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FEEDING L-CARNITINE TO GESTATING SOWS ALTERS THE INSULIN-LIKE GROWTH-FACTOR SYSTEM IN CULTURED PORCINE EMBRYONIC MUSCLE CELLS ISOLATED FROM FETAL SKELETAL MUSCLE

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Summary

The objective was to determine the effects of L-carnitine on cell proliferation and on messenger RNA (mRNA) concentrations in the insulin-like growth factor (IGF) system. Cultured porcine embryonic myoblasts (PEM) were isolated from fetuses at mid-gestation from sows fed a common gestation diet with a 50-g top dress of 0 (control, n = 6) or 100 mg of L-carnitine (n = 6). Proliferation of PEM was evaluated at 36, 48, 60, and 72 h post-plating. Real-time quantitative PCR was used to determine growth factor mRNA concentrations in culture. The number of cells/cm^2 did not differ (P>0.05) from sows fed either diet, but the number of cells/cm^2 increased (P<0.05) between each time period. There was a treatment × time interaction (P = 0.05) for number of doublings. The number of doublings was greater (P<0.01) between 36 and 48 h for PEM isolated from dams fed L-carnitine, compared with that of the controls. When PEM were incubated with L-carnitine (n = 4) at six concentrations (3.125, 6.25, 12.5, 25, 50 and 100 μmol/L) and compared with a control, no proliferation differences were detected (P>0.05). There was no treatment difference (P>0.05) for the expression of IGF-I or insulin-like growth factor binding protein 5 (IGFBP-5). But PEM isolated from sows fed L-carnitine had decreased (P<0.05) IGF-II, IGFBP-3, and myogenin (61, 59, and 67%, respectively) mRNA concentrations compared with those of controls. These data suggest that L-carnitine influences the IGF system and myogenin, resulting in enhanced proliferation and delayed differentiation of porcine embryonic myoblasts. These results show that L-carnitine plays a role in regulating proliferation and differentiation of cultured porcine embryonic myogenic cells and that fetal muscle growth and development could be increased by feeding L-carnitine.

(Key Words: Insulin-like Growth Factor, Insulin-like Growth Factor Binding Protein, L-carnitine, Myoblasts, Pigs.)

Introduction

The IGF system is complex, including growth factors, receptors, and binding proteins, all of which are distinct gene products. The IGF-I and -II growth factors are important regulators of fetal and postnatal growth. Both of these factors mediate cell proliferation, differentiation, and metabolism *in vivo* and *in vitro*. Research has reported that in porcine fetuses, IGF-I and -II were expressed and produced primarily in muscle cells, supporting the hypothesis that these growth factors modulate fetal muscle growth. The IGF in serum are invariably found in association with IGF binding proteins (IGFBP), which serve as a storage reservoir, increase the half-life, and either inhibit or potentiate IGF actions. In addition, the IGFBP have biological effects that

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are independent of the two growth factors. Evidence of the IGF-independent action of IGFBP-3 was also reported in 1997; it was found that IGFBP-3 binds specifically to the type-V transforming growth-factor-β receptor. Proliferation of PEM is suppressed in the presence of IGFBP-3, and the amount of IGFBP-3 mRNA is reduced concurrently with the expression of myogenin, suggesting that IGFBP-3 also has a role in myogenic cell differentiation. At the initiation of C2 myoblast differentiation, IGFBP-5 mRNA reportedly increases, coinciding with the onset of myogenin. In addition, in 2002, the IGF-independent actions of IGFBP-5 were reportedly attributed to localization of IGFBP-5 in the nucleus and IGFBP-5 membrane-bound receptors. These data suggest that IGFBP can effect muscle growth and development at the local level, as well as have IGF-independent effects.

Kansas State University has previously reported that supplementing pigs with carnitine was beneficial for both growth traits and carcass muscling. In a gestation study, it was found that supplementing dams with L-carnitine increased circulating IGF-I concentrations at mid-gestation. Other researchers determined that injecting L-carnitine into streptozotocin-induced diabetic rats increased serum carnitine concentrations, total serum and liver IGF-I concentrations, and expression of liver IGF-I mRNA. L-carnitine had no effect on IGF-II concentrations or IGF-II mRNA.

