Linoleate Impairs Collagen Synthesis in Primary Cultures of Avian Chondrocytes

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Abstract. **The effects of supplemental fatty acids, vitamin E (VIT E), and iron-induced oxidative stress on collagen synthesis, cellular injury, and lipid peroxidation were evaluated in primary cultures of avian epiphyseal chondrocytes. The treatments included oleic and linoleic acids (0 or 50 μ*M*) complexed with BSA and *dl*-α-tocopheryl acetate (VIT E at 0 or 100 μ*M*). After 14 days of preculture, the chondrocytes were enriched with fatty acids for 8 days then cultured with VIT E for 2 days. The chondrocytes were then treated with ferrous sulfate (0 or 20 μ*M*) for 24 hr to induce oxidative stress. Collagen synthesis was the lowest and the activity of lactate dehydrogenase (LDH) was the highest in chondrocyte cultures treated with 50 μ*M* linoleic acid and 0 VIT E. In contrast, VIT E supplemented at 100 μ*M* partially restored collagen synthesis in the chondrocytes enriched with linoleic acid and lowered LDH activity in the media. The iron oxidative inducer significantly increased the values of thiobarbituric acid-reactive substances (TBARS) in the culture medium. The data showed that linoleic acid impaired chondrocyte cell function and caused cellular injury but that VIT E reversed these effects. Results from a previous study demonstrated that VIT E stimulated bone formation in chicks fed unsaturated fat, and the present findings in cultures of epiphyseal chondrocytes suggest that VIT E is important for chondrocyte function in the presence of polyunsaturated fatty acids. VIT E appears to be beneficial for growth cartilage biology and in optimizing bone growth.** [P.S.E.B.M. 1996, Vol 212]

The epiphyseal growth plate cartilage is responsible for longitudinal bone growth and endochondral ossification (1, 2). During endochondral bone formation, chondrocytes proceed through proliferation, differentiation, and hypertrophy during their life (3, 4). Chondrocytes synthesize varying amounts of collagens (types II, VI, IX, X, and XI) and proteoglycans for cartilage matrix formation (5–7). Certain nutrients appear to influence the activity of chondrocytes. A study with human cartilage organ cultures showed that supplemental vitamin E (VIT E) resulted in a significant increase in sulfated proteoglycan synthesis (8). Ascorbic acid was found to stimulate collagen synthesis and calcium deposition in avian chondrocyte cultures (9), and retinoic acid was reported to enhance type X collagen expression *in vitro* (10).

Epiphyseal growth cartilage of young animals and humans exhibits an unusual fatty acid composition. Adkisson *et al.* (11) first reported that normal epiphyseal cartilage contains a low concentration of n-6 polyunsaturated fatty acids (PUFA) but a high amount of Mead acid (20:3n-9) relative to other tissues. Xu *et al.* (12) confirmed this unique fatty acid profile in growth plate cartilage and further demonstrated that the content of 20:3n-9 in chondrocytes and matrix vesicles is not affected by dietary linoleate.

The role of VIT E in protecting cellular peroxidation in membrane-bound lipids has been well documented (13). Recent investigations support a role for

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antioxidants in normal bone metabolism. Ebina *et al.* (14) showed that VIT E supplementation increased trabecular bone volume, decreased osteoid volume, and decreased osteoclast number and percentage bone surface in rats supplemented with VIT E. Matsumoto *et al.* (15) found that the activities of superoxide dismutase (SOD) and catalase were higher in the preminerализed zone of growth cartilage but the enzyme activities were lower in the mineralized zone. Therefore, the report from Matsumoto *et al.* (15) suggests that the mineralized region of growth cartilage may have a limited capacity for handling lipid peroxidation since SOD and catalase activities are low in this region. Further, enrichment of cartilage with dietary PUFA may be detrimental to cartilage biology and bone growth. Recently, Watkins *et al.* (16) reported that chicks fed saturated fat (butter oil) had increased cortical bone formation rates compared with chicks fed highly unsaturated oils (soybean oil). Xu *et al.* (17) reported that VIT E enhanced the development of the mineralized zone in growth plate cartilage and stimulated formation and mineralization of trabecular bone *in vivo*. These findings suggest that VIT E plays an important role in chondrocyte biology and in bone modeling of the young.

