

# Effects of amylin on bone development and egg production in hens

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**ABSTRACT** Amylin is a member of the calcitonin family of hormones cosecreted with insulin from the pancreatic  $\beta$ -cells that can act as an osteoblast mitogen and as an inhibitor of bone resorption in mice and humans. The aim of this study was to investigate the role of amylin on bone formation and some egg parameters in hens. The study was performed in 60 hens aged 10 wk. Thirty hens constituting the treatment group were s.c. injected with amylin at a 75  $\mu$ g/kg dose every other day. The remaining hens were used as the control group. Five birds from the treatment and control groups were slaughtered at 14, 16, 18, and 20 wk of age and serum and bone parameters were compared between the

treatment and control groups. The remaining 20 hens were fed without any amylin injection until 35 wk. All hens at the end of the 35th week were slaughtered and then serum, bone, and egg parameters were assessed. In the treatment group, bone calcium levels increased, whereas serum calcium levels decreased. This dose of amylin also increased the cortical width of tibiotarsuses in hens. Eggshell thickness was found thicker in the treatment group than in the control group. Overall, the results of this study suggest that amylin may stimulate the bone and eggshell quality by increasing calcium uptake from the bloodstream and may influence the sustainability of yield in hens.

**Key words:** amylin, chicken, bone, calcium, osteoporosis

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## INTRODUCTION

Amylin is a 37-amino acid peptide hormone cosecreted with insulin from the  $\beta$ -cells of the pancreatic islets (Ogawa et al., 1990; Moore and Cooper, 1991; Koda et al., 1992; Cornish et al., 2001). Nearly 60 different effects have been reported in various experiments using amylin or pramlintide in a variety of species. Amylin has a physiological role in coordinating regulation of nutrient uptake via several mechanisms, including inhibition of food intake, slowing gastric emptying, regulation of acid and digestive enzyme secretion, and inhibition of nutrient-stimulated glucagon secretion (Young, 1997). Since its discovery, much interest has focused on its possible role in pathogenesis and treatment of diabetes. On the other hand, it is structurally related to calcitonin (13%), calcitonin gene-related peptide (43 to 49%), and adrenomedullin (20%). Thus, it also has effects on bone metabolism, being a potent osteoblast mitogen and acting like calcitonin as an inhibitor of bone resorption (Reid and Cornish, 1996). It produces hypocalcemia and this effect of amylin appears to be at least partly explicable by a direct effect on osteo-

clasts to inhibit bone resorption (Alam et al., 1993). In this way, amylin also increases bone calcium levels. The actions of amylin to stimulate bone formation and inhibit bone resorption make it an attractive candidate for the therapy of osteoporosis and repair of local bone defects (Cooper, 1994). In poultry generally, osteoporotic hens show evidence of widespread loss of structural bone throughout the skeleton. This loss starts when hens reach sexual maturity and continues throughout the laying period (Wilson et al., 1992). Egg-laying hens require substantial amounts of calcium to support eggshell formation. Over time, small amounts of structural bone are catabolized to provide some of the calcium required. The structural bone is not replaced as long as the hen remains in production, and as the hen ages, cortical thinning can result in osteoporosis (Whitehead and Fleming, 2000). We carried out this study to examine whether amylin has an effect on calcium metabolism, cortical width, bone density and quality, and sustainability of yield in hens for better and long-term productivity.

## MATERIALS AND METHODS

### *Housing and Feeding*

Sixty Super Nick hens aged 10 wk were used in this study. Thirty hens were used as the treatment group

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and the remaining hens were used as the control group. The treatment and control groups were housed in the same room but in different compartments having separate deep litter systems and had ad libitum access to feed and water in each group. The chickens were kept at 20 to 27°C and 12 h/d of lighting was applied during the rearing period. Lighting was increased 1 h/wk during the laying period and kept constant when 16 h of light daily was reached. The treatment and control groups were fed the same diet that was formulated according to the age of the birds and included the following: grower (10 to 12 wk), developer (13 to 18 wk), prelay (18 to 19 wk), and layer (>20 wk) (Table 1).

