer, let stand for 30 min, to be completely cracked, centrifuged 30 min (4 ℃, 25,000 g). The supernatant was used to detect the total protein content of different samples by BCA method. In addition, part of the supernatant, adding the buffer solution [loading buffer / sample (V / V), 1: 4], 100 ℃ boiling water bath for 5 min, protein denaturation. 12,000 g centrifugation 5 min (4 ℃), take the supernatant, 20 ℃ storage equipment.

*2)****SDS-polyacrylamide gel electrophoresis***

Preparation of 10% separation gel, 4% concentrated gel. The total protein of TRPV6 sample was 80μg, CaBP-D28K PMCA and 1b protein amount were 40, 30 μg. SDS-PAGE electrophoresis was performed at 100 V and 2550 mA, 80 min, 50 min, and 90 min, respectively. Then, the control protein standard Marker and cut the adhesive tape to the right size and put it in the membrane buffer.

*3)****Transfer***

Cut 6 sheets of the large same as adhesive tape filter paper and nitrocellulose membrane (NC film), and soak in the transfer buffer for about 30 min. In the environment of 4 ℃, 100 V, 200300 mA conditions were transferred 80 min, 60 min and 90 min.

*4)****Immune response***

Place NC film in 5% skimmed milk powder closed liquid at room temperature of 2 h. Discard the enclosed liquid and wash the film with TBST.

First antibody incubation: adding antibodies diluted at an appropriate proportion (using 5% skimmed milk powder, respectively, TRPV6, CaBP-D28K, Ca^{2+}-ATPase, GAPDH antibodies at 1:200, 1:200, 1:300 and 1:10000 ratio dilution), 4 degrees a bed overnight. Restore to room temperature, TBST wash film.

Secondary antibody incubation: add horseradish peroxidase coupling two resistance (TRPV6 two, anti sheep, rabbit IgG, 1:5000 dilution; GAPDH, CaBP-D28K, Ca^{2+}-ATPase two, anti Sheep anti rat IgG, 1:10000 dilution), room incubation 2 h. TBST washing film.

*5)****Chemiluminescence reaction***

The reagents A in the ECL kit were mixed with the reagent B in equal volume and then dropped on a nitrocellulose membrane, and the reaction is 5 min.

*6)****Take photos and analyze***

Western-blot results were taken by scanning and the gel imaging system software was used to

quantitatively analyze the gray value and determine the relative content of target protein in the sample.

F. Data Analysis and Statistics

All data were expressed as $\overline{X} \pm SE$, and SPSS 16.0 statistical software was used for data analysis. One-way ANOVA (LSD) was used for the significant difference test.

III. RESULTS AND ANALYSIS

A. Changes in the Expression of TRPV6, CaBP-D28K, PMCA, 1b and mRNA in Eggshell Gland During the Process of Egg Laying

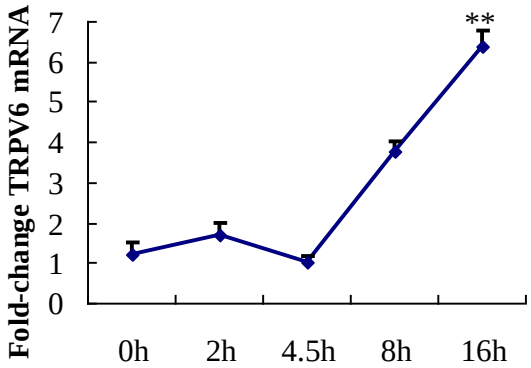


Fig. 1 Dynamic Changes of TRPV6 and mRNA Expression in Eggshell GLand During the Process of egg Laying Cycle (n=8)

"**" indicates a significant difference compared with 0h (P <0.01)

After egg laying, the ovum is excreted from the ovary to the calcification process of eggshell. TRPV6 mRNA expression in the shell gland in 4.5 h initial level is relatively low, reached the lowest at 4.5 h, then the expression level increased gradually, reached the maximum at 16 h after the egg shell calcification process, compared with the 0 h difference highly significant (P < 0.01)(Figure 1).