The present study was conducted to describe the effect of fatty acid enrichment on chondrocyte function to understand how VIT E improved growth cartilage morphometry and trabecular bone formation in animals fed unsaturated fat (17). The objectives of this study were to determine the effects of supplementing fatty acids and VIT E in primary cultures of epiphyseal chondrocytes on collagen synthesis, cell membrane damage, and lipid peroxidation. The iron oxidative inducer (20 μM $FeSO_4 \cdot 7H_2O$ + 100 μM ascorbate) employed for this work has been used extensively to induce lipid peroxidation *in vitro* for studies on oxidative stress and antioxidant effects in biological systems (18–20). Ascorbate converts Fe^{3+} to the more reactive Fe^{2+} for initiating lipid peroxidation (21). Measurement of LDH activity in the medium was done to evaluate cell damage (22).

Materials and Methods

Chemicals and Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco BRL (Grand Island, NY) for cell culture study. Cell culture dishes were purchased from Corning Costar Co. (Cambridge, MA). *L*-Ascorbate, bovine serum albumin (BSA, essentially fatty acid free), butylated hydroxytoluene (BHT), calf thymus DNA (Type I), high purity collagenase (EC 3.4.24.3, type VII), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), tannic acid, *dl*-α-tocopheryl acetate, trichloroacetic acid (TCA), trypsin (EC 3.4.21.4, type III-S), Triton X-100, LDH assay kits, and other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Crude collagenase (CLS II) was obtained from Worthington Biochemical (Freehold, NJ). Oleic acid (18:1n-9), linoleic acid (18:2n-6), and the standard mixtures of fatty acid methyl esters were purchased from Nu-Chek-Prep (Elysian, MN). 1,1,3,3,-Tetraethoxypropane and thiobarbituric acid were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). [2,3,4,5-^{3}H]Proline (107 Ci/mmol) was purchased from Amersham Life Science (Arlington Heights, IL) and Hoechst 33258 dye was obtained from American Hoechst Co. (Somerville, NJ).

Cell Isolation. Chondrocytes were isolated from tibiotarsal growth plate cartilage of 6-week-old broiler chickens following the method described by Ishikawa *et al.* (23) with slight modification. After exsanguination, chicken leg quarters were surgically removed and placed on ice. The growth plate cartilage was exposed and cut into 1-mm cubes under sterile conditions. The cartilage tissues were washed three times with 10 ml of ice-cold synthetic cartilage lymph (SCL) (24) and digested using 10 ml of 0.25% trypsin (in SCL) at 37°C for 25 min. After removing the trypsin buffer, cartilage tissues were further digested with 0.05% crude collagenase in 10 ml of DMEM (100 U/ml penicillin and 100 μg/ml streptomycin) and supplemented with 1 mg/ml of BSA at 37°C for 16 hr. The isolated chondrocytes were incubated at 37°C with citrate buffer (8.0 mM citric acid in 80.5 mM Na_3 citrate, pH 6) to dissolve residual minerals. The cells were then filtered through a 100-μm cell strainer and washed twice with DMEM and counted (Coulter Counter; Coulter Electronics, Inc., Hialeah, FL).

Cell Culture. Two milliliters of chondrocytes (2.2×10^5 cells/ml DMEM with 10% FBS) were plated into 35-mm dishes and incubated at 37°C with 5% CO_2. Each treatment included six replicate cell culture wells. From Day 3, the medium was replaced with DMEM containing 5% FBS and 50 μg/ml *L*-ascorbate to promote collagen synthesis. The medium was replaced every 3–4 days. After 14 days of preculture, the chondrocytes were cultured with BSA (25 μM) complexed with oleic or linoleic acid at 0 or 50 μM for 8 days and VIT E for 2 days. The serum free DMEM containing 50 μg/ml ascorbate and 25 μM BSA was used as the control. Chondrocytes were treated with the iron oxidative inducer (0 or 20 μM of $FeSO_4 \cdot 7H_2O$ + 100 μM of ascorbate) with or without 100 μM of VIT E for 24 hr. The medium and cells were collected for the following assays: (i) TBARS value and LDH activity determined in the culture medium; (ii) DNA content and the enrichment of fatty acids quantified in the chondrocytes.

The effects of supplemental fatty acids and VIT E on collagen synthesis were evaluated following the

preculture period in isolated chondrocytes enriched with 50 μM of 18:1n-9 or 18:2n-6 for 8 days and with VIT E for 2 days. Chondrocyte cultures not supplemented with fatty acids served as a control. The chondrocytes were incubated with 2 ml of DMEM containing 2.5 μCi/ml of [2,3,4,5-^{3}H]proline and oxidative inducer (0 or 20 μM $FeSO_4 \cdot 7H_2O$ + 100 μM ascorbate) for 24 hr. The medium and cells were harvested for the determination of collagen synthesis.

