

Carnitine and Dehydroepiandrosterone Sulfate Induce Protein Synthesis in Porcine Primary Osteoblast-Like Cells

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Abstract. Age-related bone loss eventually leads to osteopenia in men and women. The etiology of age-related bone loss is currently unknown; however, decreased osteoblast activity contributes to this phenomenon. In turn, osteoblast proliferation and function is dependent on energy production, thus the loss of energy production that occurs with age may account for the deficient osteoblast activity. Carnitine and dehydroepiandrosterone-sulfate (DHEAS), both of which decline with age, promote energy production through fatty acid metabolism. Thus, we hypothesized that carnitine and DHEAS would increase osteoblast activity *in vitro*. Accordingly, we measured the effect of carnitine and DHEAS on palmitic acid oxidation as a measure of energy production, and alkaline phosphatase (ALP) activity and collagen type I (COL) as indices of osteoblast function in primary porcine osteoblast-like cell cultures. Carnitine (10^{-3} and 10^{-1} M) but not DHEAS (10^{-9} , 10^{-8} , and 10^{-7} M) increased carnitine levels within the cells. Carnitine alone and in combination with DHEAS increased palmitic acid oxidation. Both carnitine and DHEAS alone and in an additive fashion increased ALP activity and COL levels. These results demonstrate that in osteoblast-like cells *in vitro*, energy production can be increased by carnitine and osteoblast protein production can be increased by both carnitine and DHEAS. These data suggest that carnitine and DHEAS supplementation in the elderly may stimulate osteoblast activity and decrease age-related bone loss.

Key words: Osteoblast — Carnitine — Dehydroepiandrosterone sulfate — Alkaline phosphatase — Collagen Type I.

Bone loss is often divided into two broad mechanisms: (1) postmenopausal bone loss, characterized by increased osteoclast activity, and (2) age-related bone loss, characterized by decreased osteoblast activity. Osteoblast activity is dependent on energy production. Thus, it is plausible that the loss of energy production, which accompanies age, may account for decreased osteoblast activity. Most cells use both glucose and fatty acid oxidation pathways for ATP synthesis, although some cell types show a substrate pref-

erence. For instance, some studies have suggested that glucose is the major fuel substrate for bone [1, 2]. However, more recent work indicates that cell populations enriched with osteoblast-like cells generate 40–80% of the energy demands through fatty acid oxidation [3]. Osteoblasts are responsible for bone formation and it follows that they maintain a high rate of ATP utilization to support the requisite protein synthesis. Cells with a high rate of protein synthesis generate ATP from fatty acid oxidation, the most efficient metabolic process for energy production [4–6]. Thus, modulation of fatty acid oxidation may regulate the amount of energy available for protein synthesis in osteoblasts.

A number of factors are required for fatty acid oxidation. For example, carnitine is the requisite carrier for the transport of long-chain fatty acids across the inner mitochondrial membrane into the mitochondrial matrix [7]. In muscle, carnitine concentration decreases with advancing age [8–10]. This decline may contribute to diminution of mitochondrial function associated with age. We have observed that the ratio of esterified to free carnitine in the peripheral blood increases with age [11], suggesting a declining ability for fatty acid transport. Accordingly, this decline may contribute to reduced energy availability with age, culminating in cardiomyopathy, hepatic encephalopathy, or skeletal muscle weakness [12]. This loss of energy availability could also compromise osteoblast activity and bone production in an age-related manner.

Another factor indirectly related to fatty acid oxidation is dehydroepiandrosterone (DHEA), an intermediate in the pathway for the synthesis of testosterone, estrone, and estradiol [13]. DHEA and its sulfated derivative, DHEAS, are interconvertible and DHEAS is the major source of circulating DHEA [14]. The physiologic roles of DHEA and DHEAS have not been conclusively defined. However, DHEA and DHEAS increase lipid metabolism and mitochondrial respiration [15, 16]. This may be accomplished through DHEA's ability to increase the synthesis and activity of carnitine acyltransferases, enzymes which catalyze the reversible transfer of the fatty acid moiety from fatty acyl CoA to fatty acyl-carnitine [17, 18]. Additionally, our laboratory has demonstrated that DHEAS administration increases hepatic and cardiac carnitine levels and carnitine acetyl transferase activities in young adult oophorectomized rats [19]. Taken together, these observations stimulate ques-

tions about potential roles that DHEA, through promotion of energy metabolism, might play on osteoblast activity.

