EFFECT OF SUPPLEMENTED PROTEIN AND Ca NUTRITION ON FLUORIDE-INDUCED DISTURBANCE OF RIB COL1A1 GENE EXPRESSION IN RABBITS

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SUMMARY: The effect of high fluoride (F) and supplemented protein and calcium nutrition on rib COL1A1 gene expression was explored in 32 healthy one-month-old New Zealand rabbits divided randomly into four equal groups of 8 in a 1:1 female/ male ratio. With one group left untreated as a control with a marginally low protein (Pr, 8.58%) and low calcium (Ca, 0.49%) diet, the other three groups were given, respectively, for up to 120 days: (1) high fluoride (442 mg NaF [=200 mg F^-]/kg dry feed plus the same low Pr and low Ca control diet, (2) high F plus high Pr (18.41%) and low Ca (0.46%) in their diet, and (3) high F plus low Pr (8.58%) and high Ca (2.23%). After 60 and 120 days, two male and two female rabbits were randomly selected from each group and sacrificed. With the help of quantitative real-time polymerase chain reaction (QRT-PCR) the rib COL1A1 gene expression levels of the four groups were quantified. The rib COL1A1 gene expression level in the high F group *decreased* compared to the control, whereas in the high F plus high Pr and the high F plus high Ca groups 2 and 3, this gene expression *increased* compared with the high F group 1 with the low Pr and low Ca.

Keywords: COL1A1 gene; Collagen gene expression; Polymerase chain reaction (PCR); Rabbit rib collagen; Real-time PCR; Supplemented nutrition.

INTRODUCTION

Collagen is a major component of bone and cartilage. Type I collagen gene (COL1A1), which encodes two α1(I) polypeptide chains assembled into a collagen molecule, is the main collagen gene in bone.[1] Susheela was one of the first to observe that collagen protein is damaged by excessive fluoride (F) ingestion.[2] This finding provided an important breakthrough in our concept of fluorosis and led to significant advances not only in fluoride research but also in our understanding of many other pathological phenomena, especially those related to collagen diseases.[3]In recent years, a number of reports have shown that F affects collagen metabolism of cartilage and bone.[4-6]

The carboxyterminal cross-linked telopeptide of type I collagen (ICTP), a marker of bone resorption, has been directly linked to circulating thyroid hormone and thyroid stimulating hormone (TSH). The latter is identified as a single molecular switch in the independent control of both bone formation and resorption, and could inhibit type I collagen gene expression.[7-9] Some reports have also shown that F behaves as a TSH analogue and may influence expression of collagen gene by interfering with thyroid hormone metabolism.[8-13]

In our previous reports, we have shown that industrial fluoride pollution can increase the expression levels of the COL2A1 and COL1A2 genes in Inner Mongolia cashmere goats[14,15] when the soluble F content of herbage was 30-80

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mg/kg in the dry grass seasons but the increase was less when it was lower in green grass seasons.[16-18] However, the F dosage researchers often use in experimental laboratory studies is higher than the soluble F content of herbage in an industrial fluoride polluted area.

Are the effects different between high and low doses of F? In our previous study we found that supplying protein-enriched feed during dry grass seasons could relieve excessive wear on the teeth. We also noted that deficiencies of certain nutrient factors such as protein (Pr) and calcium (Ca) are involved in skeletal fluorosis.[19] We therefore ask: Are there any effects of protein or calcium on type I collagen gene expression? Focusing on this question, we conducted the present investigation of the effect of high F and supplemented Pr and Ca nutrition on rib COL1A1 gene expression in rabbits.

MATERIALS AND METHODS

Experimental materials: Thirty-two one-month old New Zealand rabbits (16 males and 16 females) with an average weight of 1.07 kg were obtained from Rabbit Breeding Farm of Taigu County for use in this study. For the high F exposed groups, 442 mg of NaF (= 200 mg F^-) was added per kg of dry diet to give an estimated daily mean intake of 20 mg F/rabbit, based on an average consumption of 100 g of ration/day. Table 1 lists the F, protein (Pr), and calcium (Ca) content in the feed for each group of rabbits.