Preparation of Fatty Acid and BSA Complex. The preparation of BSA complexed fatty acids (50 μM) was as follows: fatty acids (Nu-Chek-Prep) were dissolved in ethanol and mixed with 6 M NaOH to form soaps (25). The mixture then was evaporated under nitrogen and finally redissolved in the medium containing 25 μM of BSA. The pH of the BSA complexed with fatty acids was adjusted to 7.4 immediately after preparation and then added to the cultured chondrocytes.

Harvesting Chondrocytes. After removing the medium, the cell layer was rinsed with 2 × 0.5 ml of cold PBS to remove the remaining media. The cell layer was scraped from each culture dish with 3 × 1 ml of cold PBS and transferred into a 15-ml centrifuge tube. Cells were pelleted at 800g for 10 min and rinsed with an additional 2 ml of PBS and pelleted again. The final pellet was then lysed with 500 μl (1 N NH_4OH and 0.2% of Triton X-100) solution for 20 min at 37°C. The cell lysate was then diluted to 2 ml with PBS, in which, 200 μl of aliquot was used for DNA determination and 1800 μl was used for fatty acid analysis or radioactivity measurement for determining collagen synthesis.

Analysis of Fatty Acids. The method for fatty acid analysis was described earlier (12). Briefly, total lipids were extracted with chloroform:methanol (2:1, v/v), saponified, and fatty acid methyl esters (FAME) prepared using 14% boron trifluoride (Alltech Associates Inc., Deerfield, IL). The FAME were analyzed by gas-liquid chromatography using a DB 23 column (J & W Scientific Co., Folsom, CA). Fatty acids were identified by comparison of retention times with authentic standard mixtures of FAME (Nu-Chek-Prep).

DNA Determination. The DNA content of each cell culture well was measured following the assay procedure of Downs and Wilfinger (26). The total DNA was labeled with Hoechst 33258 dye in cell lysate and then measured by fluorescence spectrophotometry (JD-490, SLM Instruments, Inc.) with the excitation and emission wavelength at 350 and 455 nm, respectively (26). The DNA standard was prepared from calf thymus DNA.

Collagen Synthesis. Collagen synthesis was determined according to the method described by Peterkofsky and Diegelman (27) and Peterkofsky (28). The chondrocytes were labeled with 5 μCi [^{3}H]proline for 24 hr in 2 ml of serum free DMEM supplemented with different fatty acids and 0 or 100 μM VIT E. After incubation, the cells were harvested and the cell pellets washed twice with 0.5 ml of PBS. The cell lysate was mixed with 20% trichloracetic acid (final TCA concentration was 10%) to precipitate the protein. The acid insoluble precipitate was centrifuged at 1000g for 5 min and was resuspended with 10% TCA and washed twice to remove free [^{3}H]proline. The acid insoluble precipitate was digested with high purity collagenase for 16 hr and radioactivity in the collagenase digestible protein mixed with scintillation fluid (EcoLite, ICN, Costa Mesa, CA) determined (TRI-CARB 1900 TR; Packard Instrument Co., Meriden, CT). The total noncollagenous protein in the precipitate was dissolved into 2 ml of 0.2 N NaOH and the radioactivity determined. Both collagen and noncollagen synthesis were expressed as dpm/μg DNA.

LDH Assay. Culture medium for LDH (EC 1.1.1.27) determination was collected immediately and stored at −70°C. A LDH assay kit was used to measure LDH activity in culture medium for evaluating cellular membrane leakage. The activity of LDH in the medium was normalized by the DNA content in the cells and was expressed as mU/μg DNA.

Determination of Lipid Peroxidation. Lipid peroxidation was evaluated by determining TBARS value. Malondialdehyde (MDA) standard was prepared from 1,1,3,3,-tetraethoxypropane and TBARS value was determined as described previously (22). The assay mixture contained 0.01% of BHT to prevent additional lipid peroxidation during the assay procedure. The MDA standard curve was used to calculate the MDA-equivalent substances, and lipid peroxidation was expressed as nmol TBARS (MDA equivalents)/μg DNA.

Statistical Analysis. All data were subjected to a three-way analysis of variance (ANOVA) and when significant F values were obtained a Tukey's mean separation test was performed (29) and measures of variation were determined as standard error of the mean (SEM) or pooled SEM.