### Experimental Design and Laboratory Analyses

Thirty hens constituting the treatment group were s.c. injected with rat amylin (lot no. T01194X1; American Peptide Company, Sunnyvale, CA) at 75 µg/kg dose in the loose skin at the nape of the neck every other day. Five birds from the treatment and control groups were slaughtered at 14, 16, 18, and 20 wk of age, respectively, and their blood was taken to investigate blood parameters. Blood samples were taken by cutting the neck veins of chickens and spilling their blood into previously labeled sterile tubes. Serum was separated by centrifugation at  $3,000 \times g$  for 10 min and stored at -20°C until the time of analysis. The right tibiotarsuses from each of the slaughtered hens were excised and defleshed without boiling. The tibias were individually sealed in a plastic bag to minimize moisture loss and stored at -20°C until analysis. After slaughtering 40 hens, the remaining 10 hens in each group were fed until 35 wk without any amylin injection. All remaining hens at the end of the 35th week were slaughtered and then parameters of serum and bone were evaluated. The weight of each bird was recorded at the start of the experiment and slaughterings.

Serum samples were immediately analyzed for calcium using an enzymatic calorimetric kit (catalog no. C 503-480; Teco Diagnostics, Anaheim, CA; Cali, 1972), following the directions of the manufacturer. Concentrations of serum calcium were determined by measuring the color change using a spectrophotometer (Schimadzu UV 1601, Kyoto, Japan).

### Bone Quality Analyses

The right tibiotarsuses were marked in the middle region (diaphysis). At this region, 3 points were determined and bone density was measured in these points using quantitative computed tomography (QCT; Select Marconi/SP-16 multi slayt, thickness: 1.5 mm, voltage: 120 kV, mAS: 84, Brussels, Belgium) and bone density was calculated to be the average of these points. Cortical width of tibiotarsus was also calculated from the outer to the inner side in 3 different portions (right, middle, and left) by QCT in the same slice as bone density was calculated. Then, finally, the cortical width was calculated as the mean value.

To determine the bone ash and calcium levels, tibiotarsus was boiled in HCl (27.66%) for 5 min and bone marrow and surrounding tissue were completely removed. Tibiotarsuses were soaked in a Soxhlet extractor (Gerhardt/Soxtherm Automatic, Königswinter, Germany) for 24 h (removing water and polar lipids). Bones were then further extracted in anhydrous ether for 24 h (removing nonpolar lipids). After the second extraction, bones were dried at room temperature for 24 h. After that, they were weighed to obtain a dry weight and ashed in a muffle furnace (Gallenkamp FR 612, Leicestershire, UK) overnight at 600°C in porcelain crucibles and weighed again. The percentage of tibia ash was calculated by dividing tibia ash weights by tibia dry weight and multiplying by 100 (Hall et al., 2003). One hundred milligrams of bone ash was dissolved in 2 mL of 37% HCl and diluted with deionized water and then the bone calcium levels were detected with the glyoxal bis method using a spectrophotometer (Kashiwa, 1970).

### Egg Parameters

The first egg was obtained within 19 wk of age in the treatment group and 20 wk of age in the control group. After slaughtering 40 hens, the remaining 20 birds were used to determine the egg parameters of the chickens. The eggs obtained from the remaining chickens in both groups were collected daily during the research period of 15 wk from 20 to 35 wk of age. Egg production records were taken daily in each group and expressed weekly as eggs produced per hen per day. Eight to 10 freshly laid eggs were collected from each group every