Table 1. F^- dosage (mg/kg dry diet), protein（Pr), and Ca levels (%) in the diet of the rabbits[a]

	"Control" (LPr+LCa)	High F (HiF+LPr+LCa)	High F plus high Pr (HiF+ HiPr+LCa)	High F plus high Ca (HiF+LPr+HiCa)
F in diet	20.1	200	200	200
Pr in diet	8.58%	8.58%	18.41%	8.58%
Ca in diet	0.49%	0.49%	0.46%	2.23%

[a]F^- is from NaF; Pr in diet is soybean; Calcium in diet is $Ca_3(PO_4)_2$ and $CaCO_3$ (Ca:P = 2:1). (A standard rabbit diet contains 12-16% protein and 1% Ca.)

Animal test model: The 32 one-month old New Zealand rabbits were randomly divided into four equal groups (1:1 female:male) and were maintained on the diets shown in Table 1 with unrestricted access to F-free drinking water under standard conditions of temperature (22–25ºC), 12/12-hr light/dark cycle, ventilation, and hygiene.

Total RNA extraction and analysis: At days 60 and 120, two male and two female rabbits were randomly selected from each group and sacrificed by jugular vein exsanguination. Rib tissues were quickly collected and stored in liquid nitrogen after being washed three times with saline solution. Total cellular RNA was extracted from the rib tissue by a modified technique using Trizol Reagent (Invitrogen, USA) and XHF-1 High-speed Dispersator (Scientz, China). The RNA extracts were treated with RNase-free DNase I to remove contaminating DNA, quantified on a spectrophotometer (Eppendorf, Germany), and stored at –80ºC.

Quantitative real-time polymerase chain reaction (QRT-PCR): Two pairs of specific primers (Table 2) were designed according to the alignments of the published cDNA sequences of β-actin and COL1A1 genes in rabbit and humans. These two pairs of primers were tested for their specificity by conventional

reverse transcription polymerase chain reaction (RT-PCR) before being used for the QRT-PCR studies.

Table 2. Primer sequences with their corresponding PCR product size and position

Gene	Primers (5'→3')	Primer locations	Product (base pairs)	Gene bank Accession No.
β-actin	CGTGCGGGACATCAAGGA AGGAAGGAGGGCTGGAACA	244－420	177	AF309819
COL1A1	TGCCATCAAAGTCTTCTGC AATCCATCGGTCATGCTCT	3901-4043	143	NM_000088

The expression level of COL1A1 gene was quantified by real-time amplification of COL1A1 gene and the house-keeping gene β-actin as control from the above RNA preparation using the Mx3000P™ QRT-PCR system (Stratagene, USA) and One-Step SYBR® QRT-PCR kit (Takara, China). The QRT-PCR was performed in a 20 μL reaction mixture. Relative quantification of QRT-PCR product was performed using the comparative $\Delta\Delta C_T$ method and SYBR green fluorescent labeling.[20] Thermocycling conditions were as follows: an initial reverse transcription step of 15 min at 42ºC and 40 cycles at 95ºC for 5 sec, 60ºC for 20 sec, and 72ºC for 6 sec. The reaction was then subjected to a melting protocol from 55ºC to 95ºC with a 0.2ºC increment and 1 sec holding at each increment to check the specificity of the amplified products.

The representative QRT-PCR products were purified with a DNA wizard cleanup kit (Promega, USA) and sequenced using an ABI Prism 377 automated DNA sequencer (Applied Biosystems, USA).

RESULTS

Conventional RT-PCR for COL1A1 and β-actin: Amplification products of the expected size were obtained from each pair of primers (Figure 1). Sequencing of the purified products showed that the β-actin gene fragment was 98% homologous with the rabbit β-actin gene, and the COL1A1 gene fragment was 97% homologous with the human COL1A1 gene.

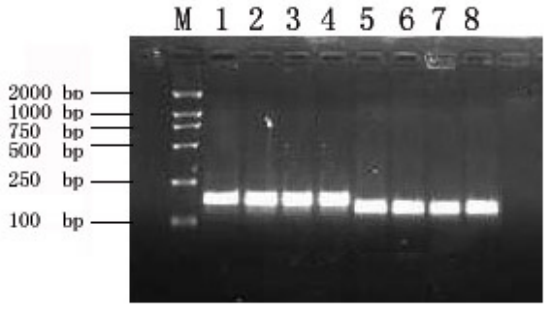

Figure 1. Electrophoresis of conventional RT-PCR products of β-actin and COL1A1 genes from control and experimental rabbit ribs. M: molecular weight markers (DL2000); Lanes 1–4 show β-actin (177 base pairs) of four groups, respectively; Lanes 5–8 show COL1A1 (143 base pairs) of four groups, respectively.