Results

Chondrocytes supplemented with different fatty acids were significantly enriched by their respective fatty acid treatment (Fig. 1). The content of 18:1n-9 was greatly enriched in chondrocytes cultured with oleic acid compared to chondrocytes in the control and linoleic acid groups. Similarly, 18:2n-6 was enriched in chondrocytes cultured with linoleic acid compared to the chondrocytes in the control and oleic acid groups.

Chondrocytes incubated with iron oxidative inducer had higher TBARS values compared to those not treated with iron (Table I). Chondrocytes enriched with 18:2n-6 tended to exhibit a higher TBARS value

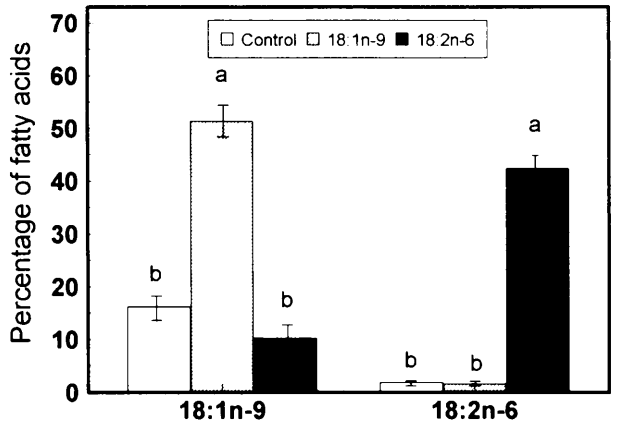

Figure 1. Bar graph indicating the enrichment of chondrocytes with oleic acid (18:1n-9) or linoleic acid (18:2n-6) for 8 days when supplemented in the medium at 50 μ*M*. Bars representing fatty acid values (mean weight percentages) having different superscripts are significantly different ($P < 0.05$) within the 18:1n-9 and 18:2n-6 groups. Error bars represent SEM.

and supplementation of VIT E had no effect on TBARS values in this experiment (Table I).

Fatty acid and VIT E treatments effected the values for LDH activity in the medium (Fig. 2). In the absence of iron oxidative inducer as indicated in Panel A of Figure 2, cellular LDH release was higher in chondrocytes enriched with linoleic acid compared to chondrocytes in the control and oleic acid groups. VIT E containing DMEM (100 μ*M*) significantly lowered LDH leakage from chondrocytes in the linoleic acid treatment group (Fig. 2A). When chondrocytes were incubated with DMEM containing 20 μ*M* of iron oxidative inducer, a similar pattern was observed as shown in Panel B of Figure 2. Therefore, the enrichment of linoleic acid resulted in higher LDH leakage from chondrocytes, and VIT E was found to protect against the damage caused by linoleic acid.

Chondrocytes were labeled with [^{3}H]proline for the last 24 hr of the culture period. The incorporation of [^{3}H]proline into the total collagen fraction was used to determine collagen synthesis. Enrichment of chondrocytes with linoleic acid greatly decreased collagen synthesis (Fig. 3) and non-collagen synthesis (Table I) independent of Fe treatment. In the absence of VIT E, chondrocytes enriched with 18:2n-6 had lower collagen synthesis as indicated in panel A (Fig. 3). However, when chondrocytes were treated with DMEM containing 100 μ*M* of VIT E, collagen synthesis was restored in chondrocytes enriched with 18:2n-6 even though collagen synthesis was still lower than chondrocytes in the control and oleic acid groups as indicated in panel B. DMEM with added VIT E (100 μ*M*) significantly increased noncollagen synthesis compared with 0 μ*M* of VIT E (Table I).

Discussion

This study examined the effects of supplemental fatty acids and VIT E on collagen synthesis, cellular damage, and lipid peroxidation in primary cultures of epiphyseal chondrocytes. After 8 days of fatty acid enrichment with 18:1n-9 or 18:2n-6, the chondrocyte fatty acid composition reflected the respective fatty acid treatments. These results are consistent with previous studies that cell cultures can be enriched by supplementing with exogenous sources of fatty acids (30, 31).