After egg laying, the ovum is excreted from the ovary to the calcification process of eggshell, The dynamic changes of CaBP-D28K mRNA expression in eggshell gland were consistent with the expression of TRPV6 mRNA. CaBP-D28K mRNA expression level is low before the ovum into the shell gland (after the egg 4.5 h), the expression increased gradually after in the shell gland, reached the maximum in the process of 16 h compared with eggshell calcification, 0 h significant difference (P < 0.01) (Figure 2).

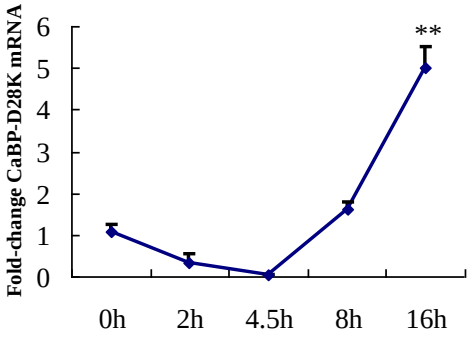


Figure 2 Dynamic Changes of Cabp-D28K and Mrna Expression in the Shell Gland of Laying Eggs During the Process of Egg Laying Cycle (N=8)

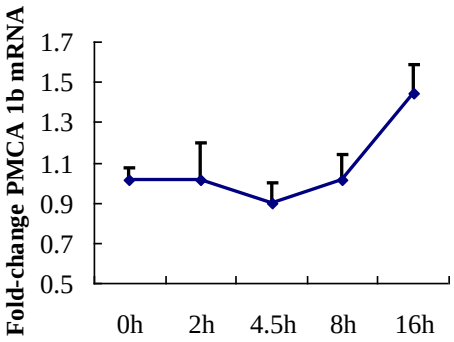


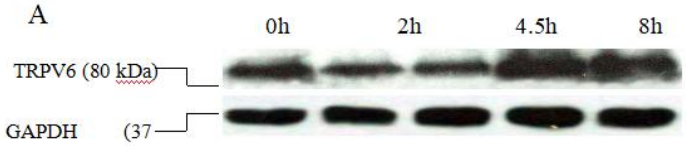
Figure 3 Dynamic Changes of PMCA, 1b and Mrna Expression in the Shell Gland of Laying Eggs During the Process of Egg Laying Cycle (N=8)

After egg laying, the ovum is excreted from the ovary to the calcification process of eggshell, The dynamic changes of the expression of PMCA, 1b and mRNA in the eggshell gland were consistent with the dynamic expression changes of TRPV6, mRNA and CaBP-D28K mRNA. It was express lower before the ovum into the shell gland, and reached the maximum in 16 h eggshell calcification, but the difference not significant (P > 0.05).

B. Changes of TRPV6、CaBP-D28K、PMCA 1b Protein Expression in Eggshell Gland During Egg Laying Cycle

The dynamic changes of TRPV6, CaBP-D28K and PMCA 1b proteins during egg production were quantitatively detected by protein immunoblotting, and the specific changes of TRPV6, CaBP-D28K, PMCA 1b After incubation, the specific bands of TRPV6, CaBP-D28K and PMCA 1b were predicted to be close to 80KD (4 A), 28 KD (5 A) and 138 KD (6 A) And a specific band at 37 KD after incubation with GAPDH antibody, consistent with the results of the GAPDH protocol (see Figure 4 A, 5 A, 6 A).

GAPDH as the internal reference, gray analysis results indicate that: (4 B) after laying eggs from the ovaries to discharge the eggshell calcification process, eggshell gland TRPV6 in the first 08 H expression level is low, but increased gradually, the eggshell calcification (16 h, TRPV6 after egg laying) expression of the most high. But the difference was not significant (P > 0.05); Figure 5 B pointed out that after laying 02 h, during egg formation from ovary to protein, the concentration of CaBP-D28K protein in the eggshell gland is inconvenient, with the backward movement of the germ cell, the concentration of CaBP-D28K in the shell gland gradually increased in calcification to eggshell and egg laying reached the maximum. Compared with 0 h after laying, the difference was significant (P < 0.05), showed that CaBP-D28K plays an important role in the eggshell calcification process ;Figure 6 B shows that the expression of PMCA 1b protein in the egg shell gland is consistent with that of TRPV6. With the backward movement of the germ cell, the expression of PMCA 1b gradually increases, and the egg shell formation process reaches the maximum value, but compared with 0 h , The difference was not significant (P> 0.05). The increase of calcium ion transport related protein concentration during eggshell formation indicates that Ca2 + trans cellular transport pathway plays an important role in eggshell calcification.