As osteoblasts have high-energy requirements and both carnitine and DHEAS play an important role in energy metabolism we propose that both carnitine and DHEAS promote fatty acid oxidation and energy metabolism in osteoblasts. Accordingly, the aim of this study was to investigate the effects of carnitine and DHEAS on primary cultures of porcine bone marrow stromal cells (BMSC).

Materials and Methods

Cell Isolation and Culture

Porcine bone marrow stromal cells were isolated using the methods described by Thomson et al. [20]. Briefly, cultures consisting of bone marrow stromal cells (progenitor osteoblast cells) and trabecular bone cells (mature osteoblast cells) were prepared from distal femurs of young, 3 to 4-week-old pigs. Soft tissue was removed, the bone was immersed in 70% ethanol, and then transferred to a flow hood for aseptic collection of bone cells. Bones were sectioned at the metaphysis, and tissue from trabeculae and medullary cavity was transferred into a flask containing Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY) plus 10% fetal bovine serum (FBS) (Biowhittaker, Inc. Walkersville, MD). Cells were grown to confluence, then nonadherent cells were discarded and adherent cells were subcultured to a new flask following treatment with trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA-4Na) (Gibco, Grand Island, NY). Medium was completely changed twice weekly and adherent bone marrow stromal cells were passed approximately weekly when cultures became confluent. These cell cultures were heterogeneous and included osteoblast-like cells at various stages of differentiation [20].

Mineralized Bone Nodule Formation

Cultures were grown with 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10⁻⁸ M dexamethasone for 7–10 days, as previously described by Maniopoulos et al. [20]. Under these conditions, mineralized bone nodules were identified by staining the cell cultures with Alizarin red S.

Histochemical Staining for Alkaline Phosphatase

Bone marrow stromal cells were seeded at 2 × 10⁵ cells in a 35 mm culture dish with 10% FBS/DMEM, and cultured for 24 hours. The medium was then removed and BMSC were fixed in 10% neutral buffered formalin. Alkaline phosphatase was stained using ImmunoPure Fast Red TR, (Sigma Chemical Co., St. Louis, MO) the naphthol phosphate AS-MX.

Determination of Cell Number and Viability

DNA Incorporation. Cells were seeded in 96-well plates (4000 cells/well) in 0.1% FBS/DMEM for 24 hours to synchronize the cell cycle. DMEM with 10% FBS and [³H]thymidine (0.2 µCi/ml) (New England Nuclear, Boston, MA) were added and the cultures were incubated for 20 hours at 37°C. At the end of this 20-hour incubation period, the medium was removed and cell layers were washed three times with PBS. Cells were then trypsinized for 20 minutes and harvested onto fiberglass filter papers, and the radioactivity was counted with a β-scintillation counter (1600TR, Parkard, Meriden, CT).

Cell Viability

Trypan blue exclusion. Cells were plated in 35 mm culture dishes at an initial density of 2 × 10⁵ cells/dish in 0.1% FBS/

DMEM. After a 24-hour attachment period, the medium was changed to 10% FBS/DMEM for another 24-hour incubation at 37°C. The cells were then released by trypsin, a 0.04% (w/v) trypan blue solution in phosphate-buffered saline (PBS) was added, and the number of viable cells (nonstained) were counted using a hemacytometer.

MTT [3, (4, 5-Dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium Bromide] Assay. MTT assay is an indirect measurement of cell growth. Cells were cultured in 96-well plates (4000 cells/well) in 0.1% FBS/DMEM for 24 hours. DMEM supplemented with 10% FBS was added and the cultures were incubated for another 24 hours at 37°C. MTT [3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide] (Sigma) was added and after 4 hours of incubation, cells were extracted with an extraction buffer (N, N-dimethyl formamide/sodium dodecyl sulfate) to lyse the cells and solubilize all formazan dye grains [22]. After an overnight incubation, optical densities at 570 nm were measured by automated ELISA plate reader (Dynatech MR650, Alexandria, VA).