Consistent with the effect of linoleic acid on chondrocyte injury, the enrichment of 18:2n-6 lowered chondrocyte collagen synthesis. This is in agreement with an abstract reporting that arachidonic acid de-

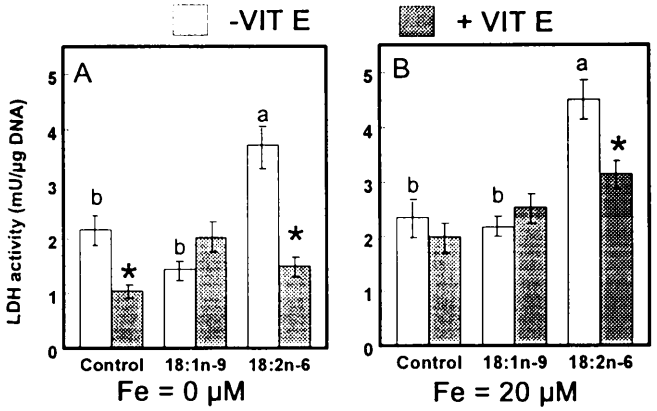

Figure 2. Effect of vitamin E (VIT E) on lactate dehydrogenase (LDH) activity in chondrocytes ($n = 6$). The chondrocytes were enriched with no fatty acids (control), 50 μ*M* of oleic acid (18:1n-9), or 50 μ*M* of linoleic acid (18:2n-6). VIT E was supplemented at 0 or 100 μ*M*. (A) The effects of no Fe. (B) The effects of Fe treatment. Bars having different letters (^{a,b}) represent significant differences ($P < 0.05$) between fatty acid treatments. *Differences between VIT E treatments ($P < 0.05$). Error bars represent SEM.

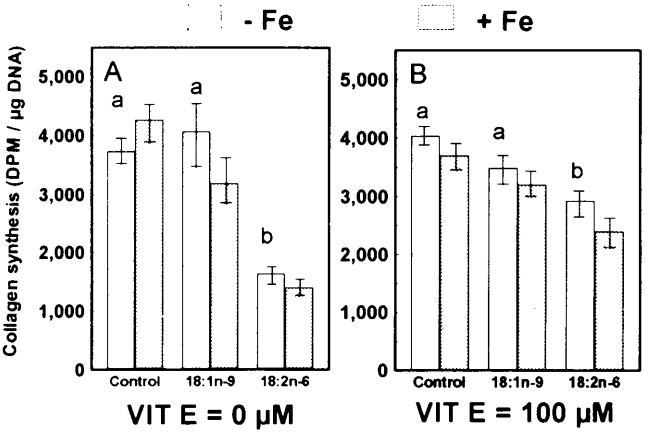

Figure 3. Fatty acid effects on collagen synthesis in chondrocytes ($n = 6$). The chondrocytes were enriched with no fatty acids (control), 50 μ*M* of oleic acid (18:1n-9), or 50 μ*M* of linoleic acid (18:2n-6). VIT E was supplemented at 0 or 100 μ*M* (Panel A and B, respectively). Iron (Fe) was supplemented at 0 or 20 μ*M*. Bars having different letters (^{a,b}) represent significant differences between fatty acid treatments ($P < 0.05$). Error bars represent SEM.

Table I. Effects of Fatty Acids, Vitamin E, and Iron on the Average Treatment Values for Lipid Peroxidation, LDH Activity, Collagen (Col), and Noncollagen (NCol) Protein Synthesis

Treatment[a]			Measurements[b]			
FA	VIT E	Fe	TBARS	LDH	Col	NCol
Control	0	0	0.46	2.17	3724	47149
Control	100	0	0.28	1.03	4028	56728
Control	0	20	0.48	2.35	4256	58841
Control	100	20	0.46	1.99	3693	72350
18:1n-9	0	0	0.27	1.44	4058	54356
18:1n-9	100	0	0.32	2.02	3476	53782
18:1n-9	0	20	0.62	2.17	3179	55604
18:1n-9	100	20	0.56	1.99	2894	45075
18:2n-6	0	0	0.45	3.72	1633	26056
18:2n-6	100	0	0.29	1.50	2920	53440
18:2n-6	0	20	0.52	4.50	1399	17827
18:2n-6	100	20	0.72	3.14	2386	32281
Pooled SEM			0.08	0.42	549	6061
FA effect						
Control			0.43	1.85[c]	3925[d]	58176[d]
18:1n-9			0.45	2.01[c]	3402[d]	52205[d]
18:2n-6			0.50	3.22[d]	2084[c]	32401[c]
VIT E effect						
0			0.48	2.68[d]	3041	43306[c]
100			0.45	2.01[c]	3233	51702[d]
Fe effect						
0			0.35[c]	1.93[c]	3306	48585
20			0.55[d]	2.79[d]	2968	46272
ANOVA						
FA			NS	0.0002	0.0001	0.0001
VIT E			NS	0.01	NS	0.0137
Fe			0.0001	0.002	NS	NS
FA * VIT E			NS	0.003	NS	0.01

