低カルシウム飼料給餌による卵殻質の低下と小腸及び卵殻腺におけるカルシウム結合タンパク質(CaBP-D28k)遺伝子発現に与える影響

誌名	日本家禽学会誌
ISSN	00290254
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発行元	日本万国家禽学会
巻/号	36巻5号
掲載ページ	p. 295-303
発行年月	1999年9月

Effect of Low Calcium Diet on Messenger Ribonucleic Acid Levels of Calbindin-D_{28K} of Intestine and Shell Gland in Laying Hens in Relation to Egg Shell Quality

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We investigated the effects of short-term feeding low calcium diet on egg shell quality (specific gravity, relative weight of egg shell, egg shell thickness) and mRNA levels of calbindin-D_{28K} (CaBP-D_{28K}) in the shell gland and intestine. After feeding low calcium diet (0.5%), hens laid fragile eggs with low shell quality. Plasma calcium concentration also decreased significantly after feeding of low calcium diet. When the hens received a moderate calcium diet (3.5%) after low calcium diet for 5 days, shell quality was improved and plasma calcium concentrations increased. Levels of CaBP-D_{28K} mRNA in the shell gland did not change after feeding low calcium diet for 5 days. Levels of CaBP-D_{28K} mRNA in the intestine of the low calcium diet group significantly increased 2 fold when compared to those of the control group. We infer that low calcium concentration in plasma may stimulate parathyroid and 1,25$(OH)_2D_3$ production in the kidney and induce CaBP-D_{28K} mRNA expression in the intestine. It is supposed that a low calcium diet influences egg shell quality, plasma calcium concentration and intestinal CaBP-D_{28K} gene expression by calcium homeostasis. In the shell gland, however, the levels of CaBP-D_{28K} mRNA may not be influenced by low plasma calcium.

*(Jpn. Poult. Sci., **36** : 295-303, 1999)*

Key words : calbindin-D_{28K}, low calcium diet, gene expression, egg shell calcification

Introduction

Laying hens require a large amount of calcium for egg production. Dietary calcium is the main source for egg shell and is absorbed efficiently from the intestine into the blood. Calcium binding protein plays an important role for this intestinal transport of calcium. Biosynthesis of calcium binding protein with a molecular weight of 28 K dalton (CaBP-D_{28K}) is mainly regulated by 1α,25-dihydroxy vitamin D_3 [1,25$(OH)_2D_3$] (NORMAN *et al.*, 1982 ; CORRADINO and FULLMER, 1991 ; NYS *et al.*, 1992 a). 1,25$(OH)_2D_3$ is the hormonal form of vitamin D_3 and its action mediated with vitamin D_3 receptor (VDR), a member of the nuclear receptor superfamily. In the laying hens CaBP-D_{28K} is also considered to be involved in calcium transport in the shell gland

Received January 13, 1999 Accepted April 15, 1999

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(CORRADINO *et al.*, 1968 ; CLEMENS *et al.*, 1989). Many reports demonstrated that mRNA levels of CaBP-D_{28K} in the intestine is kept fairly constant during the ovulation cycle in the chicken. But mRNA levels of CaBP-D_{28K} in the shell gland remain low when there is no calcification (i.e. approximately 5 hr after the ovulation), but they remain high during the shell formation period of about 8–20 hr after ovulation. CaBP-D_{28K} mRNA expression in the shell gland changes remarkably in relation to the states of calcification and associates with changes in sex steroid hormones during ovulation cycle (STRIEM and BAR, 1991 ; NYS *et al.*, 1992 b ; IEDA *et al.*, 1995 ; IEDA *et al.*, 1998).

Calcium flux is regulated by many factors such as plasma levels of ionized calcium itself (ETCHES, 1996). If the diet contains enough calcium (more than 3.56%), most of the egg shell calcium is derived directly from the diet via the intestine (HURWITZ and BAR, 1969). If the content is 1.95%, bone supplies 30–40% of the shell calcium. Therefore, on calcium-free diets, the skeleton is the principal source for egg shell (MUELLER *et al.*, 1969). However, it is likely that these relationships vary depending on the time of day. Hens rarely eat food at night, so that they experience a temporary calcium deprivation (STURKIE and MUELLER, 1976 ; ETCHES, 1996).

Although regulation mechanisms for calcium storage and consumption have been well documented in the laying hen, there is no information on CaBP-D_{28K} mRNA expression in the intestine and shell gland during an emergency in calcium supply. Calcium deprivation from the diet has been employed as a typical experimental manipulation to study physiological mechanisms in the regulation of shell formation in the laying hen. This study aims to evaluate the effect of feeding low calcium diet on CaBP-D_{28K} mRNA expression in the intestine and shell gland and the correlation of these variables with shell quality.

