Dietary Conjugated Linoleic Acids Decrease Leptin in Porcine Adipose Tissue¹–³

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Abstract

We investigated the effects of dietary conjugated linoleic acids (CLA) on white adipose tissue (WAT) in heavy pigs. Twelve pigs were assigned to 1 of 2 groups supplemented with either 0 or 0.75% of a CLA preparation (isomeric mixture) and were slaughtered at 159 ± 2.3 kg live weight. Their subcutaneous WAT was analyzed by both chemical and microanatomical methods. The WAT of CLA-fed pigs tended to have a higher protein content (P = 0.064) and smaller adipocytes (P = 0.053) than that of control (CTR) pigs. The number of proliferating preadipocytes tended to be greater (P = 0.076) in pigs fed CLA, whereas the number of apoptotic adipocytes was greater (P < 0.01) than in CTR pigs. Immunohistochemistry revealed that leptin (Ob) expression was lower (P = 0.048) in adipocytes from treated pigs and Western blot quantification of Ob revealed lower levels (P < 0.05) in CLA-fed pigs. The Ob receptor was not affected by dietary CLA supplementation. Tyrosine hydroxylase activity was higher (P < 0.001) in WAT of CLA-fed pigs than in CTR. It is conceivable that the increased noradrenergic activity due to dietary CLA decreases the Ob expression, although it does not diminish the lipid content of WAT, at least in heavy pigs. This article describes the interaction between CLA and Ob in the WAT of heavy pigs and we hypothesize that there is an increased noradrenergic stimulation of lipolysis directly in the target tissue. J. Nutr. 139: 1867–1872, 2009.

Introduction

The conjugated linoleic acids (CLA)⁵ are a group of positional and geometric isomers of linoleic acid that are characterized by the presence of conjugated dienes and differ in both the position and stereochemistry of their double bonds. The 2 isomers that have been shown to exhibit biological activity are trans-10, cis-12 (t-10, c-12) CLA and cis-9, trans-11 (c-9, t-11) CLA (1), which have been largely employed as food supplements in commercial CLA isomeric mixtures (2). Several pieces of data demonstrate that dietary CLA reduces the accumulation of adipose tissue in several mammalian species (3), although there are species- and breed-specific differences (4–7). The mechanisms of action of CLA on white adipose tissue (WAT) are not fully clarified at present because of both discrepancies between in vitro and in vivo studies and the possibility that CLA act in different ways and produce different consequences for target structures when administered in the diet to different species (8). Mammalian WAT produces several hormonal proteins, including leptin (Ob), which is produced and secreted by the white adipocytes with numerous effects on metabolism, immune response, reproduction, and feeding behavior in rodents and humans (9,10). Ob has direct autocrine or paracrine effects on adipocytes, which in turn may contribute to the weight- and fat-reducing activity of this hormonal messenger (10).

Adipocytes synthesize several membrane-bound and soluble isoforms of Ob receptors that are involved in the interactions of Ob with other membrane-bound receptors in the tissue (11). Immunohistochemical studies show a widespread distribution of long isoforms (Ob-Rb), which are considered to be the major signaling form of the receptor detected in a variety of porcine tissues, including WAT (12).

Several hormones and chemical messengers can regulate Ob synthesis and release both in vivo and in vitro. The most important of these messengers are insulin and norepinephrine (NE), and it is known that fatty acids with a chain length equal or superior to 8 carbons mimic the noradrenergic stimulation of lipolysis (13).

Considering that, to our knowledge, the interaction between CLA and adipocyte Ob has not been studied in mammalian WAT, the aim of this work was to describe the effects of CLA
(isomorphic mixture) dietary intervention upon Ob, Ob-Rb, and noradrenergic innervation in the porcine WAT.

### Methods

Morpho-functional and chemical aspects of subcutaneous WAT were evaluated after 3 mo of dietary treatment in pigs slaughtered at 9 mo of age at a live weight (LW) of 160 ± 10% kg (Council Regulation [EEC] no. 2081/92 for Parma Ham production), characterized by a greater fat deposition (14%) than pigs slaughtered at a lighter weight (110–120 kg LW) for meat production.