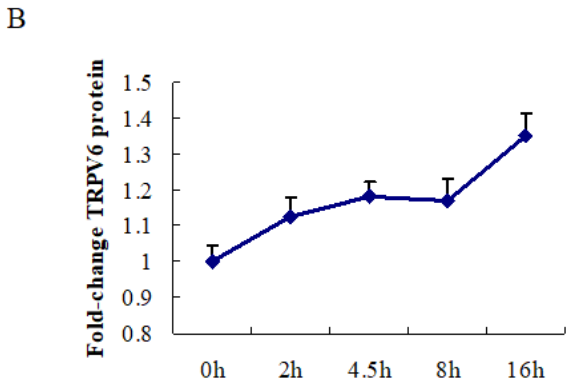


Fig.4 Dynamic Changes of TRPV6 Protein Expression In Eggshells During Egg Circulation (N = 8)

A: TRPV6 and GAPDH specific immunoblot; B: data analysis, all values are compared with 0h

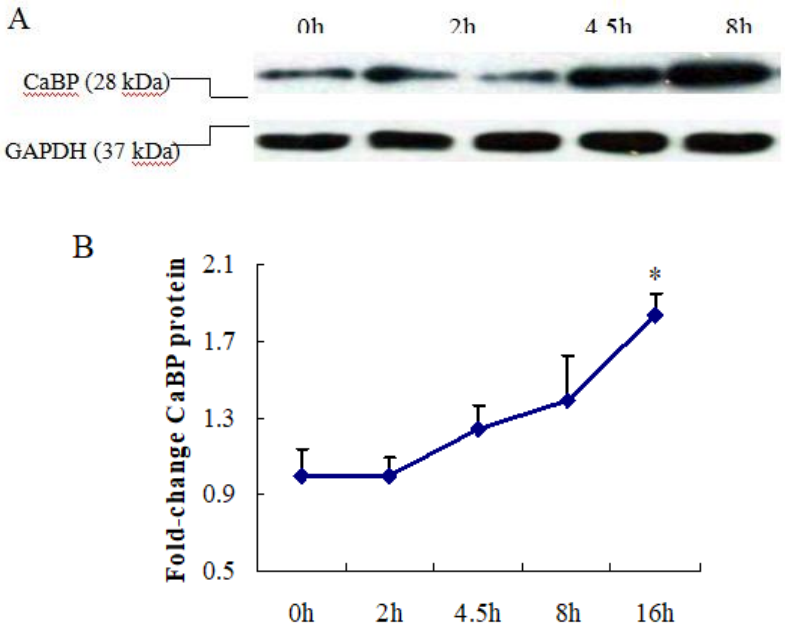


Fig.5 Dynamic Changes of Cabp-D28K Protein Expression in Eggshells During Egg Circulation (N = 8)

A: Specific immunoblotting of CaBP-D28K and GAPDH; B: Data analysis, all values compared to 0 h

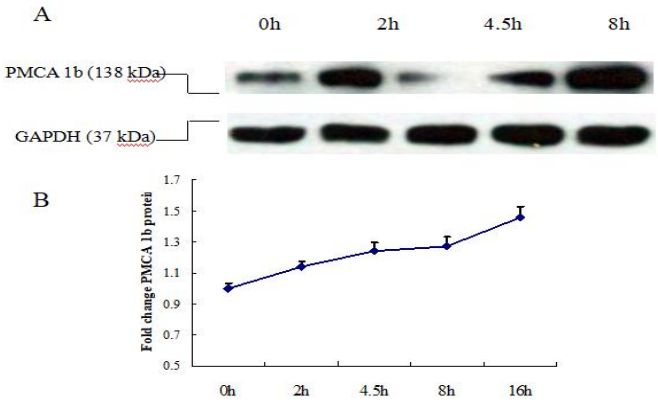


Fig.6 Dynamic Changes of PMCA 1b Protein Expression in Egg Shell Gland During Egg Circulation (n = 8)

A: PMCA 1b and GAPDH specific immunoblot; B: data analysis, all values were compared with 0 h.