Materials and Methods

Animals and feeding programs

Seventy two Single-comb White Leghorn hens (10–13 months old, 1.5–2.0 kg) were used in this study. Hens were housed in individual cages with separate feeders and subjected to LD 14 : 10. Water was provided *ad libitum*. Hens were offered control (3.5% calcium) or low calcium (0.5% calcium) diets (Table 1), 120 g per day. Leftover food (less than 10 g) was removed every day. Hens were divided into 3 groups, (1) control group (N=22) ; those fed regular diet containing 3.5% calcium (Table 1) for fifteen days, (2) low calcium group (N=25) ; those fed control diet for the first five days and then low calcium diet for the second five days and (3) normal calcium replacement group (N=25) ; those fed control diet for first five days, low calcium diet for second five days and control diet for third five days. The time of oviposition was recorded daily by a digital-switch clock system. Hens laying relatively regular sequences of more than four eggs were used in this study. Eggs were collected daily and shell quality was assessed by the traits such as specific gravity, the weight of egg shell in relation to whole egg weight (relative weight of egg shell, %) and shell thickness.

Hens were killed 8 hours after ovulation on day 10 (low Ca group and control) or day 15 (normal Ca replacement group). The mucosal layers of the intestine and shell

Table 1. Composition of experimental diets

Ingredient and composition	Low calcium diet	Control diet
	%	
Corn	60.68	58.86
Wheat bran	1.5	1.46
Corn gluten feed	7.0	6.79
Soybean meal	14.9	14.45
Rapeseed meal	3.0	2.91
Corn germ meal	5.0	4.85
Corn gluten meal	2.5	2.43
Fish meal	1.0	0.97
Meat and bone meal	2.5	2.43
Animal fat	1.0	0.97
Calcium phosphate	0.4	0.39
Salt	0.25	0.24
Methionine	0.05	0.05
Lysine	0.03	0.03
Vitamin mix	0.07	0.07
Mineral mix	0.08	0.08
Lime stone	0	3
Calculated analysis		
ME, kcal/g	2,929.7	2,841.81
Crude Protein	18.13	17.59
Calcium	0.54	3.52
Total phosphate	0.61	0.59
Sodium	0.16	0.16

gland were collected. The tissues were snap frozen in liquid nitrogen and were kept at −80℃ until the extraction of total RNA. Plasma was separated from blood and kept at −30℃ until plasma calcium measurement was performed.

Northern blot analysis

Total RNA were extracted using TRIzol Reagent (Gibco BRL/Life Technologies, Gaitherburg, MD) and Northern blot analysis was accomplished as described previously (CHOMCZYNSKI, 1993 ; IEDA *et al.*, 1998). A 767-base pair (bp) fragment of chicken CaBP-D_{28K} cDNA (a gift from Dr. B.S. KOMM) was used as a probe for CaBP-D_{28K} mRNA assay (MANGELSDORF *et al.*, 1987) and a 400-bp fragment of S17 ribosomal protein cDNA (a gift from Dr. B. TRUEB) was used for an internal control. cDNAs were labeled with [a^{32}P]-dCTP (3,000 Ci/mmol, ICN Biomedicals inc., Costa Mesa, CA) using a rediprime DNA labelling system (Amersham Inc., UK). The membranes were hybridized with labeled cDNAs for 18 hours. The final washing condition was 0.1×SSC containing 0.1% SDS at 60℃ (15 min). The intensity of the ^{32}P-DNA-RNA hybridization on the membrane was quantified by scanning radioactivity using the BAS-2000 Bio-Imaging analyzer (Fuji Photo Film Co., Ltd., Japan). Values were normalized by ribosomal protein S17 mRNA levels.

Plasma calcium measurement and statistics

Plasma calcium concentration was measured by calcium E-test wako (Wako Pure Chemical Industries, LTD. Japan).

All data were analyzed by Sheffes F test using StatView (Abacus Concepts, Inc., USA).

Results

Effects of feeding of low calcium diet and refeeding of 3.5% calcium diet on egg production rate, egg shell quality and plasma calcium concentrations

Egg production rate decreased after 5 days feeding of low calcium diet and further decreased after refeeding of calcium for 5 days (data not shown). Next day after feeding of low calcium diet, hens laid thin and fragile shelled eggs. Some of these eggs were cracked partially or completely broken which could not be collected. The number of these cracked or completely broken eggs within eggs increased day by day and some hens ceased laying after feeding of low calcium diet. In control group, egg production rates were relatively constant and there were no cracked eggs.