It has long been established that the transport of long-chain fatty acids across the mitochondrial membrane into the mitochondrial matrix where β-oxidation occurs is L-carnitine-dependent. To our knowledge, however, there is no data representing how sow supplementation with L-carnitine affects PEM and the expression of IGF-system mRNA. In this experiment, L-carnitine was fed to gestating sows, and PEM were isolated from fetuses at mid-gestation. The main goal of this study was to use an *in vitro* model (PEM) to determine if there is a direct or indirect effect of L-carnitine on embryonic cell proliferation and if mRNA concentrations of the IGF-system are affected.

Procedures

Animals and Feeding Protocol. Twelve fourth-parity sows (PIC, Franklin, KY; C 22 sows; BW = 552.7 lb) were artificially inseminated (PIC; 327 MQ) 12, 24, and 36 h after the onset of estrus. Sows were randomly allotted to one of two dietary treatments. All sows were fed once daily 4.4 lb of a gestation diet based on corn-soybean meal (Table 1) and received a 50-g top dress containing either 0 (control, n = 6) or 100 mg of L-carnitine (Carniking 10; 10% L-carnitine; Lonza, Inc., Fairlawn, NJ) from d 1 to 54.5 of gestation. Day 1 of gestation was considered 12 h after the first insemination. Sows were allowed *ad libitum* access to water.

Table 1. Composition of Diet (As-fed Basis)

Item	Gestation Diet, %
Corn	85.06
Soybean meal (47% CP)	10.89
Mono-calcium phosphate	1.85
Limestone	1.05
Salt	0.50
Minerals and vitamins	0.65
	100.0
Calculated Analysis, %	
Lysine	0.55
Crude protein	12.30
ME, Mcal/lb	1.50
Calcium	0.80
Phosphorus	0.70
Fat	3.64
Fiber	1.67

Eighteen hours before hysterectomy, sows were transported from Kansas State University Swine Teaching and Research Center to the

surgery suite on campus where sample collections were performed 24 h after the last feeding.

A hysterectomy was completed on each sow between d 55 and 59 of gestation. Sows were anesthetized and the uterus was removed. Once the uterus was removed, the muscle layers and skin were closed with absorbable sutures. Fetal pigs were immediately removed under aseptic conditions and rapidly transported to a laminar flow hood where myogenic cells were isolated. Isolated cells were suspended in solution, frozen at -80°C, and stored in a liquid nitrogen tank.

Cell Culture. Proliferation rates of the porcine embryonic myoblasts isolated from each of the sows were determined at 36, 48, 60 and 72 h post-plating. Growth factor and myogenin mRNA concentrations were determined from the same breakouts, at 96 h post-plating.

To establish cultures from frozen stocks, rapidly thawed cell suspensions were diluted with 25 mL of Dulbecco's Modified Eagle Medium containing 10% (v/v) fetal calf serum. One mL and 10 mL of cell solution were plated on 4-well plates (2-cm^2-wells) and 100-mm dishes, respectively, coated with Basement Membrane Matrigel (diluted 1:27 (v/v) in DMEM. All cultures were maintained at 37°C, 5% CO_2, 95% air in a water-saturated environment. After a 24-h attachment period, the 100-mm plates were rinsed twice with 5 mL of DMEM. Plates were refed with DMEM containing 10% FCS (0.5 mL/2-cm^2 well or 7 mL/100-mm plate). At the desired post-plating time, [^{3}H]thymidine (1 μCi/mL final concentration; NEN Life Science, Boston, MA) was added to the culture media on the 4-well plates and allowed to incubate at 37°C for 3 h. After radioisotope exposure, cells were rinsed three times with 0.5 mL cold DMEM, and 5% cold trichloroacetic acid (TCA) was added. Four random microscope fields were counted and averaged for each well to determine the number of PEM. Plates were incubated at 4°C overnight and then rinsed again with cold TCA. [^{3}H]thymidine incorporation into cellular DNA was measured by dissolving cell material in 0.5 mol/L sodium hydroxide and counting in a scintillation counter. All data were averaged from triplicate wells.