Addition of Treatments

The cells were cultured in 2% FBS/DMEM in 96-flat-bottom-well plates (4000 cells/well). After an attachment period of 24 hours, either or both carnitine and DHEAS were added at the indicated concentrations and the cells were cultured for an additional 72 hours. L-carnitine (Austin Chemical Co., Chicago, IL) was dissolved in 2% FBS/DMEM to provide final concentrations in culture media of 10⁻¹ M, 10⁻² M, and 10⁻³ M. DHEAS (Sigma) was dissolved in 100% ethanol to provide a 10⁻³ M stock solution, and then diluted into 10⁻⁷ M, 10⁻⁸ M, and 10⁻⁹ M final concentrations with 2% FBS/DMEM. Carnitine and DHEAS were added to the cell culture separately or in the following combinations: 10⁻¹ M carnitine plus 10⁻⁷ M DHEAS, 10⁻¹ M carnitine plus 10⁻⁸ M DHEAS, or 10⁻¹ M carnitine plus 10⁻⁹ M DHEAS. For the control, only 2% FBS/DMEM was added to the cell culture.

Alkaline Phosphatase Assay (ALP)

At the end of the 72 hour incubation period, the medium was removed. The cells were then rinsed with PBS and lysed by three freeze-thaw cycles in lysate buffer (0.1% Triton X-100, 10 mM Tris, and 0.5 mM MgCl₂). ALP activity was measured at 37°C following the addition of 2 mg/ml of *p*-nitrophenylphosphate (Sigma) substrate [19]. The plates were read at 410 nm using a plate reader (Dynatech MR650). Bovine intestinal mucosal ALP (Sigma) was used to construct a standard curve. One unit of enzyme activity equals one µmole product formed per minute.

Collagen Type I Assay (COL)

The COL concentration was measured by ELISA [23] and modified from the immunocytochemistry method from Thomson et al. [20]. At the end of the 72-hour incubation period, the medium was removed. The cells were then rinsed with PBS and fixed with 4% formalin for 10 minutes at room temperature. The cells were then rinsed with PBS-T twice. Goat anti-type I collagen antibody (0.4 µg/100 µl/well) (Southern Biotechnology Associates, Inc., Birmingham, AL) was added and incubated for 2 hours at room temperature, then rinsed with PBS-T three times. Anti-goat IgG conjugated with ALP (0.135 µg/150 µl/well) (Sigma) was used as a second antibody and incubated for another 2 hours at room temperature. The cells were then rinsed with PBS-T three times. *p*-Nitrophenylphosphate (1 mg/ml) was added and the absorbance at 410 nm was recorded as described above. Interference from endogenous cellular ALP activity, determined by using the ALP-conjugated secondary antibody alone, was minimal.

Carnitine Assay

Incubation was terminated, the medium was decanted, and the cell

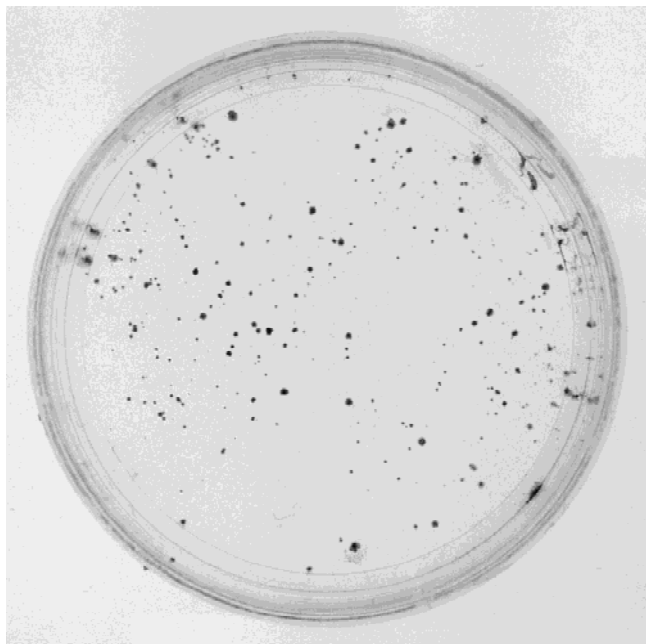


Fig. 1. Cells harvested from the porcine distal femur are capable of mineralization. Cells were harvested from the distal femoral metaphyseal trabeculae of pigs. They were then grown with 50 $\mu\text{g/ml}$ ascorbic acid, 10 mM β -glycerophosphate and 10^{-8} M dexamethasone in 10% FBS/BGJ₆ medium for 7–10 days at which time they were then stained with Alzarin red.