[a] The treatments included BSA (25 μM) complexed fatty acids (18:1n-9 or 18:2n-6) at 50 μM, the serum free DMEM containing 25 μM BSA was used as the control, *dl*-α-tocopheryl acetate (VIT E) at 0 or 100 μM, and ferrous sulfate (Fe) at 0 or 20 μM.
[b] TBARS, thiobarbituric acid reactive substances (nmol/μg DNA); LDH, lactate dehydrogenase (mU/μg DNA); Col, collagen synthesis (dpm/μg DNA); NCol, noncollagen synthesis (dpm/μg DNA).
[c,d] Means (n = 6) in a column within a main effect with different superscripts[c,d] are significantly different ($P < 0.01$).

pressed collagen synthesis of chondrocytes isolated from chicken articular cartilage (32). The mechanism by which linoleic acid impaired chondrocyte function remains unclear. Perhaps an overproduction of PGE_2 inhibits collagen synthesis, since PGE_2 was found to inhibit collagen synthesis in chondrocyte cultures (33). The decrease in collagen synthesis observed with PUFA enrichment also may be related to membrane damage and impaired cell function, for which VIT E is protective.

VIT E seemed to exert a protective effect on LDH release from chondrocytes enriched with 18:2n-6. The enrichment of 18:2n-6 in chondrocytes resulted in cellular injury and impaired the function of chondrocytes compared with those in the oleic acid and control groups. The response with 18:1n-9 in chondrocytes follows that reported previously (30) where supplemental 18:1n-9 protected endothelial cells from oxidative injury as measured by LDH activity, but supplementation with γ-linolenic acid (18:3n-6) enhanced oxidative injury. Data from the present study suggest that growth plate cartilage and chondrocytes may be sensitive to n-6 PUFA which might explain the low n-6 PUFA content in normal growth plate cartilage of the young animal and human (11, 12). The reason for linoleic acid inducing LDH leakage from chondrocytes is not clear; however, the present data could suggest that 18:2n-6 perturbed cell membrane integrity or perhaps increased oxidative stress in the cells because VIT E reduced LDH activity in the medium. Since the onset of peroxidative reactions within biological membranes can impair cell behavior and function, VIT E and antioxidant systems designed to protect chondrocytes may be limiting in growth plate cartilage (15, 17). VIT E, which serves as a free-radical scavenger to inhibit peroxidation of membrane lipids (13), may maintain cell membrane integrity and function in chondrocytes. To our knowledge, this is the first report to describe

the effects of fatty acid enrichment and VIT E supplementation on chondrocyte function.

Lipid peroxidation, as measured by TBARS analysis, was significantly increased in chondrocytes incubated with 20 μM iron-oxidative inducer. These data are in agreement with other reports that iron oxidative inducer can stimulate lipid peroxidation in cell cultures (18, 20). It is noteworthy that there was a trend for higher TBARS values in chondrocytes enriched with 18:2n-6.

It was hypothesized that lipid peroxidation may be important for stimulating collagen gene expression in cultured fibroblasts (34, 35). The explanation proposed for this mechanism was that ascorbic acid induced an increase in lipid peroxidation which upregulated collagen gene expression (34, 35). In the present study, an iron oxidative inducer had no effect on collagen and noncollagen synthesis, independent of fatty acid enrichment and VIT E treatment, in primary cultures of avian chondrocytes. This result supports a recent report (36) which showed that lipid peroxidation had variable effects on collagen synthesis.

In summary, the results from this study showed that supplemental linoleate impaired collagen synthesis in and caused cellular injury to cultured chondrocytes, and that VIT E partially restored the loss of chondrocyte function. These data provide in part an explanation for the low concentration of n-6 PUFA in growth cartilage and chondrocytes of young animals and the human (11, 12). Epiphyseal chondrocytes may be sensitive to excessive amounts of 18:2n-6 and they may have a lower tolerance to oxidative stress. Further, these results suggest that VIT E may be vital for protecting membranes of chondrocytes during maturation and differentiation as well as benefiting chondrogenesis and bone growth as reported previously by this laboratory (17).

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