**Pigs and diets.** Twelve castrated male crossbred pigs (Goland × Hypor) with an initial LW of 106.4 ± 1.8 kg were assigned to 1 of 2 LW-matched groups. Pigs were housed in individual cages with totally slatted floors under environmentally controlled conditions (temperature, 16°C; relative humidity, 60%). The CLA group received a diet supplemented with 0.75% CLA. The control group (CTR) received an isonitrogenic diet containing 0.75% soybean oil instead of CLA. The diets were formulated to meet NRC requirements (15) (Table 1). The CLA preparation contained 50% CLA isomers (equal quantities of α-9, 9-11, and 9-10, c, c-12 isomers) in the form of FFA (CLA oil, Silo s.r.l.). The diets were administered twice daily for 3 mo at 9% of LW. The pigs were slaughtered at 159 ± 2.3 kg LW, as prescribed by Parma ham regulation (Council Regulation [EEC] no. 2081/92). The amounts of feed offered were recorded and unconsumed feed was weighed daily. The carcass weights were recorded and the dressing percentage (hot carcass weight/weight at slaughter) was calculated. The pigs used in this study were cared for in accordance with European Union guidelines (no. 86/609/EEC), which were approved by the Italian Ministry of Health (L.116/92).

**Blood and tissue samples.** At the end of the trial, blood samples were collected by vena cava puncture before the morning feeding. The blood samples were collected in 10-mL vacutainer glass tubes (Venoject, Terumo Europe N.V.), immediately placed on ice, and subsequently centrifuged at 8500 × g for 15 min. Sera were partitioned into aliquots and stored at −20°C pending analysis.

### TABLE 1 Diet composition (as fed basis)†

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/kg</th>
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<tbody>
<tr>
<td>Maize</td>
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</tr>
<tr>
<td>Barley</td>
<td>220</td>
</tr>
<tr>
<td>Wheat middings</td>
<td>140</td>
</tr>
<tr>
<td>Soybean meal</td>
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</tr>
<tr>
<td>Corn gluten meal, 60%</td>
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<td>Vitamin premix2</td>
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<td>L-lysine HCl</td>
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<td>Oil2</td>
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<tr>
<td>Ether extract</td>
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<tr>
<td>Crude fiber</td>
<td>4.05</td>
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<tr>
<td>Ash</td>
<td>4.62</td>
</tr>
</tbody>
</table>

† Diets formulated to contain 13.15 kJ/g metabolizable energy, 0.68% lysine, 0.27% methionine, 0.27% cystine, 0.51% threonine, and 0.14% tryptophan.
² Providing the following per kg: Fe (FeSO₄·H₂O), 100 mg; Zn (ZnO), 100 mg; Cu (CuSO₄·5H₂O), 15 mg; Mn (MnO), 25 mg; I (CaI₂·2H₂O), 1 mg; Se (Na₂SeO₃), 0.1 mg; retinol, 300 μg; cholecalciferol, 25 μg; α-tocopheryl acetate, 30 mg; menadione bisulfate, 2 mg; thiamine, 2 mg; riboflavin, 3 mg; pyridoxine, 6 mg; cyanocobalamin, 0.02 mg; biotin, 0.1 mg; nicotinic acid, 25 mg; folic acid, 1 mg; and choline, 500 mg.
³ The CTR diet containing 0.75% soybean oil instead of 0.75% CLA oil. The fatty acid composition of the soybean oil (g/100 g total fatty acids) was: palmitic acid, 12%; stearic acid, 4.40%; oleic acid, 22%; linoleic acid, 54%; and linolenic acid, 7.60%. The fatty acid composition of the CLA oil (g/100 g total fatty acids) was: palmitic acid, 11%; stearic acid, 3.5%; oleic acid, 33%; linoleic acid, 2%; CLA, c-9, t-11, 24.5%; and CLA t-10, c-12, 25.5%.