layers were rapidly rinsed three times with cold PBS. The cells were lysed by three freeze-thaw cycles in lysate buffer (0.1% Triton X-100, 10 mM Tris and 0.5 mM MgCl_2). The cell lysates were stored at -80°C until analysis. Carnitine was measured in triplicate by quantitative radioenzymatic assay slightly modified from that reported by Parvin and Pande [24]. Free carnitine was measured in untreated samples whereas total carnitine was determined after alkaline hydrolysis. Results were expressed as nmol/mg noncollagen protein (NCP). NCP was determined by Biorad micro protein assay kit with globulin as a standard (#500–0001 protein assay kit, Bio-Rad Laboratories, Inc., Hercules, CA) [25].

Fatty Acid Oxidation

The cells were seeded in 35 mm culture dishes (2×10^5 /dish) in 2% FBS/DMEM. After an attachment period of 24 hours, carnitine and DHEAS were added separately or in combination to the cultures. $[9,10^{-3}\text{H}]$ -Palmitic acid (0.5 $\mu\text{Ci/ml}$, specific activity 56.5 Ci/mmol) (Du Pont-New England Nuclear, Wilmington, DE) and nonradioactive palmitate were prebound to fatty acid free albumin (Sigma) and added to provide a final concentration of 0.4 mM palmitate. After 24-hours of incubation at 37°C , the medium and cell lysate were used to measure the tritiated water production as an estimate of exogenous fatty acid oxidation. Tritiated water, formed during β -oxidation of labeled $[9,10^{-3}\text{H}]$ -palmitic acid, served as a tracer for exogenous fatty acid transport and oxidation. The medium or cell lysate was added to 5 ml of Dole-Meinertz extraction solution [26], vortexed, and then allowed to stand for 15 minutes. After adding 2 ml of water and 3 ml of heptane and vortexing, it was allowed to stand for 15 minutes and then the upper organic phase was removed. The lower aqueous phase, which contained $^3\text{H}_2\text{O}$ and acid-soluble products of fatty acid metabolism, was then counted on a Parkad Tricarb liquid scintillation counter.

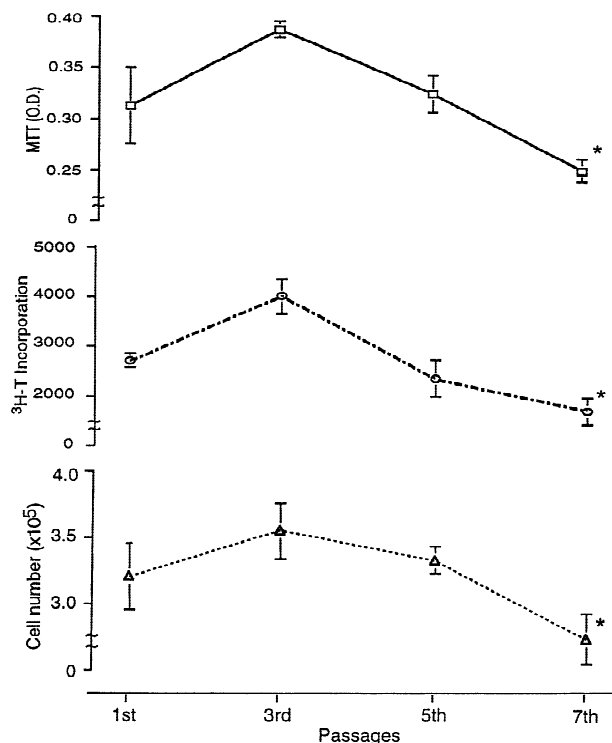


Fig. 2. Osteoblast-like cell proliferation decreases with increasing number of passages in culture. Cells were maintained in 10% FBS/DMEM for 1st, 3rd, 5th, and 7th passages. At each passage, cells were treated as described in Materials and Methods. Each value (mean \pm SEM) represents the average of five trials. * $P \leq 0.002$ compared with the 3rd passage by ANOVA.