Quantification of COL1A1 gene expression: The standard curves obtained by correlation of the Ct values (threshold cycles) with the dilution series of the COL1A1 and β-actin genes exhibited a relatively low intra-assay variation (Figures 2 and 3). The amplification efficiencies of the COL1A1 and β-actin genes were 91.3% and 96.2%, respectively.

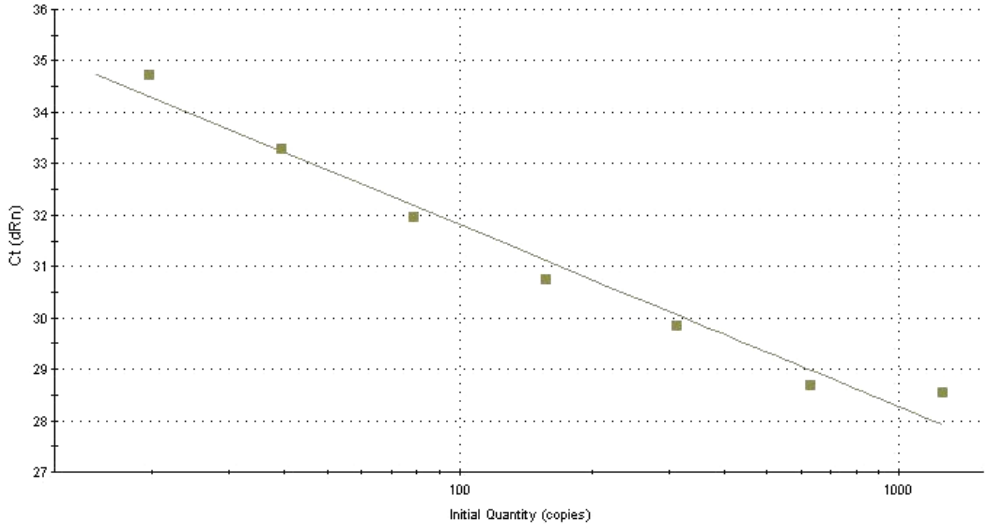

Figure 2. Standard curve for COL1A1 gene obtained by the correlation of the Ct values with the dilution series of the COL1A1 gene. Standard Curve: Logfit values; SYBR Standards, RSq:0.973; SYBR, Y=-3.551*LOG(X) + 38.92, Eff.=91.3%

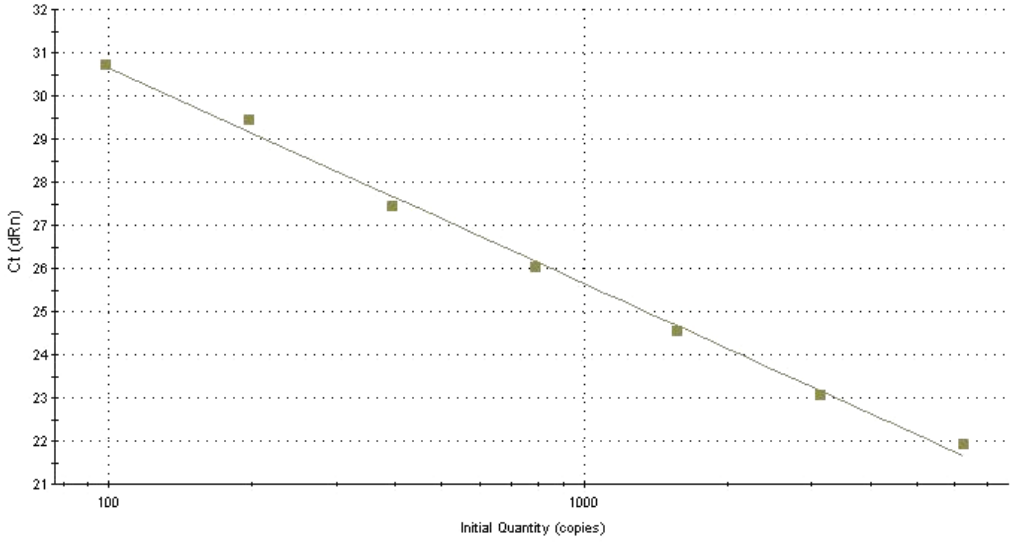

Figure 3. Standard curve for β-actin gene obtained by the correlation of the Ct values with the dilution series of the β-actin gene. Standard Curve: Logfit values; SYBR Standards, RSq:0.996; SYBR, Y=-4.9991*LOG(X) + 40.63, Eff.=96.2%

Specificity of QRT-PCR amplification was verified by melting curve profile analysis (Figure 4).