Direct Effects of L-carnitine on PEM *In Vitro*. A separate set of experiments evaluating the range of 3.125 to 100 μmol/L L-carnitine were undertaken to determine any potential direct effects of L-carnitine on PEM. To establish cultures from frozen stocks, the same procedure as previously described was followed, with differences being 1-mL of cell solution was plated on a 24-well plate (2-cm^2 wells) coated with Basement Membrane Matrigel (diluted 1:27 (v/v) in DMEM). After a 24-h attachment period, the wells were aspirated and refed with 0.5 mL DMEM containing 10% FCS. At 48-h post-plating, the wells were rinsed three times with 0.5 mL DMEM, and 0.5 mL of desired test media was added to each well. To make stock solutions, L-carnitine was dissolved in DMEM. The L-carnitine stock solutions were diluted with DMEM containing low serum (2% v/v swine serum) to make test media (3.125, 6.25, 12.5, 25, 50, and 100 μmol/L). In addition, a control media was made with DMEM replacing the addition of a L-carnitine stock solution. At 72 h post-plating, the wells were incubated for 3 h with [^{3}H]thymidine. The remaining steps of the radioisotope exposure were completed as described previously. All data points were averaged from triplicate wells.

Isolation of RNA and Real-time Quantitative PCR. At 96-h post-plating, total RNA was isolated from cells on the 100-mm plates. The concentration of RNA was determined by absorbance at 260 nm. TaqMan reverse transcription reagents and MultiScribe Reverse Transcriptase were used to produce complimentary DNA (cDNA) from 1 μg of total

RNA. Random hexamers were used as primers in cDNA synthesis.

Real-time quantitative PCR was used to measure the quantities of IGF-system mRNA, relative to the quantity of 18S ribosomal RNA (rRNA) in total RNA isolated from cultured cells. Measurement of the relative quantity of cDNA was carried out by using TaqMan Universal PCR Master Mix, 900 nmol/L of the appropriate forward and reverse primers, 200 nmol/L of appropriate TaqMan detection probe, and 1 µl of the cDNA mixture. The porcine-specific forward and reverse primers and TaqMan detection probes were synthesized according to published GenBank sequences for the specific genes of interest.

Relative quantities of target mRNA were normalized to 18S rRNA with the eukaryotic 18S rRNA endogenous control. Assays were performed in an ABI Prism 7000 sequence detection system, using thermal cycling parameters recommended by the manufacturer (50 cycles of 15 s at 95ºC and 1 min at 60ºC). Relative expression of the porcine genes was normalized with the 18S endogenous control by using the Δ-CT method, and is expressed in relative units. Titration of 18S, IGF-I, IGF-II, IGFBP-3, IGFBP-5, and myogenin primers against increasing amounts of cDNA gave linear responses with slopes of -3.3 to -3.9.

Statistical Methods. A single-factor (0 or 100 mg of L-carnitine) experiment in a completely randomized design was conducted. Statistical analyses for cell-proliferation data and gene-expression levels were performed with the Mixed procedure of SAS, with the dam as the experimental unit. The statistical model for the proliferation data included the fixed effects of treatment and time and the random effect of animal. The statistical model for the gene-expression data included the fixed effect of treatment and the random effect of animal. The Mixed procedure of SAS was also used for the analysis of the PEM L-carnitine titration experiment, and the L-carnitine concentration was the fixed effect with assay as the random effect. All main-effect and interaction means were separated ($P<0.05$), unless otherwise noted, by using the Least Significant Difference procedure when the respective F-tests were significant.

Results and Discussion

To determine the effect of feeding L-carnitine to gestating sows, plasma L-carnitine concentrations were determined. At mid-gestation, circulating total carnitine was 30% greater ($P<0.05$) in the sows supplemented with L-carnitine compared with that of the control sows (27.20 and 20.97 µmol/L, respectively). This indicates that supplemental L-carnitine increases circulating carnitine concentrations.

The average number of cells/cm^2 did not differ ($P>0.05$) between PEM from sows fed a control diet or fed a diet with supplemental L-carnitine (Figure 1, Panel A); as expected, however, there was a time effect ($P<0.05$). As the number of hours post-plating increased, the average number of cells/cm^2 also increased ($P<0.05$) between each of the evaluated time-periods. At the conclusion of the experiment (72 h), there were 11% more cells in the L-carnitine treatment than in the control.