Statistical Analysis

For total and free carnitine concentrations and palmitate oxidation, analysis of variance (ANOVA) was used to compare the means of control and treated groups. Data were expressed as the mean \pm standard error of the mean (SEM). For ALP activities and COL levels, ANOVA on rank-transformed data for randomized complete block design was performed to compare the different groups [26]. Data were expressed as median (first quartile-third quartile). Data analyses were conducted using SAS (SAS Institute Inc., Cary, NC) and Statview version 4.02 Software (Abacus Concepts, Berkeley, CA). Significant difference was set at $P \leq 0.05$.

Results

Model System

This model system has been previously well described [20]. However, mineralizing capability and the ALP activity were measured to confirm the presence of osteoblast-like cells in our cultures. Mineralized bone nodules were formed in the primary cultures indicating that osteoblast-like cells were present (Fig. 1). Immunochemical ALP-positive staining also supported the presence of osteoblast-like cells in the cultures (data not shown). The three assays (^3H thymidine, trypan blue exclusion, and MTT) used to assess DNA incorporation and viability of the bone marrow stromal cells

Table 1. Effect of carnitine and DHEAS on total and free carnitine concentrations in pig osteoblast-like cells

| Parameter | Control | L-carnitine (M) | | | DHEAS (M) | | | L-carnitine (10^{-1} M) + DHEAS (M) | | |
|------------------------------|------------------------|-----------------|-----------|------------|-----------|-----------|-----------|--|------------|------------|
| | | 10^{-3} | 10^{-2} | 10^{-1} | 10^{-9} | 10^{-8} | 10^{-7} | 10^{-9} | 10^{-8} | 10^{-7} |
| Total carnitine ^a | 2.1 ± 0.4 ^b | 6.4 ± 1.4* | 43 ± 10** | 225 ± 71** | 2.1 ± 0.6 | 2.0 ± 0.5 | 1.9 ± 0.2 | 218 ± 35** | 196 ± 60** | 185 ± 43** |
| Free carnitine ^a | 1.7 ± 0.5 | 4.9 ± 1.0* | 39 ± 11** | 211 ± 75** | 1.9 ± 0.5 | 2.0 ± 0.6 | 1.9 ± 0.3 | 201 ± 37** | 200 ± 49** | 184 ± 51** |

^a nmole/mg noncollagen protein, replicates

^b All data are expressed as mean ± SEM. Each value represents the average of 7 replicates

* $P \leq 0.05$; ** $P \leq 0.01$

in cultures showed similar responses across the number of passages. The maximal DNA incorporation and cell viability occurred in the third passage (34 ± 1.3 days old) and gradually declined through the seventh passage (Fig. 2). There was a 40–50% decline in both of these measures from the third passage to the seventh passage ($P = 0.002$).

Cellular Total and Free Carnitine Concentrations

Total and free intracellular carnitine levels were measured to confirm that *in vitro* administration of carnitine was effective. The total and free carnitine concentrations in the third passage of BMSC, cultured with 10^{-1} M carnitine alone or in the presence of DHEAS, increased above control cultures approximately 90–100-fold whereas cells from cultures treated with 10^{-2} M or 10^{-3} M carnitine had a 20-fold or 3-fold increase in cellular carnitine concentrations, respectively (Table 1). DHEAS did not effect the total or free carnitine levels.

Fatty Acid Oxidation

To determine if carnitine and DHEAS, either alone or in combination, promotes energy production, fatty acid oxidation was measured using palmitate as the substrate. The amount of palmitate oxidized by the cell cultures with 10^{-1} M carnitine alone or combined with DHEAS ($P \leq 0.01$), was greater than oxidation by control cultures (Table 2). The combination of carnitine and DHEAS was additive. Palmitate oxidation ($P \leq 0.088$) tended to increase in cultures treated with 10^{-2} M carnitine or 10^{-8} M and 10^{-7} M DHEAS.