IV.DISCUSSION

During the peak period of egg production, the eggshell gland transfers a large amount of calcium ions to form eggshells. This study shows that in the circadian cycle of egg, the egg is released from the ovary, fallopian tube after entering the shell gland, calcium ions across a cell membrane associated protein TRPV6, CaBP-D28K and PMCA 1B in the eggshell calcification stage, gene and protein expression levels were significantly increased. The results suggest that Ca^{2+} Trans cell transport plays an important role in the calcium transfer process of eggshell gland.

TRPV6 is a member of the transient receptor potential channel TRP super family, is a special epithelioid calcium channel with high Ca^{2+} selectivity. Studies have shown that TRPV6 is the gating channel for calcium ion Tran's cellular transport. At present, there are few reports about the role of TRPV6 in eggshell gland at home and abroad. The former sixth chapters confirm the existence and distribution of TRPV6 in eggshell gland tissues. In this study, we found that the expression level of TRPV6 mRNA and protein reached the maximum at the time of egg laying, and the expression level of TRPV6 mRNA and protein reached the maximum in the process of calcification. Studies have shown that, compared with the estrous cycle, the expression of TRPV6 in the endometrial of pregnant pigs is higher. In addition, the levels of TRPV6 mRNA in the uterus increased at the middle and late stages of pregnancy, and the placental labyrinth and corpus cavernosum TRPV6 were also significantly expressed in the second trimester. These results suggest that important reproductive functions, such as pregnancy or eggshell formation, can regulate the expression of TRPV6 in the uterus.

In the laying hens, the expression of CaBP-D28K in a large number of calcium transport organization: intestine, kidney and eggshell gland; at the same time, there are also low concentration expression in bone and tooth tissue; its main function is to play a role in calcium ion transport buffer in the process of maintaining calcium ion concentration near the TRPVS channel pore low, followed by cells high concentration of calcium ions to the basement membrane, prevent high calcium ions to form toxic side effects and prevention of apoptosis in cells. In the eggshell gland, CaBP-D28K is mainly absorbable epithelium and tubular glandular epithelium. In this study, the expression of CaBP-D28K mRNA and protein in the process of calcification were significantly increased, and the changes of CaBP-D28K mRNA in egg shell gland were from 0 to the highest during the process of egg laying. But the data show that the eggshell gland CaBP-D28K protein did not show fluctuations in egg production cycle, which may be due to differences in the formation of different detection methods, this experiment used Western blot method to detect the protein expression of was more sensitive and accurate, the data show that the egg shell formation on expression of CaBP-D28K in the shell gland, while the latter in the shell gland calcium ions play an important role in transferring.

In the cells, PMCA 1b is responsible for storing energy in the form of ATP, and the Ca^{2+} is excreted through the electrochemical gradient, mainly in the basement membrane of the intestinal and renal cells associated with calcium transport in the eggshell gland tissue, PMCA 1b mainly distributed in the top of the tubular cell surface microfilm, rather than the basement membrane. In this study, PMCA 1b was expressed in the egg shell gland tissues, and the mRNA level and protein expression increased during eggshell formation, but there was no significant change. Studies have reported that egg formation process can make the PMCA 1b activity increased, but in eggshell formation after compression can downgrade its activity, because the experiment did not detect significant changes in expression, the amount of PMCA 1b after the formation of the PMCA 1B in the eggshell, egg form changes in the process is still in dispute. This study showed that eggshell formation had no significant effect on the expression of PMCA 1B in eggshell gland.

V. CONCLUSION

The eggshell gland calcium ions across a cell membrane associated protein TRPV6, CaBP-D28K and PMCA 1b showed dynamic changes in the corresponding production cycle, especially, in the process of egg shell formation, the expression of three proteins increased, showed that the laying cycle has regulation function on the three kinds of protein, also suggested that calcium ions across a cell membrane with an important role in the eggshell calcification process, but did not show significant changes due to increased expression of TRPV6 and PMCA 1b protein, suggesting that other calcium ion transport mechanism of eggshell calcification process, needs to be further proved.

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