Stevioside counteracts the alpha cell hypersecretion caused by long-term palmitate exposure

Abbreviated title for the running head: Stevioside counteracts alpha cell hypersecretion

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Reprints are not available.

Keywords: fatty acids, glucagon, gene expression, alpha TC1-6 cells.

Abbreviations: ACC, acetyl-CoA carboxylase-1 · Alpha TC 1-6 cells, alpha tumor cell 1 clone 6 cells · CPT-1, carnitine palmitoyl transferase-I · KRB, Krebs-Ringer buffer · PEPCK, phosphoenol pyruvate carboxykinase · PPAR gamma, peroxisome
proliferator-activated receptor-gamma · SCD, stearoyl-CoA desaturase · SREBP-1c, sterol regulatory element-binding protein-1c · TG, triglyceride

Abstract

Long-term exposure to fatty acids impairs beta cell function in type 2 diabetes while little is known about the chronic effects of fatty acids on alpha cell. We therefore studied the prolonged impact of palmitate on alpha cell function and on the expression of genes related to fuel metabolism. We also investigated if the antihyperglycaemic agent, stevioside, was able to counteract these effects of palmitate. Clonal alpha TC1-6 cells were cultured with palmitate in the presence or absence of stevioside. After 72h we evaluated glucagon secretion, glucagon content, triglyceride content and changes in the gene expression. Glucagon secretion was dose-dependently increased after 72h culture with palmitate at concentrations ≥ 0.25 mM (P<0.05). Palmitate (0.5mM) enhanced triglyceride content of alpha cells by 73% (P<0.01). Interestingly, stevioside (10^-8 and 10^-6 M) reduced palmitate-stimulated glucagon release by 22% and 45%, respectively (P<0.01). There was no significant change in glucagon content after 72h culture with palmitate and/or stevioside. Palmitate increased CPT-1 mRNA level while stevioside enhanced CPT-1, PPAR gamma and SCD gene expressions in the presence of palmitate (P<0.05). In conclusion, long-term exposure to elevated fatty acids leads to a hypersecretion of glucagon and an accumulation of triglyceride content in clonal alpha TC1-6 cells. Stevioside was able to counteract the alpha cell hypersecretion caused by palmitate and enhanced the expression of genes involved in fatty acid metabolism. This indicates that stevioside may be a promising antidiabetic agent in treatment of type 2 diabetes.
Introduction

The pathogenesis of type 2 diabetes is complex and usually requires defects both in islet cell function and insulin sensitivity. The islet cell dysfunction is characterised by failure of beta cells to compensate for insulin resistance and by a concomitant enhanced glucagon release from alpha cells. Diabetic patients are usually obese with ectopic fat deposition in pancreatic islets, liver, heart, skeletal muscle, and blood vessels (1-4). Growing evidence suggests that fatty acid accumulation in non-adipose tissues may play an essential role in the pathogenesis of diabetes (1-4). Considerable attention has been paid to the deleterious effect of fatty acids on pancreatic beta cell. In vitro observations have shown that elevated fatty acids increase basal insulin secretion but inhibit glucose-induced insulin release (5-9). In addition, moderately elevated fatty acids, especially palmitate, a predominant fatty acid in human plasma, are toxic to beta cells(8-12).

Recent studies using microarray technology indicate that fatty acids change the expression of hundreds of beta cell genes (9; 12). Palmitate modifies a series of gene expressions in beta cells depending on the concentration and exposure time of palmitate (8; 9). Disruption of the lipid metabolism is implicated as a key contributor to fatty acid accumulation (1-4; 6). Chronically elevated levels of fatty acids dramatically influence beta-cell lipid metabolism via changes in the activity and expression of enzymes that are involved in glucose and lipid metabolism, as well as in the expression level of key transcription factors (3; 5; 6; 9; 12-14).

Thirty years ago, Unger and Orci (15) proposed the bihormonal-abnormality hypothesis, which highlighted that both deficient insulin secretion and excessive glucagon level contribute to the hyperglycemic state. Although considerable studies on beta-cell function have been carried out, much less attention has been focused on
the influence of fatty acids on alpha cell. Recently, we have demonstrated that acute exposure to fatty acids directly stimulates glucagon release from pancreatic alpha cells (16). The stimulatory action on glucagon of individual fatty acids is influenced by chain length, spatial configuration and degree of unsaturation of fatty acids (16). However, the long-term effect of fatty acid on alpha cell function remains to be clarified. To our knowledge, there is no report focused on the chronic effect of fatty acids on alpha cell.