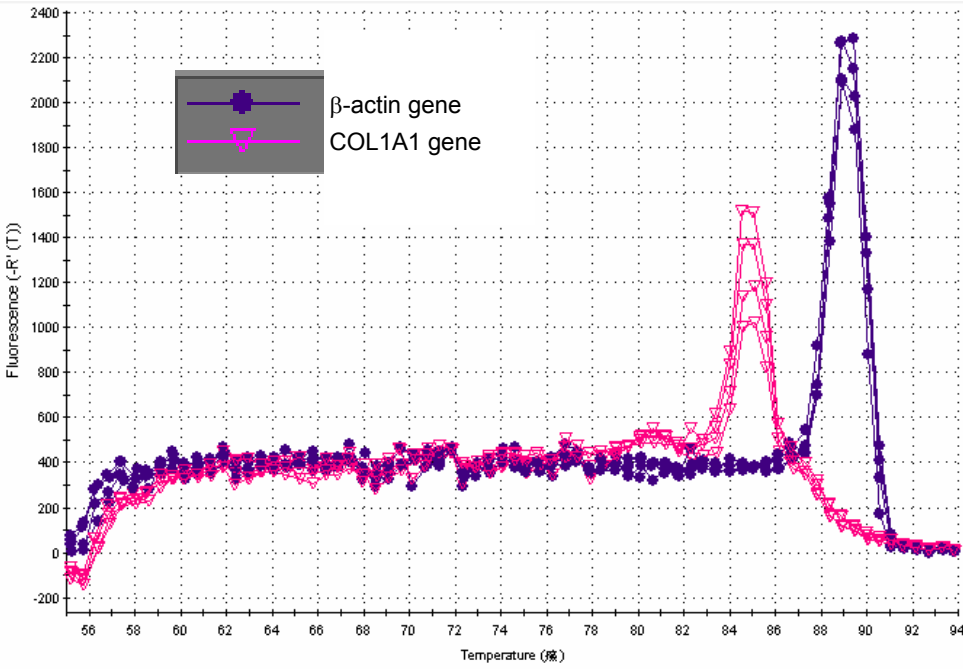

Figure 4. Melting curves for β-actin and COL1A1 genes. The single peak at 85°C for COL1A1 (first peak) and β-actin at 89°C (second peak) indicates that no other transcripts were amplified in the QRT-PCR.

The expression level of COL1A1 gene in the HiF group is reported in Table 3. The results showed that at days 60 and 120, the expression level of COL1A1 gene in the HiF group decreased by 46% and 78% compared to the control.

Table 3. Fold change of COL1A1 gene expression level relative to control (Mean±SEM)

Group	ΔC_T (Avg. COL1A C_T – Avg. β-actin C_T)		$\Delta\Delta C_T$ (ΔC_T HiF–ΔC_T control)		Fold difference in COL1A1 relative to control ($2^{-\Delta\Delta CT}$)	
	Day 60	Day 120	Day 60	Day 120	Day 60	Day 120
Control	2.36±0.89	–0.29±0.53	0.00±0.89	0.00±0.53	1	1
HiF	3.25±0.52	1.89±0.28	0.89±0.52	2.18±0.28	0.54	0.22

The expression levels of COL1A1 gene calculated by the $\Delta\Delta C_T$ method in the HiF+HiPr and HiF+HiCa groups are reported in Table 4. The results showed that at days 60 and 120 the COL1A1 gene expression level increased by 39% and 82% in the HiF+HiPr group and by 15% and 42% in the HiF+HiCa group in comparison to the HiF group.