At slaughter, a sample of WAT was immediately excised at the level of the thigh of the left pelvic limb. The samples were vacuum-packed and stored at −20°C.

For micro-anatomical analyses, additional samples (1 cm³) were removed at the same localization. These samples were promptly fixed in Bouin’s fixative for 24 h at 4°C, dehydrated in graded alcohols, cleared with xylene, and embedded in paraffin. Other 1-cm³ samples were frozen in isopentane cooled in liquid nitrogen and stored at −20°C.

### Chemical analyses.** Chemical analyses for dry matter, ether extract, protein, and ash were conducted on WAT samples (16).

**Serum metabolite analyses.** Triacylglycerol (COD 11529 Biosystems), total cholesterol (COD 11506 Biosystems), glucose (COD 11504 Biosystems), urea (Alfa Biotech), and total protein content (Alfa Biotech) were determined in serum by enzymatic spectrophotometer assays using a spectrophotometer (V-630 Bio UV-Vis Spectrophotometer Jasco).

**Western blot analysis and tissue homogenization.** Tissue specimens were homogenized in 2 mL of ice-cold lysis buffer (50 mMol/L Tris-HCl, pH 7.5, 0.2% Triton X-100, and protease inhibitor mixture) per 0.2 g of tissue using an Ultra-Turrax homogenizer (IK-Arke). The homogenate was then centrifuged at 20,000 × g for 20 min at 4°C. The supernatant was collected and the protein concentration was determined by the Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

The proteins (35 μg) were resolved by 15% SDS-PAGE, electro-transferred onto nitrocellulose membranes, and subsequently incubated with an anti-swine monoclonal antibody (1:100). Immunoreactivity was detected by chemiluminescence autoradiography according to the manufacturer’s instructions and the images were scanned (CANO SCAN 4200F). The OD of the protein bands were determined densitometrically using Scion Image software.

### Micro-anatomical analyses

**Histological and histometric analyses.** Each of the paraffin-embedded specimens was cut with a microtome to obtain serial sections (10 μm thick), which were stained with hematoxylin and eosin to assess morphology under a light microscope (Olympus BX51). For histometry, the area of 100 adipocytes from each section was determined under the microscope (magnification ×200) using the DP software for image analysis (Olympus). Cell number estimation was calculated from the mean cell volume in 1 g of tissue according to the method reported by Mersmann et al. (17).

**Immunohistochemistry: proliferating and apoptotic adipose cells.** Paraffin-embedded sections were processed to reveal adipocytes, which were in the S-phase of the cell cycle as revealed by immunostaining with a monoclonal antiserum against proliferating cell nuclear antigen (PCNA; clone PC10, Sigma) (dilution, 1:3000) as described previously (18). The PCNA-immunoreactive nuclei will, for brevity, be designated as belonging to proliferating (PRO) cells. The specificity of immunostaining was tested by incubating other sections with normal pig serum instead of the primary antiserum; this procedure always produced negative results. As positive controls, alimentary canal samples from calf and dog were tested; in all cases, the expected positive reactions were observed in the intestinal crypts.

Other deparaffinized sections were processed to identify adipocytes that were undergoing apoptosis. Apoptotic (APO) nuclei were identified using a modified TdT-mediated dUTP nick end–labeling technique (TUNEL; DeadEnd Colorimetric TUNEL System, Promega), as described previously (18). The TUNEL-reactive nuclei will, for brevity, be designated as belonging to proliferating (PRO) cells. The specificity of immunostaining was tested by incubating other sections with normal pig serum instead of the primary antiserum; this procedure always produced negative results. As positive controls, alimentary canal samples from calf and dog were tested; in all cases, the expected positive reactions were observed in the intestinal villi.

With the aid of the DP software for image analysis, APO and PRO cells were counted in at least 100 adipose cells per section (3 sections for each sample to yield 300 evaluated adipose cells per pig). Results were expressed as percentages of positive cells.