**Table 1.** Diet schedule and calculated nutrient analyses

Analyses	Grower	Developer	Prelay	Layer
CP (%)	18.06	15.51	16.29	18.03
ME (kcal/kg)	2.53	2.58	2.60	2.70
Calcium (%)	1.00	1.00	2.50	3.70
Phosphorus (%)	0.55	0.53	0.65	0.68
Linoleic acid (%)	1.20	1.20	1.20	1.00
Methionine (%)	0.40	0.34	0.39	0.30
Lysine (%)	0.75	0.70	0.65	0.70

other week to determine egg weight and shell thickness and were measured within 24 h. In total, 130 eggs (65 eggs per group) were collected at 22, 24, 26, 28, 30, 32, and 34 wk of age and were weighed after being stored at room temperature for 24 h. Shell thickness (without inner and outer shell membranes; membranes were removed manually) was measured at 3 areas (broad end, middle portion, and narrow end of the shell) by using a micrometer (Mitutuyo Corporation, 0.01 to 20 mm, Kawasaki, Japan).

### Statistical Analyses

The SPSS statistical software 13.00 was used for statistical analysis (SPSS Inc., Chicago, IL). Statistical significance for serum calcium, bone ash and calcium, cortical width, bone density, and BW were evaluated by the Mann-Whitney test when comparing medians. In the comparison of means (eggshell thickness and egg weight), statistical significance was evaluated by Student's *t*-test. For all of the tests in all of the studies, *P*-values <0.05 were considered significant. Data are presented as means  $\pm$  SEM.

## RESULTS AND DISCUSSION

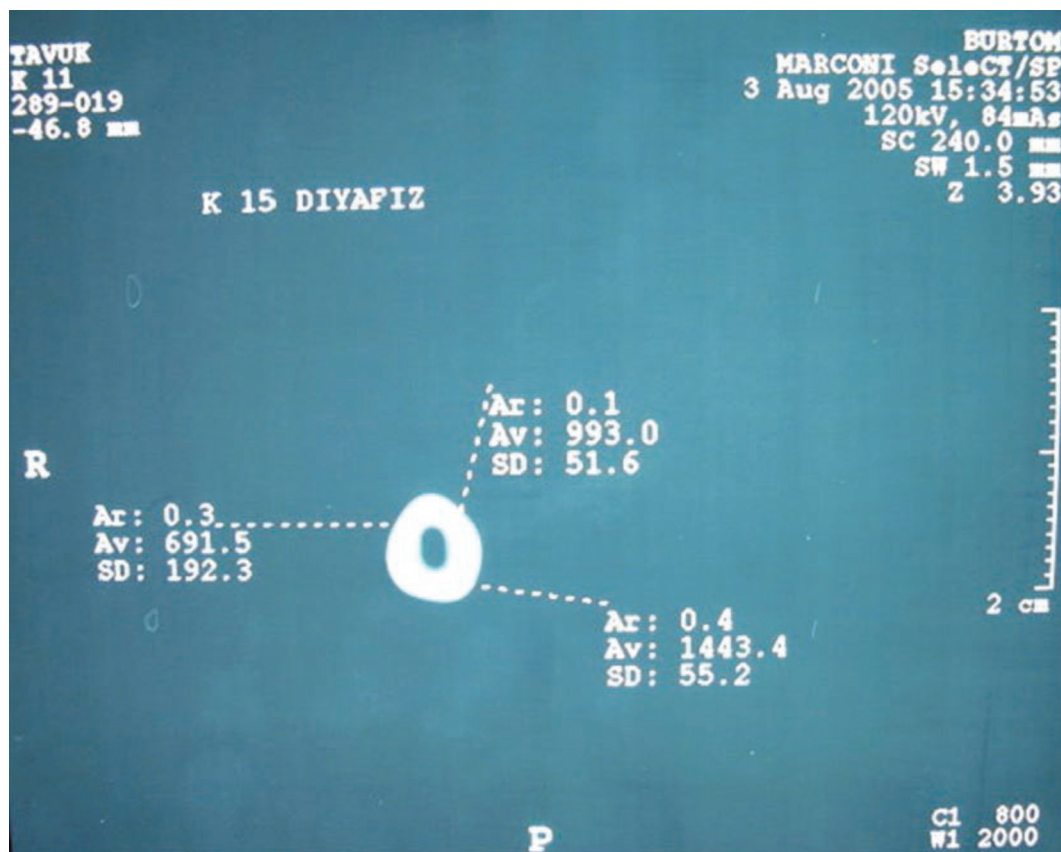
In this study, we sought to determine the effects of local amylin administration on bone development, calcium levels, and egg production in hens. Serum and bone calcium levels were determined spectrophotometrically, whereas bone density and cortical width of tibiotarsus were measured using QCT.