Table 4. Fold change of COL1A1 gene expression level relative to HiF group (Mean±SEM)

Group	ΔC_T (Avg. COL1A C_T – Avg. β -actin C_T)		$\Delta\Delta C_T$ (ΔC_T HiF+HiPr – ΔC_T HiF; ΔC_T HiF+HiCa – ΔC_T HiF)		Fold difference in COL1A1 relative to HiF ($2^{-\Delta\Delta CT}$)	
	Day 60	Day 120	Day 60	Day 120	Day 60	Day 120
HiF	3.25±0.52	1.89±0.28	0.00±0.52	0.00±0.28	1	1
HiF+HiPr	2.77±0.35	1.02±0.59	–0.48±0.35	–0.87±0.59	1.39	1.82
HiF+HiCa	3.04±0.33	1.39±0.57	–0.21±0.33	–0.5±0.57	1.15	1.42

DISCUSSION

Effect of high fluoride on the COL1A1 gene expression: The type I collagen gene (COL1A1), which is responsible for bone stability and cell biological functions, encodes two α1(I) polypeptide chains that are assembled into a collagen molecule.[21,22] A number of reports indicate that high dosage of F affects collagen metabolism of cartilage and bone[23,24] and leads to a decrease of type I collagen gene expression.[25,26]

Among body tissues, the thyroid gland appears to be the most sensitive to F[27-29], and thyroid hormone (TH) secreted by the thyroid gland plays an important role in bone growth process.[30,31] A number of studies have shown that protein or Ca deficiency induces an increase in serum triiodothyronine (T_3).[32-34] Increased serum T3 secretion induced by excessive fluoride[35,36] further inhibits osteoblast formation by the action of the thyroid hormone receptors (TRs),[37,38] which may be responsible for the decrease in collagen genes' expression.

The present study showed that high F down-regulates the expression level of the COL1A1 gene by 46% on the 60th and 78% on the 120th day in the HiF group compared with the control. This finding is consistent with what has been found in humans and rats,[25,26] but it is contrary to our previous studies in goats.[14,15] The reason for the fluoride-induced decrease in collagen expression in the present study appears to be connected with the fact that TSH, as a single molecular switch, can inhibit type I collagen gene expression.[8] And, at high levels, F, as a TSH analogue, inhibits adenyl cyclase activity, thereby ultimately affecting the intracellular levels of cAMP.[12,13] Thus, down-regulation of type I collagen gene expression in the present study may result from high F ingestion. Maciejewska found that a low F level could stimulate expression of type I collagen formation in dentin and high F had the opposite effect,[39] which supports our results in goats with low F and rabbits with high F.

In contrast to our previous studies in goats,[14,15] the gene expression results of the present study differ markedly. Here the F level was 200 mg/kg for the laboratory study, whereas in the industrial fluoride pollution area the soluble F content of herbage was lower at 30-80 mg/kg (dust F was dominant) in the dry grass seasons, and it was even less in green grass seasons (herbage grew rapidly and dust F was washed away frequently by rain). Thus there is an important dosage difference in our two studies with different animals. In addition, there are three other aspects to consider: First, the nutrition levels were different in the two experiments. In the present study, rabbits were fed protein (Pr) and Ca deficient grain forage. However, as just noted, the nutrition level of the diets in our previous study on goats in green grass season was higher than in dry grass season. Second, the manner of F ingestion was different in the two experiments. The rabbits were fed in the laboratory, and the F ingestion was continuous, but the goats were pastured in an industrial fluoride pollution area, and F ingestion was discontinuous. Finally, the rabbits were caged with relatively less activity, whereas the pastured goats move to graze here and there, which increased their bone mass.

Effect of supplemented nutrition on the COL1A1 gene expression: In earlier studies, we found that, besides the adverse effects of high F on tooth quality, sub-optimal nutrition, especially protein (Pr) deficiency, has a negative influence on tooth development and enhances the toxic effects of F.[5] Pr deficiency, by increasing the skeletal deposition and toxicity of F, induces loose and non-uniform collagen fibers in bone.[40,41] Here we have observed that increased Pr in the diet can enhance the COL1A1 gene expression level, thereby suggesting that supplemented Pr can help relieve F toxicity. Furthermore, calcium is the major component of bone and teeth and is another important nutritional factor besides protein. Ca deficiency is known to cause osteomalacia and osteoporosis.[42-44] This study showed that increased Ca in the diet increases the COL1A1 gene expression level, thereby indicating that adequate Ca nutrition can also provide protection against F toxicity.

In conclusion, we suggest that a high F content in a rabbit diet (200 mg/kg) can decrease the COL1A1 gene expression level, but supplemented nutrition with Pr or Ca can effectively increase it.

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