An indicator of PEM proliferation is the rate of [^{3}H] incorporation. Incorporation of [^{3}H]thymidine/cell was not altered ($P>0.05$) by treatment at 36, 48, 60, or 72 h post-plating (Figure 1, Panel B).

There was a treatment x time interaction ($P = 0.05$) for the number of doublings (Figure 1, Panel C). There was a 1.5-fold increase ($P<0.01$) in the number of doublings between 36 and 48 h for the PEM isolated from dams fed L-carnitine, compared with the number of doublings in PEM from control dams. At the remaining time intervals of 48/60 and 60/72 h,

there were no differences (P>0.05) detected between the two treatments.

Porcine embryonic myoblasts isolated from a commercial gilt fed a diet containing no supplemental L-carnitine were used to evaluate the direct effects of L-carnitine. No differences (P>0.05) were observed for the percentage change in counts per minute between the six L-carnitine concentrations and the control (Figure 2).

Total RNA was isolated from the PEM cultures at 96 h post-plating. The expression of IGF-I, IGF-II, IGFBP-3, IGFBP-5, and myogenin mRNA were determined (Table 2). There was no difference (P>0.05) between treatments for the expression of IGF-I or IGFBP-5. But PEM isolated from sows fed L-carnitine had decreased (P<0.05) mRNA concentrations of IGF-II, IGFBP-3, and myogenin (61, 59, and 67%, respectively) compared with those of PEM isolated from control sows.

Little is known about the effects of L-carnitine on the IGF system in primary porcine muscle cell cultures. Two different models were used to determine the direct and indirect effects of L-carnitine on PEM proliferation. A diet with no supplemental L-carnitine was fed to a gilt, and then PEM were isolated from fetal muscle and used to determine the direct effects of adding L-carnitine *in vitro*. The indirect effects of L-carnitine were evaluated by isolating PEM from sows either supplemented with L-carnitine or fed a control diet during gestation. The increased concentrations of circulating L-carnitine in the gestating sows supplemented with L-carnitine suggests that the L-carnitine is being absorbed and entering the blood stream. Previous research at Kansas State University also observed that sows supplemented with L-carnitine had increased concentrations of circulating carnitine.

At the initiation of the experiment, there was not a treatment difference in the number of cells at 36 h. At each succeeding time increment, however, a numerical increase was observed for the number of PEM isolated from sows supplemented with L-carnitine and there were 11% more cells in the L-carnitine treatment than the control at 72 h. In support of the increased number of cells at the conclusion of the experiment, there was a 1.5-fold increase in the number of doublings between 36 and 48 h post-plating in the L-carnitine cells, compared with the number of doublings in cells from the controls. This suggests that PEM isolated from sows fed L-carnitine have an initial increased rate of proliferation and that this alteration in proliferation early during the culture period is resulting in numerical increases in total cells at the conclusion of proliferation. These data support earlier reports that offspring from dams supplemented with L-carnitine had a larger cross-sectional area in the semitendinosus muscle. A concern with primary cultures is that non-myogenic cell types, which could have shorter doubling times, are present and can make interpretation of proliferation data difficult. Results of the differentiation studies (data not shown) suggest that the changes in cell proliferation were a result of treatment affecting only myogenic cells, indicating that the increased rate of proliferation in cultures derived from L-carnitine is not due to the potential for greater proliferation of non-myogenic cells overtaking these cultures.

Even though there was not a treatment effect, the uptake of [^{3}H]thymidine in the PEM isolated from sows supplemented with L-carnitine continued to increase up to 60 h post-plating, but only up to 48 h in the control cultures. At the last evaluated time (72 h), a numerically greater uptake of [^{3}H]thymidine remained in the L-carnitine cultures. This is consistent for the observed numerical increase in number of cells in the L-carnitine cultures compared with the number of cells in the controls at the end of the assay.