Figure. 1 illustrates changes of relative weight of egg shell during a sequence of the feeding program. It was fairly constant during the 5 day period of 3.5% calcium, normal diet but it immediately started to decrease from day 6 (one day after feeding of low calcium diet) and reached minimum on day 9 (4 days after feeding of low calcium diet). The lowest relative weight of egg shell was 30% less when compared to the normal shell quality (Fig. 1). The relative weight of egg shell from day 7 to day 10 significantly decreased from its initial 5 days ($P<0.05$). It recovered after refeeding of 3.5% calcium diet to normal shell quality. There was no difference in shell quality between the first 5 days and the last 5 days of 3.5% calcium diet. Other parameters (specific gravity and egg shell thickness) showed same changes in response to feeding of low calcium diet and refeeding of 3.5% calcium diet (data not shown). Plasma

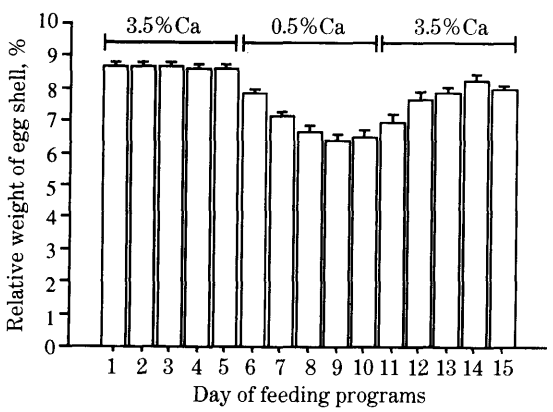

Fig. 1. Changes of egg shell quality (relative weight of egg shell) in a normal diet (day 1 to 5), a low calcium diet (day 6 to 10) and normall calcium replacement (day 11 to 15) during feeding programs. See materials and methods for details of feeding programs. Data represent means±SEM (n=9-45).

calcium concentration significantly decreased after feeding of low calcium diet for 5 days but it was restored by the replacement of 3.5% calcium diet to the calcium levels of control group (Fig. 2).

Effects of feeding of low calcium diet and refeeding of 3.5% calcium diet on mRNA levels of CaBP-D_{28K}

Figure. 3 shows the effects of low calcium diet on CaBP-D_{28K} mRNA levels in the intestine. Intestinal CaBP-D_{28K} mRNA levels in the low calcium group were significantly higher approximately 2 fold than those in the control group and high

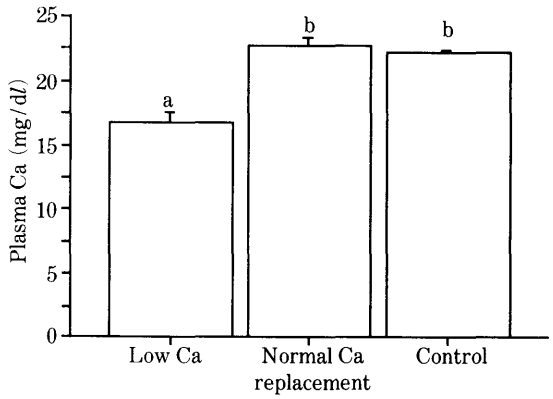

Fig. 2. Changes in plasma calcium concentrations in low calcium, normal calcium replacement and control groups. See materials and methods for detail of feeding groups and program. Data represent means ±SEM (n=8). Means with different superscripts are significantly different ($P<0.05$).

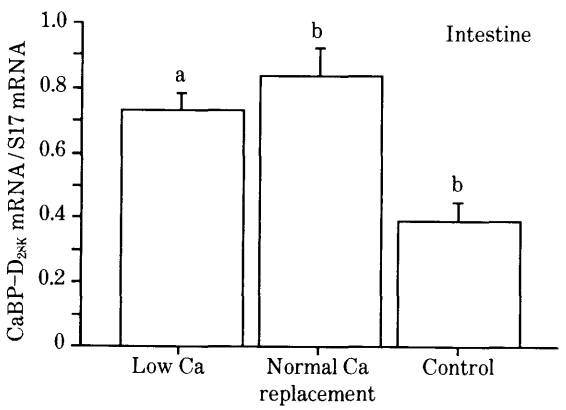

Fig. 3. Changes in mRNA levels of CaBP-D_{28K} in the intestine of laying hens in low calcium, normal calcium replacement and control groups. Same legend as in Fig. 2. Data from Northern blot analysis, which represent means±SEM (n=8). Means with different superscripts are significantly different ($P<0.05$).

levels were maintained 5 days after refeeding of 3.5% calcium diet (normal calcium replacement group). Figure. 4 illustrates the effects of low calcium diet on CaBP-D_{28K} mRNA levels in the shell gland. CaBP-D_{28K} mRNA levels in low calcium group tended to decrease, although, there was no significant difference between the control and the low calcium groups. The levels of CaBP-D_{28K} mRNA in refeeding of 3.5% calcium diet were same as those in the control group.