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Fluorescence immunohistochemistry: tyrosine hydroxylase, Ob, and Ob-Rb. Immunohistochemistry toward tyrosine hydroxylase (TH) was used to reveal noradrenergic innervation. Frozen sections (10 μm thick) were incubated with a 1:100 rabbit anti-goat TH (Chemicon) primary antiserum for 24 h at 20°C, washed in Tris buffered saline-Triton (TBS-T), and subsequently treated with the biotin-avidin blocking kit solution (Vector Laboratories). Sections were then incubated with a solution of goat biotinylated anti-rabbit IgG (Vector Laboratories; 0.01 μg/L in TBS-T) for 1 h at 20°C. After rinsing twice in TBS-T, the sections were treated with fluorescein avidin D (Vector Laboratories; 0.01 μg/L in 0.1 mol/L NaHCO₃, pH 8.5, with 0.15 mol/L NaCl). Finally, slides were embedded in Vectashield mounting medium (Vector Laboratories).

Double fluorescence labeling for goat anti-rabbit Ob (Santa Cruz Biotechnology; 1:50) and rabbit anti-goat Ob-Rb (Santa Cruz Biotechnology; 1:50) was carried out on the same sections using established protocols (19). Sections from specimens of the experimental groups were stained at the same time with excitation and barrier filters set for fluorescein (Ob) and rhodamine (Ob-Rb).

The sections were observed using a confocal laser scanning microscope (FluoView FV300, Olympus). The specificity of the immunofluorescence staining reactions was tested as follows: 1) substitution of either the Ob-, Ob-Rb, and TH antibodies by nonimmune serum (negative controls); the immunoreactions were not present; 2) application of immunofluorescence tests to rabbit stomach and subcutaneous adipose tissue sections (positive controls): the expected positive reactions were detected.

Image analysis
To quantify Ob-, Ob-Rb-, and TH-immunoreactivities, adipose tissue sections were examined using a confocal laser microscope (Olympus FV300) equipped with argon/helium/neon lasers and the FluoView software for image analysis (Olympus) according to Corino et al. (19). Prior to quantification, the images were digitally zoomed by 2.8 times. Pixel intensity was determined using the histogram/area functions of the FluoView software, which assigned gray levels (GL) within a 0–256 gray scale. Data were presented as mean fluorescence intensities.

Statistical analysis. Statistical analysis of the data were performed using SAS statistical software (SAS Institute 2000, version 8.2000). Data from the performance, chemical (WAT), serum metabolite, and Western blot analyses were analyzed using I-way ANOVA with diet as the main factor. Data from the histometric, immunohistochemical, and immunofluorescent analyses were analyzed by mixed-model ANOVA that included the fixed effects of the treatment and the random effect of each pig. Values from each pig were considered as the experimental unit of all response variables. Data are presented as least squared means ± SEM. Differences between means were considered significant at P ≤ 0.05.

Results
Dietary CLA supplementation did not affect pig carcass characteristics (Supplemental Table 1).

Chemical analyses of subcutaneous WAT. Dry matter (92 ± 0.4%), lipid content (90.2 ± 0.4% wet weight), and ash content (0.1 ± 0.03% wet weight) were not affected by dietary CLA supplementation. The protein content tended to be greater (P = 0.064) in CLA-fed pigs (1.9 ± 0.1% wet weight) than in CTR pigs (1.1 ± 0.3% wet weight).

Serum metabolites. Dietary CLA did not affect serum glucose (5.4 ± 0.1 mmol/L), cholesterol (3.1 ± 0.3 mmol/L), urea (5.2 ± 0.2 mmol/L), or protein concentrations (65.7 ± 2.5 g/L). The concentration of triacylglycerol tended to be greater (P = 0.089) in the serum of CLA-fed pigs (0.5 ± 0.05 mmol/L) than in CTR pigs (0.3 ± 0.01 mmol/L).