Serum calcium levels for hens aged between 14 and 20 wk are shown in Table 2. Serum calcium concentrations were lower in the treatment groups than in the control groups at 14 ( $P < 0.05$ ), 16, 18, and 20 (for each  $P < 0.01$ ) wk of age. Amylin injection decreased serum concentration of calcium in our study. Several studies have described a calcium-lowering effect of amylinomimetic agents, including human (Datta et al., 1989; MacIntyre, 1989; Zaidi et al., 1990a,b) and rat amylin (Fürsinn et al., 1993; Young et al., 1993, 1996). However, information about the effects of amylin in different animal models is limited with very little literature and no literature in chickens. Min et al. (1999) demonstrated that amylin was a hypocalcemic agent in lactating goats. The present results were consistent with those of previous studies with rodents, humans, and small ruminants and provided the first evidence that rat amylin is also a potent hypocalcemic agent in hens. Such effects of amylin in this study are to be expected because of it being a member of the calcitonin family. On the other hand, it is unlikely that amylin reduces circulating concentrations of calcium via enhanced renal excretion because recent studies showed that s.c. injection of amylin at a physiological dose reduced urinary excretion of calcium in conscious rats (Blakely et al., 1997). Similarly, Miles et al. (1994) conclude that amylin lowers serum calcium and increases the renal excretion of calcium indepen-

**Table 2.** Bone and serum evaluations of hens at 14 to 20 wk of age<sup>1</sup>

Parameters	14 wk		16 wk		18 wk		20 wk	
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
Serum calcium (mg/dL)	13.65 $\pm$ 0.59	11.32 $\pm$ 0.43*	12.39 $\pm$ 0.37	10.12 $\pm$ 0.27**	10.66 $\pm$ 0.18	9.62 $\pm$ 0.13**	12.51 $\pm$ 0.23	10.54 $\pm$ 0.14**
Bone ash (%)	58.71 $\pm$ 0.55	59.80 $\pm$ 0.23	58.22	58.64 $\pm$ 0.37	59.12	60.15 $\pm$ 0.47	58.87	60.61 $\pm$ 0.79
Bone calcium (%)	18.18 $\pm$ 0.61	21.31 $\pm$ 0.62*	18.50 $\pm$ 0.33	19.53 $\pm$ 0.34*	17.85 $\pm$ 0.78	20.38 $\pm$ 0.94*	19.27 $\pm$ 0.51	23.20 $\pm$ 0.87**
Bone density (g/cm <sup>2</sup> )	1,566 $\pm$ 63	1,603 $\pm$ 91	1,228 $\pm$ 45	1,357 $\pm$ 44	1,392 $\pm$ 76	1,538 $\pm$ 67	1,560 $\pm$ 29	1,612 $\pm$ 112
Cortical width (mm)	0.78 $\pm$ 0.19	0.67 $\pm$ 0.19	0.78 $\pm$ 0.03	0.87 $\pm$ 0.03*	1.01 $\pm$ 0.03	1.60 $\pm$ 0.03**	1.11 $\pm$ 0.01	1.66 $\pm$ 0.01**

<sup>1</sup>Table indicates average of all parameters in different slaughtering times and groups with  $\pm$  SEM (\* $P < 0.05$ ; \*\* $P < 0.01$ ).