Dscussion

The present study demonstrates relationship between CaBP-D_{28K} mRNA levels and egg shell quality when hens were fed low calcium diet. Egg shell quality (specific gravity, relative weight of egg shell, egg shell thickness) and plasma calcium concentration were markedly reduced by feeding of low calcium diet for 5 days. Egg shell quality and plasma calcium concentration were restored to normal levels by refeeding of 3.5% calcium (Figs. 1 and 2). Hens fed a high calcium diet were generally able to replenish the calcium loss from medullary bone during shell calcification when shell formation is not taking place, but on a low calcium diet the cortical bone of the femur is eroded (TAYLOR and MOORE, 1954). It is well known that when plasma calcium concentration decreases, calcium homeostatic mechanisms will act to maintain calcium concentration. Parathyroid hormone (PTH), which is secreted under the influence of low plasma calcium concentration, stimulates the production of the 25-hydroxy-1α-hydroxylase enzyme that converts 25$(OH)_2D_3$ to 1,25$(OH)_2D_3$ in the kidney. Both PTH and 1,25$(OH)_2D_3$ also stimulate the release of Ca^{2+} from bone (NORMAN *et al.*, 1982 ; ETCHES, 1996). In addition, 1,25$(OH)_2D_3$ facilitates the absorption of Ca^{2+} from the intestine by inducing the gene expression of CaBP-D_{28K} via its cytosolic receptor, VDR (NORMAN *et al.*, 1982). In the present study, it was found that plasma calcium concentration and egg shell quality (specific gravity, relative weight of egg shell and egg shell thickness) were markedly reduced by feeding of low calcium diet for 5 days (Figs. 1 and 2). Consequently, it is assumed that five days of feeding of low calcium diet might have induced reabsorption of calcium from medullary bone in order to maintain plasma calcium levels (STURKIE and MUELLER, 1976). However, plasma calcium levels were not high enough to form the complete shell (Figs. 1 and 2, LENNARDS and ROLAND, 1981 ; KESHAVARZ, 1986).

CaBP-D_{28K} mRNA levels in the intestine were compared between the groups given low or sufficient amount of calcium. The mRNA levels of CaBP-D_{28K} significantly increased about 2 times higher in low calcium and normal calcium replacement groups than in the control group (Fig. 3). Although plasma calcium levels were returned to normal levels after refeeding of 3.5% calcium diet, CaBP-D_{28K} mRNA were still maintained higher levels than its in the control group. This may be attributed to that homeostatic mechanisms for plasma calcium levels may be involved in raising CaBP-D_{28K} mRNA production. It is supposed that feeding of low calcium diet for 5 days may increase the production of 1,25$(OH)_2D_3$ by PTH, which in turn induces the increase in CaBP-D_{28K} mRNA levels in the intestine. PTH and 1,25$(OH)_2D_3$ may keep high after refeeding of 3.5% calcium diet, because more calcium is demanded for rebuilding bone.

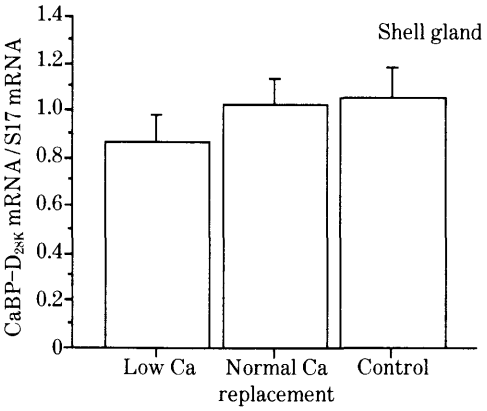

Fig. 4. Changes in mRNA levels of CaBP-D_{28K} in the shell gland of laying hens low calcium, normal calcium replacement and control groups. Same legend as in Fig. 2. Data from Northern blot analysis, which represent means±SEM (n=8).