Western blot analysis. Western blot analysis with an Ob species-specific antibody detected a band of 16 kDa in pig WAT (Fig. 1). Quantification of the Ob band OD revealed that CTR pigs had more Ob in WAT (486 ± 69 OD; P < 0.05) than did CLA-fed pigs (195 ± 42 OD).

Histological, histometrical, and immunohistochemical analyses. Histological examination revealed that WAT of the pigs of both CLA and CTR groups had well-structured adipocytes; the typical, mature unilocular adipocytes (or signet-ring cells) and scarce, small multilocular cells (preadipocytes) were evident in all of the examined samples.

Adipocyte area tended to be less (P = 0.053) in WAT of CLA-fed pigs than in CTR pigs (Table 2). The estimated adipocyte number did not differ between the 2 groups.

PCNA-positive nuclei were found in preadipocytes only. TUNEL-positive nuclei were found in adipocytes. The number of PRO preadipocytes tended to be greater (P = 0.076) in pigs fed CLA and the number of APO adipocytes was higher (P < 0.01) in CLA-fed pigs than in CTR pigs (Table 2).

Fluorescence immunohistochemistry: Ob, Ob-Rb, and TH. Immunofluorescence revealed that both Ob and the Ob-Rb were evident in all of the examined samples. Immunofluorescence reactions was tested as follows: 1) substitution of either the Ob-, Ob-Rb, and TH antibodies by nonimmune serum (negative controls); the immunoreactions were not present; 2) application of immunofluorescence tests to rabbit stomach and subcutaneous adipose tissue sections (positive controls): the expected positive reactions were detected.

Discussion
WAT. Histology revealed that CLA did not produce any detrimental effects on pig WAT. As we have already observed in previous studies in both this species (19) and rabbits (20), tissue structure was similar in the specimens from both groups. Histometry showed that CLA supplementation tended to reduce adipocyte area, as also observed by Noto et al. (21) and Azain et al. (22) in rats and by Zhou et al. (23) in pigs. Moreover, we observed a numerically, but not significantly, greater protein content and a slightly numerically lower lipid content in WAT of CLA-fed pigs than in CTR pigs; this result may have been due to the numerically greater number of adipocytes per g of tissue. We suggest that the greater protein content, likely associated with a higher cellularity (DNA, protein, and collagen), is consistent with a smaller adipocyte area and more adipocytes. Because the P-values for these comparisons (protein content, P = 0.064; adipocyte area, P = 0.053) were fairly close to 0.05, it is possible that more replications (n = 8) would be needed to obtain a significant difference between CTR and treated groups. On the
other hand, CLA dietary intervention did not reduce the chemically identified lipid content. This may depend upon sex, LW, genetics, degree of fatness, or a combination of these (24,25). We suggest that the applied dose of 0.75% CLA did not produce an evident chemical reduction of WAT lipid content in the heavy pigs used in the production of Parma ham, as they are characterized by greater fat deposition than pigs slaughtered at lighter weight, as previously observed (19).

Immunohistochemical analyses showed that the number of APO adipocytes was higher in CLA-fed pigs, thus confirming our previous results (19) and data from Tsuboyama-Kasaoka et al. (26) in mice.

**Serum metabolites.** The serum concentration of triacylglycerol tended to be greater in CLA-fed rabbits in one of our previous studies (20). It has been reported that CLA increases glycerol release in cultured 3T3-L1 adipocytes (27) and that this effect is induced by both CLA isomers. This result is consistent with a numerically lower lipid content and a smaller adipocyte area in CLA fed pigs; these data may be explained by a CLA-induced reduction in lipoprotein lipase activity in WAT, as found in vitro by Lin et al. (28) and in neonatal pigs by Corl et al. (29).

**Ob expression.** Ob and Ob-Rb were colocalized to the thin peripheral cytoplasmic rim of the adipocytes, making it possible for Ob to act directly on adipocytes and preadipocytes to regulate growth and metabolism (10). Lee and Fried (30) showed that larger adipocytes from obese rats contained 5 times the amount of Ob stores of smaller fat cells from nonobese rats. They also speculated that the high Ob release from a larger intracellular pool may contribute to the higher basal secretion and rapid increases in Ob release after meals.