To determine if the effects of L-carnitine on the proliferation data were a direct effect of L-carnitine, another experiment using PEM isolated from a commercial pregnant gilt was conducted by incubating the PEM with differing L-carnitine concentrations. At the six L-carnitine concentrations evaluated, no consistent differences between the concentrations and the control were found for the incorporation of [^{3}H]thymidine into the PEM. There was no trend amongst the L-carnitine concentrations for [^{3}H]thymidine incorporation. This indicates that L-carnitine is most likely not directly affecting the proliferation of the PEM, and that the increased number of doublings from the L-carnitine treatment is a result of an indirect effect. Another explanation comes from a previous study in which it was reported that human skeletal muscle in culture had two different affinities for carnitine uptake: a high-affinity uptake between 0.5 and 10 μmol/L and a low-affinity uptake between 25 and 200 μmol/L carnitine. Therefore, in the current study, the lack of a trend between the proliferation rate of the PEM and the L-carnitine concentration may be caused by the differing affinities for carnitine uptake by the PEM.

The effect of feeding L-carnitine on carcass muscle characteristics has been studied extensively. Research has reported that feeding sows 100 mg/d of L-carnitine resulted in a 90% increase in maternal circulating IGF-I concentration at mid-gestation compared with that of control sows. But little is known about the interactive effects of L-carnitine and the growth factors or about the singular effects of L-carnitine on porcine fetal muscle growth and development. It was hypothesized that L-carnitine may be influencing the IGF system. Many cell types, including myogenic cell lines, have been shown to synthesize both IGF and IGFBP. Previous studies have identified that multiple components of the IGF system affect the proliferation and differentiation of PEM. In the current study, growth factor, binding protein, and myogenin mRNA concentrations were determined in PEM at 96 h in culture. Even though not significant, the expression of IGF-I was increased 82% in PEM isolated from dams supplemented with L-carnitine, compared with expression in non-supplemented sows. High concentrations of IGF-I increased [^{3}H]thymidine incorporation into primary porcine cell cultures, and IGF-I has been shown to extend proliferation of muscle cells. Therefore, the observed numerical increase of IGF-I mRNA in L-carnitine cultures suggests that L-carnitine increases local IGF-I production. The increased IGF-I availability in the L-carnitine cultures explains why the rate of [^{3}H]thymidine incorporation/cell continued to increase for an additional 12 h, compared with that of the controls. In addition, there was an accumulation of cyclin D1 (10-fold increase) by mid-G_1 phase of the cell cycle in satellite cells isolated from IGF-I transgenic mouse muscle, in comparison with accumulations in muscle from control littermates. This suggests that IGF-I enhances the replicative life span of myogenic cells, which is also observed in the current study because of the increased doublings and the total number of cells at the end in the L-carnitine cultures.

During fetal muscle development, the progression of events in myogenesis is that mononucleated proliferating myoblast exit the cell cycle and fuse to form multi-nucleated cells or myotubes. Myoblasts withdrawal from the cell cycle activates transcription factors (MyoD family regulatory factors), with one being myogenin, which is expressed at the onset of myogenesis. Myogenin is not expressed in proliferating myoblasts, but only under differentiation conditions, and is often used as an early marker of differentiation in muscle cell cultures.

In addition to the transcription factors, IGF-II has been found to modulate fetal growth and development. Myoblasts themselves can secrete IGF-II, which can itself induce differentiation, or IGF-II can promote differentiation through paracrine and endo-

crine mechanisms. In C2 myoblasts, IGF-II mRNA concentrations rise dramatically (4-fold) during differentiation, compared with those expressed during proliferation. It is suggested that the autocrine secretion of IGF-II is essential for the process of terminal differentiation in these cells because the rate of differentiation correlates with the level of expression of IGF-II. In the current study, IGF-II mRNA concentrations were decreased 61% and myogenin was decreased 67% in L-carnitine PEM, compared with those of the control PEM. Taken together, the IGF-II and myogenin data suggest that there is a delay in the onset of terminal differentiation in PEM isolated from sows fed L-carnitine, thus allowing these cells to remain in a proliferative stage longer. Furthermore, the numerical difference in IGF-I mRNA would be consistent with cells having increased proliferative capacity.