**Figure 1.** Measurement of bone density of tibiotarsus (diaphysis) by quantitative computed tomography in the control group. The three points on which bone density was measured are indicated by dashed lines.

dent of calcitonin in the conscious dog. In contrast, there is increasing evidence from rodents that hypocalcemic actions of amylin are due to a marked reduction of bone resorption by osteoclasts (Datta et al., 1989; Alam et al., 1993).

In contrast, bone calcium levels were higher in the treatment groups than in the control groups in 14, 16, and 18 wk ( $P < 0.05$ ) and 20 wk ( $P < 0.01$ ). In the 16th and 18th weeks, bone calcium levels in the control and treatment groups decreased (Table 2). This decrease may be related to the adaptation to the egg laying cycle. The present study also showed that amylin injection increased bone calcium levels and this finding is in agreement with that reported by Cornish et al. (1995). The calcitonin family of hormones, of which amylin is a member, stimulates the bones to draw calcium from the bloodstream and inhibits bone resorption and, consistent with this, the observed high bone calcium levels

in the amylin-administered group may be due to the increased calcium uptake by bones or the inhibition of bone resorption, or both.

Cortical widths of tibiotarsuses were higher in the treatment groups than in the control groups at 16 ( $P < 0.05$ ), 18, and 20 ( $P < 0.01$ ) wk of age (Table 2). Similarly, a further effect of systemic administration of amylin is an increase in tibiotarsal cortical width. In a previous study, systemic infusion of amylin (1–8) increased tibial cortical width by 8% (Cornish et al., 2000). In our study, we have used another derivative of amylin, intact amylin peptide, in the same dose and found a significant increase in the tibiotarsal cortical width of hens in 16, 18, and 20 wk. Similarly, Cornish et al. (1998a,b) also reported that intact amylin increased tibial cortical width in adult male mice.

**Table 3.** Egg evaluations of hens at 20 to 35 wk of age ( $n = 65$ )<sup>1</sup>

Egg parameters	Control	Treatment
Egg weight (g/hen)	54.49 ± 0.57	55.97 ± 0.86
Eggshell thickness (mm × 100)	35.29 ± 0.30	36.69 ± 0.45*
Egg production (% hen-day)	82.1 ± 0.35	85.4 ± 0.62

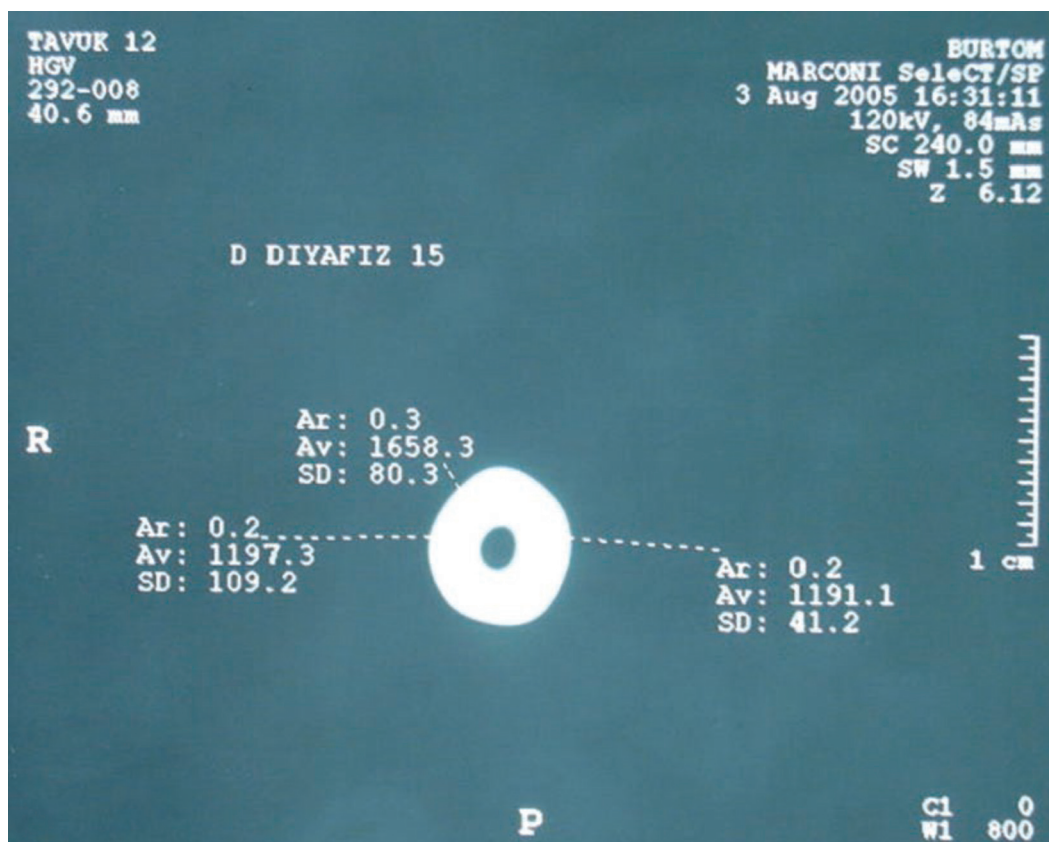
<sup>1</sup>Table indicates average of egg parameters in the control and treatment groups with ± SEM (\* $P < 0.05$ ).