In the present study, we found that Ob-Rb expression was not affected by CLA supplementation, despite the lower Ob expression in the adipocytes of CLA-fed pigs than of CTR pigs, that was observed by both immunofluorescence and Western blot. These findings are consistent with the previously mentioned smaller adipocyte area of WAT in CLA-fed pigs compared with CTR pigs. The fact that Ob expression is reduced in the adipocytes of CLA-fed pigs compared with CTR pigs suggests that it increases leptinemia through a negative feedback mechanism. This mechanism may originate in the central nervous system, where Ob-releasing neurons are present in the hippocampus and cerebral cortex (31). Accordingly, previous work from our group has demonstrated that dietary CLA is associated with a marked increase in the serum Ob concentration in sows (32) as well as in rabbits (33).

**TH expression.** Mammalian WAT receives abundant sympathetic innervation. There is now strong neurochemical evidence for the role of the sympathetic nervous system in the functional dynamics of WAT (34). NE, acting through the stimulation of the adipocyte β-adrenoceptors, is recognized as a major stimulator of lipolysis both in vitro and in vivo (35), as observed studying a β-adrenergic agonist (36).

NE may participate in a negative feedback loop by preventing the excessive activation of the sympathetic nervous system and energy expenditure (37). Interestingly, in vitro studies using isolated rat adipocytes have shown that NE stimulates lipolysis and exerts a strong inhibitory effect on insulin-stimulated Ob secretion (13). Furthermore, a negative correlation between NE

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>CTR</th>
<th>CLA</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocyte area, μm²</td>
<td>9008 ± 98</td>
<td>8750 ± 98</td>
<td>0.053</td>
</tr>
<tr>
<td>Adipocyte, n x 10⁻⁷/g WAT</td>
<td>1.6 ± 0.01</td>
<td>1.7 ± 0.01</td>
<td>0.499</td>
</tr>
<tr>
<td>PRO cells, % of total</td>
<td>9.2 ± 0.6</td>
<td>10.7 ± 0.5</td>
<td>0.076</td>
</tr>
<tr>
<td>APO cells, % of total</td>
<td>8.7 ± 1.3</td>
<td>10.7 ± 1.3</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n = 6.

**FIGURE 2** Ob and Ob-Rb immunofluorescence in the WAT of barrows fed a CLA-supplemented diet. Ob (A) and Ob-Rb (B) are colocalized (C) in the thin peripheral cytoplasmic rim of adipocytes from CLA-fed pigs. Magnification 400×. A color version of this figure is available (Supplemental Fig. 1).
In this study, we show micro-anatomical evidence that the isomeric CLA mixture used decreases pig adipocyte area and that this result may arise from the enhancement of a noradrenergic mechanism, possibly via an enhanced catecholamine release at the local level (WAT). Ob expression is also diminished as a consequence of CLA dietary administration and this is possibly a merely local effect linked to the reduction of the adipocyte areas, which in turn is able to elicit increasing leptinemia of a possible origin from neurons of the central nervous system. Clearly, further study in the CLA-altered fat stores is needed to better understand the mechanism of action of the 2 isomers and provide insight into the regulation of WAT metabolism.

### Literature Cited


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### TABLE 3 Ob, Ob-Rb, and TH immunofluorescence peaks of the WAT of barrows fed a CTR or CLA-supplemented diet

<table>
<thead>
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<th></th>
<th>CTR</th>
<th>CLA</th>
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<td>Ob</td>
<td>19.4 ± 2.0</td>
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</tr>
<tr>
<td>Ob-Rb</td>
<td>27.9 ± 3.9</td>
<td>31.4 ± 3.9</td>
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</tr>
<tr>
<td>TH</td>
<td>162.7 ± 14.6</td>
<td>273 ± 14.6</td>
<td>&lt; 0.001</td>
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</table>

1 Values are means ± SEM, n = 6.