The IGF binding proteins are known for their ability to bind growth factors to increase their stability and half-life, as well as to modulate the action of the growth factors. In this study, the mRNA concentrations of IGFBP-3 and IGFBP-5 were reduced 59% and 50%, respectively, in the PEM isolated from the sows supplemented with L-carnitine, compared with their concentrations in the control sows. This suggests that there would be less binding capacity in PEM from supplemented sows, and that the action of IGF-I may be enhanced, ultimately increasing cell proliferation. The decrease in IGFBP-3 mRNA concentrations in L-carnitine cultures, compared with those of the controls, indicates that the L-carnitine cultures were at the initiation of differentiation at 96 h in culture. It has been reported that in extensively fused cultures (144 h in culture) there was a three-fold increase in IGFBP-3 mRNA concentration, compared with that of the nonfused cultures. This also supports the current proliferation data, that L-carnitine cultures proliferate longer and, hence, would have lower IGFBP-3 mRNA concentrations. The decrease in the expression of IGFBP-5 in the PEM isolated from the L-carnitine treatment also supports that there would be increased proliferation in this treatment because IGFBP-5 is secreted within 12 h of the onset of differentiation in myoblasts (14) and is associated with terminal differentiation.

We have shown that L-carnitine influences muscle growth through an indirect mechanism, as evidenced by the prolonged proliferation and suppressed differentiation in the PEM isolated from sows supplemented with L-carnitine. The increased proliferation rates and the 82% increase in IGF-I mRNA are reported characteristics specific for myogenic cells. The observed expression of EGFBP-3 is further evidence in the current study that the prolonged proliferation was not the result of contaminating non-myogenic cells because IGFBP mRNA is not produced by fibroblasts.

In summary, the enhanced IGF-I concentration and decreased IGFBP-3 could potentiate IGF-I actions on proliferation. At the same time, IGF-II and myogenin mRNA concentrations are reduced, resulting in suppressed differentiation. These results, in combination with the developmental pattern of the growth factors, show that L-carnitine plays a role in regulating proliferation and differentiation of cultured porcine embryonic myogenic cells, and that fetal muscle growth and development could be increased.