Osteoblast Proteins

To determine if carnitine and DHEAS-mediated energy availability was associated with increased osteoblast protein production, we measured ALP and COL accumulation in culture. Both carnitine and DHEAS alone significantly increased ALP activity (Table 3). Compared with the control cultures, ALP activities increased in cultures treated with 10^{-2} M– 10^{-1} M carnitine ($P < 0.01$). DHEAS (10^{-7} M– 10^{-9} M) also stimulated ALP activities ($P \leq 0.05$), but the response was not dose dependent. Treatments with both carnitine and DHEAS were additive in increasing ALP activities when compared with the control cultures ($P \leq 0.01$).

Over a twofold increase in COL concentration was ob-

served in cultures treated with 10^{-1} M carnitine and a significant increase in cultures treated with 10^{-2} M carnitine compared with the control ($P \leq 0.01$) (Table 3). Compared with controls, the combination of 10^{-1} M carnitine and DHEAS (10^{-7} M– 10^{-9} M) also increased the COL levels ($P \leq 0.01$). DHEAS (10^{-7} – 10^{-9} M) did not increase COL levels significantly. Carnitine and DHEAS treatment did not increase viable cell counts compared with control groups (data not shown). Thus, the observed differences in ALP and COL were not due to increased cell proliferation between the various treatments, but more likely to increased expression of ALP and COL per a cell.

Discussion

In this study, carnitine promoted energy production through increased fatty acid oxidation in cell cultures containing osteoblast-like cells. Furthermore, ALP activity and COL levels, proteins indicative of osteoblastic activity, increased concomitant with the increased energy availability in carnitine-treated cells.

Lower mitochondrial carnitine concentrations are associated with diminished activity of enzymes involved in fatty acid oxidation [9, 28, 29]. In addition, the rate of fatty acid oxidation and the removal of potentially toxic intracellular acyl CoA esters are dependent upon carnitine [28, 30, 31]. We observed that carnitine treatment elevated the total carnitine concentration that was associated with greater fatty acid oxidation. These findings support the fact that carnitine supplementation increases osteoblast-like cell activity by increasing transfer of fatty acids into mitochondria for oxidation.

With advancing age, carnitine and acetyl carnitine levels are markedly reduced in certain tissues, including muscle [8–10]. The potential for carnitine deficiency/insufficiency appears to increase with age, and is more prevalent among women [11]. If there is a severe deficiency, cardiomyopathy, hepatic encephalopathy, or skeletal muscle weakness could ensue. This metabolic defect might also affect bone formation since osteoblast activity and bone mineral density (BMD) decline with advancing age [32, 33]. In elderly people, if osteoblasts are carnitine deficient, less energy may be available at the cellular level and thus limit osteoblast function. Cellular energy deficits caused by a decline in mitochondrial function can impair normal cellular activities and affect the cell's ability to adapt to various physiological stresses [34].

In our study, high concentrations of carnitine are added to stimulate palmitate oxidation, ALP activities, and COL levels in the osteoblast-like cell cultures. Physiologic serum

Table 2. Effect of carnitine and DHEAS on palmitate oxidation in pig osteoblast-like cells

| | L-carnitine (M) | | DHEAS (M) | | L-carnitine (10^{-1} M) + DHEAS (M) | |
|----------------------------------|------------------|----------------|----------------|----------------|--|---------------------|
| | 10^{-3} | 10^{-2} | 10^{-9} | 10^{-8} | 10^{-9} | 10^{-8} |
| Control | 18.7 ± 1.4^b | 22.5 ± 1.2 | 21.7 ± 1.5 | 22.6 ± 1.2 | $31.4 \pm 0.9^{**}$ | $32.1 \pm 1.2^{**}$ |
| Palmitate oxidation ^a | 21.5 ± 1.2 | 22.5 ± 1.2 | 21.7 ± 1.5 | 22.6 ± 1.2 | $31.4 \pm 0.9^{**}$ | $32.1 \pm 1.2^{**}$ |

^a 10^{-2} pmol/ 10^5 cells^b All data are expressed as mean \pm SEM. Each value represents the average of 7 replicates** $P \leq 0.01$

total carnitine levels in men and women are 55 ± 12 and 47 ± 11 μ moles/liter, respectively [36]. Tissue total carnitine levels (skeletal muscle, heart, and liver) are 14 ± 3 , 9 ± 2 , and 10 ± 3 nmol/mg noncollagen protein, respectively [36]. However, high doses of carnitine therapy, from 100 up to 600 mg/kg daily, are required to treat carnitine deficiency [35, 36]. Our demonstration that high levels of carnitine were required to observe a significant effect on energy metabolism protein production in osteoblasts agrees with these studies. One possible explanation for the requirement of high carnitine levels in our model is that the *in vitro* environment results in inefficient utilization of carnitine as opposed to the *in vivo* environment. Further studies are required to explore this issue.