In contrast to the intestine, mRNA levels of CaBP-D_{28K} in the shell gland after feeding of low calcium diet were not significantly different from those in control and normal calcium replacement groups (Fig. 4). Our previous study as well as others show that mRNA levels of CaBP-D_{28K} in the shell gland oscillate during ovulatory cycle (NYS *et al.*, 1989 ; STRIEM and BAR, 1991 ; IEDA *et al.*, 1995). It seems that shell gland has own regulatory mechanisms of CaBP-D_{28K} gene expression which is related to calcium deposition and gonadal sex steroid action. Since the gene expression of CaBP-D_{28K} mRNA was suppressed with poor egg shell quality by gonadal steroid inhibitor, gonadal steroid may be involved in an importantrole for CaBP-D_{28K} gene expression and calcium transport in the shell gland (IEDA *et al.*, 1998). NYS *et al.* (1992) suggests that low calcium diet (1.7% calcium) affect protein synthesis in the shell gland. Accordingly, low plasma calcium level may affect not mRNA levels of CaBP-D_{28K} but protein synthesis in the shell gland.

Acknowledgements

Authors wish to thank Dr. B.S. KOMM (Arizona University) and Dr. B. TRUEB (Eidgenossische Technische Hochschule) for the gift of CaBP-D_{28K} and S17 cDNAs, respectively. We also thank Dr. T. OKUMURA (Nagoya University), Mr. K. NODA and Mr. H. MIYAKAWA (Aichi Agricultural Research Center) for providing us of laying hens, Mr. M. HOSOYA (Hosoya Co., Ltd.) for providing us of calcium carbonate and Mr. KAWAMURA (Chubu Shiryo Co., Ltd.) for providing us of low calcium diet. We thank Nagoya University Radioisotope Center for the use of facilities for hybridization works and BAS-2000 Bio-Imaging analyzer. We appreciate Dr. T.I. KOIKE (University of Arkansas for Medical Sciences, USA) for helpful suggestions and English corrections.

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低カルシウム飼料給餌による卵殻質の低下と小腸及び卵殻腺におけるカルシウム結合タンパク質（CaBP-D_{28K}）遺伝子発現に与える影響

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本研究は短期間の低カルシウム飼料が，卵殻質（比重，卵殻比率，卵殻厚）と小腸及び卵殻腺カルシウム結合蛋白質（CaBP-D_{28K}）mRNA量に与える影響を検討した。5日間，低カルシウム飼料（0.5%カルシウム，低カルシウム給餌群）を産卵鶏に与えると，産卵鶏は卵殻質の悪い，壊れやすい卵を産むようになった。卵殻質の指標として卵殻比率（卵重に対する卵殻乾燥重量比率）を検討したところ，低カルシウム飼料を与えて2日目には有意に卵殻比率が減少した。他の比重，卵殻厚の指標も卵殻比率と同様な変化となった。低カルシウム飼料給餌後5日目には血中カルシウム濃度もコントロール群に比べ有意に減少した。5日間低カルシウム飼料を与えた後，5日間3.5%カルシウムを含む標準カルシウム飼料を与えたところ，卵殻質は改善され，血中カルシウム濃度もコントロール群と同じレベルにまで上昇した。低カルシウム飼料を5日間与えた後，次の排卵8時間後に小腸及び卵殻腺粘膜を採取し，CaBP-D_{28K} mRNA量をノーザンブロット法によって定量した。カルシウム再給餌群は低カルシウムを5日間給餌した後，5日間標準カルシウム飼料を給餌し，最終日の排卵後8時間にサンプリングを行った。小腸では低カルシウム給餌群とカルシウム再給餌群におけるCaBP-D_{28K} mRNA量はコントロール群に比べ約2倍有意に増加した。これは，低カルシウム飼料により血中カルシウムが減少した結果，パラサイロイドホルモン（PTH）が分泌され，腎臓における1,25$(OH)_2D_3$の合成を刺激したためと考えられる。カルシウム再給餌群では，血中カルシウム濃度はコントロール群と同じレベルに戻っていたにもかかわらず，CaBP-D_{28K} mRNA量が高かったのは骨の再生とカルシウム貯蔵により多くのカルシウムが必要だったためと考えられる。

しかし，卵殻腺CaBP-D_{28K} mRNA量は5日間の低カルシウム飼料を与えたにもかかわらず有意な変動はみられなかった。この結果より血中カルシウム低下による卵殻質低下は卵殻腺のCaBP-D_{28K} mRNA産生には影響しないと考えられた。

（家禽会誌，***36*** *: 295-303, 1999*）

キーワード：カルビンディン，低カルシウム飼料，遺伝子発現，卵殻形成