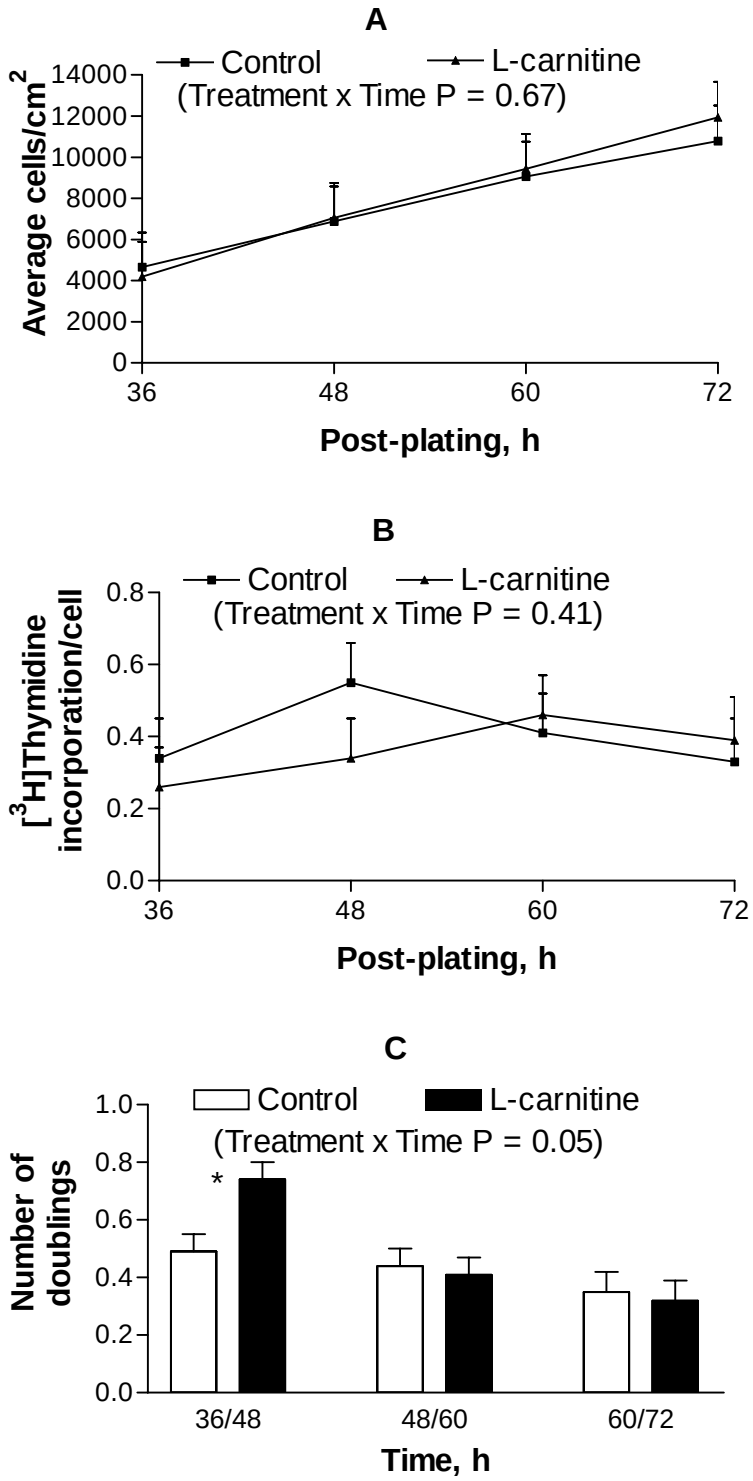

Figure 1 The Average Number of Cells per cm^2 (panel A), [^{3}H]thymidine Incorporation per Cell (panel B), and the Number of Doublings (panel C) at Designated Times Post-plating (36, 48, 60, 72 h post-plating) in Porcine Embryonic Myoblasts (PEM) Isolated from Dams Fed a Control Diet or Supplemented 100 mg/day of L-carnitine. Values are means and SEM, n = 6 per treatment. There was a treatment x time interaction (P = 0.05) for the number of doublings. The number of doublings was greater (P < 0.05) between 36 and 48 h for the PEM isolated from dams fed L-carnitine, compared with those of the controls, and the asterisk indicates this difference.

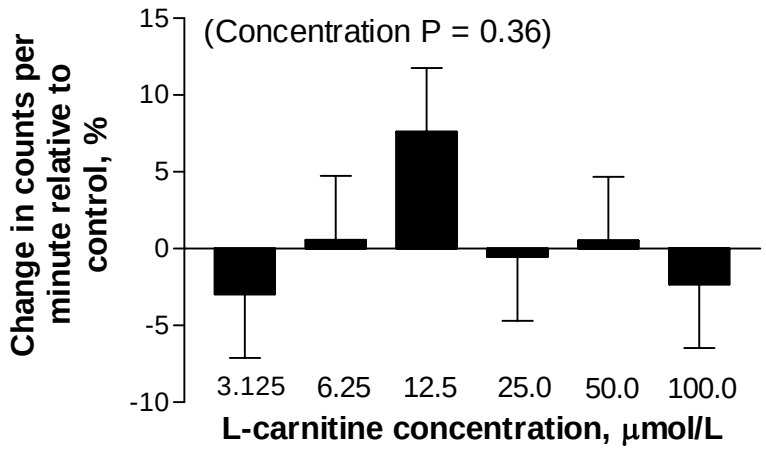

Figure 2 The Effect of Differing Concentrations of L-carnitine in 2% Swine Serum (v/v) and Dulbecco's Modified Eagle Medium on the Percentage Change of Counts per Minute in Comparison with those of the Control for Porcine Embryonic Myoblasts (PEM). The PEM were incubated with the L-carnitine for 24 h, between 48 and 72 h post-plating. Values are means and SEM, n = 4 per concentration.

Table 2. Growth-Factor Messenger RNA Content in Porcine Embryonic Muscle Cells at 96 h of Culture[a]

Gene	Control	L-carnitine	SEM	P-value
IGF-I	3.47	6.31	1.89	0.31
IGF-II	21.68	8.44	3.54	0.02
IGFBP-3	5.62	2.30	0.90	0.03
IGFBP-5	22.49	11.24	4.85	0.13
Myogenin	4.02	1.33	0.79	0.04

[a]Gene expression levels are expressed in relative units; n = 6 per treatment.