The exact physiologic role that DHEAS plays in bone metabolism is unclear. However, several reports suggest that DHEAS promotes bone density. For example, DHEAS levels were correlated with skeletal maturation [37]. Additionally, DHEAS is positively correlated to BMD and declines with age [38–41]. Finally, exogenous administration of DHEA reduced cancellous bone osteopenia in oophorectomized rats [42]. The mechanism through which DHEAS mediates its effects on bone is currently unknown, but could include direct or indirect modulation of hormonal mechanisms [43]. Our data suggest that DHEAS promotes osteoblast activity which was not due to increased cellular carnitine concentration, increased energy metabolism, or differences in cell viability. Our observation that DHEAS increases liver total and free carnitine concentrations in rats [19] suggests that it may also do so in bone, which would lead to energy production. However, we could not document that DHEAS induced either carnitine levels or fatty acid metabolism in the osteoblast cultures. Thus, our findings suggest that DHEAS promotes protein production in osteoblasts through other mechanisms, such as increasing mitochondrial respiration [16, 44], protein synthesis [18, 45], and enzyme activities [17, 18, 46, 47].

A limitation of our study is the use of an *in vitro* model that contains a heterogeneous population of cells. However, this well-documented system has been previously used for evaluation of osteoblast-like cells [20]. Furthermore, we confirmed the presence of osteoblast-like cells in the cultures through the identification of mineralized bone nodule formation and ALP staining cells.

In conclusion, carnitine alone and in combination with DHEAS stimulated the metabolic activity of early passage osteoblast-like cells *in vitro*. Carnitine achieved this through promotion of fatty acid metabolism culminating in increased osteoblast protein production. Furthermore, DHEAS increased osteoblast protein production by an undefined mechanism. These findings suggest that carnitine and DHEAS may promote osteoblast function. Thus, because carnitine and DHEAS levels decline with advancing age, carnitine and DHEAS supplementation for promotion of osteoblast activity and bone formation in elderly persons may be worth investigating.

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Table 3. Effect of carnitine and DHEAS on osteoblast proteins in pig osteoblast-like cells^c

| Parameter | Control | L-carnitine (M) | | | DHEAS (M) | | | L-carnitine (10 ⁻¹ M) + DHEAS (M) | | |
|------------------|------------------|-------------------|----------------------|-----------------------|--------------------|-------------------|--------------------|--|-----------------------|-----------------------|
| | | 10 ⁻³ | 10 ⁻² | 10 ⁻¹ | 10 ⁻⁹ | 10 ⁻⁸ | 10 ⁻⁷ | 10 ⁻⁹ | 10 ⁻⁸ | 10 ⁻⁷ |
| ALP ^a | 5.9 (3.6–7.3) | 6.4 (4.5–8.3) | 7.1** (6.5–8.3) | 7.7** (6.0–10.8) | 6.8** (4.4–9.0) | 6.6* (4.0–8.8) | 6.7* (4.9–8.4) | 8.8** (8.4–11.1) | 8.7** (8.6–11.0) | 8.2** (6.8–11.8) |
| COL ^b | 8.0 (5.0–9.5) | 8.0 (4.0–10.0) | 11.5** (8.5–15.0) | 18.5** (12.5–22.0) | 9.5 (7.5–12.0) | 9.5 (6.5–11.0) | 10.0 (7.5–11.0) | 20.5** (18.0–24.0) | 21.0** (18.0–28.5) | 21.5** (17.5–25.0) |

^a Alkaline phosphatase (×10⁵ U/μg protein)

^b Collagen type I concentration (×10⁻⁴ O.D./μg protein)

^c All data are expressed as mean (first quartile–third quartile). Each value represents the average of 5 or 4 replicates for ALP and COL, respectively.

* $P \leq 0.05$; ** $P \leq 0.01$

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