**Table 4.** Bone and serum evaluations of hens at 35 wk of age<sup>1</sup>

Parameters	35 wk	
	Control	Treatment
Serum calcium (mg/dL)	12.51 ± 0.23	12.64 ± 0.27
Bone ash (%)	60.33 ± 0.37	60.83 ± 0.24
Bone calcium (%)	20.17 ± 0.22	20.24 ± 0.37
Bone density (g/cm <sup>2</sup> )	1,510 ± 22	1,560 ± 61
Cortical width (mm)	1.38 ± 0.03	1.93 ± 0.03*

<sup>1</sup>Table indicates average of all parameters at 35 wk of age in the control and treatment groups with ± SEM (\* $P < 0.05$ ).





**Figure 2.** Measurement of bone density of tibiotarsus (diaphysis) by quantitative computed tomography in the treatment group. The three points on which bone density was measured are indicated by dashed lines.

There were no significant differences regarding bone density and ash between the control and treatment groups. In the 16th and 18th weeks, bone density values in the control and treatment groups were decreased slightly such as bone calcium levels (Table 2). Several studies reported that QCT is an effective technique to measure bone mineral density and cortical thinning in hens (Whitehead and Fleming, 2000; Korver et al., 2004). We also measured bone density with QCT (Figure 1 and 2). Increased bone calcium levels without any change in bone density may be concluded as a result of increasing cortical width parallel to bone calcium levels.

Body weights were not affected by the local injection of amylin. The 11-, 14-, 16-, 18-, 20-, and 35-wk BW of hens in the control and treatment groups were 870, 1,161, 1,286, 1,410, 1,660, and 1,824 g and 882, 1,177, 1,296, 1,411, 1,791, and 1,857 g, respectively, and not statistically different. This is in support with results obtained by Cornish et al. (2000).

Egg numbers and egg weights of hens fed from the 20th through 35th week of age were not statistically significant. There were, however, significant differences in eggshell thickness when comparing the control and treatment groups ( $P < 0.05$ ; Table 3). No significant difference was observed on bone and serum parameters of the 35th week except cortical width of tibiotarsus ( $P < 0.05$ ; Table 4).

In conclusion, we observed an increase in bone calcium and cortical bone width but not in bone density after amylin treatment. In addition, an increase in eggshell thickness (although very minor) was observed. The rationale behind the hypothesis is that because amylin is a mitogen of osteoblasts causing bone formation and reduced bone resorption in mammals, it may be used to reduce osteoporosis and improve eggshell quality in layers.

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