The present study was designed to elucidate the chronic effects of palmitate on glucagon release, glucagon content and triglyceride (TG) content of clonal alpha TC1 clone 6 cells (alpha TC1-6) as well as on the expression of genes involved in fuel metabolism. In addition, we want to evaluate the potential modifying impact of stevioside, an antihyperglycaemic, insulinotropic and glucagonostatic substance (17; 18), on palmitate-induced effect on alpha cells.

**Materials and methods**

**Alpha TC1-6 cell and culture conditions**

The alpha tumor cell 1 line was derived from an adenoma in transgenic mice expressing the SV40 large T-antigen oncogene. The alpha TC1-6 cells were cloned by a limiting dilution method and only secrete glucagon without detectable insulin (19). Alpha TC1-6 cells (passage number 26-49) were cultured in DMEM (GIBCO BRL, Paisley, UK) containing 18 mM glucose and 10% FCS (GIBCO BRL) under an atmosphere of 95% humidified air / 5% CO₂ at 37°C. Cells were passaged once a week after trypsinization and replaced with new medium twice weekly. They were seeded in wells at a density of 0.25x10⁶ cells per culture well (24-well plate) and
0.75x10^6 cells per culture well (6-well plate) 24h before use in the following experiments.

**Glucagon secretion**

Alpha TC1-6 cells were incubated for 72h at 37°C in DMEM with 18 mM glucose in the presence of 0.25-1.0 mM palmitate (Sigma Chemical, St. Louis, MO), 0.5% BSA (fatty acid-free) (Roche, Mannheim, Germany) with or without 10^{-8} to 10^{-6} M stevioside (Sigma). The solution of palmitate was prepared as previously described (9; 16). Briefly, palmitate was prepared by dissolving and heating to equal molar amounts of NaOH and palmitate supplemented with distilled water to a concentration of 500 mmol/l. They were further diluted with 5% BSA (fatty acid free) to 50 mmol/l palmitate. The stock solution was filter sterilized and stored at −20°C and palmitate solution was freshly prepared before each experiment.

After 72h, the cells were preincubated for 30 min in Krebs-Ringer buffer (KRB) containing 3.3 mM glucose and 0.5% BSA (pH 7.4) and subsequently incubated for 2h with KRB containing 18 mM glucose, 0.5% BSA, with or without 0.25-1.0 mM palmitate.

After incubation, supernatants were collected and kept frozen at −20°C for glucagon by radioimmunoassay kit (Linco Research Inc., St Charles, MO).

**Glucagon content**

Cells were cultured in 6-well plates for 72h using the same incubation protocol as described above. Then the media was replaced by glycine-BSA (glycine (Sigma) 100 mM, 0.25% BSA (Sigma), pH 8.8). Alpha-cells were disrupted by sonication twice (Branson Sonifier 250, Danburg, CT, USA) and kept on ice for 15 sec. After centrifugation at 16,000 rpm for 30 min, the supernatants were collected and stored at −20°C for assay.
Gene expression

After 72h culture, total RNA was extracted from alpha TC1-6 cells using TRIzol (GIBCO). To eliminate possible contamination of genomic DNA, the RNA was treated with DNase I (Invitrogen A/S, Taastrup, DK). The cDNA was synthesised from 1 \( \mu \)g of total RNA using iScript reverse transcriptase (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions.

The primers for selected genes were designed using Primer Select program of the Lasergene software package (DNA-STAR, Madison, WI) (Table 1).

Prior to routine use, the optimal annealing temperature and predicted size of the PCR product for each gene were verified by gradient tests and electrophoresis. Samples were analyzed by real time RT-PCR on an i-Cycler PCR system (Bio-Rad, Hercules, CA) using SYBR green detection. The thermal cycling reactions included an initiating hot start at 95°C for 3 min, 45-50 cycles at 95°C for 15s, 55-65°C for 15s, and 72°C for 25s, followed by an elongation step at 72°C for 5 min. A single fluorescence measure was taken after each annealing step. Following amplification, the melting curves of the amplicon were determined to examine the specificity of amplification. The relative quantitative expression was normalized to GAPDH mRNA level.

TG assay

Cells were exposed to 18mM glucose with or without 0.5 mM palmitate as well as stevioside (10^{-7} or 10^{-6} M) in 24-well plates. After 72h, the cells were washed once with 1.2ml PBS (GIBCO), and the amount of cells were estimated by using nuclear staining with SYTO 24 reagents (Roche) (24\( \mu \)l /well) and measured by FLUOstar Galaxy (BMG, ramconA/S, DK). The medium was removed and the cells were frozen 1h at –80 C. Subsequently, cells were incubated for 20 min with TG reagents (Roche) (270\( \mu \)l/well). TG content was determined by a TG GPO-PAP kit (Roche),
and was normalized to cell number. The recovery of TG content was approximately 90%.

**Protein assay**

After the secretion study, the medium was removed and cells were lysed in 0.1M NaOH (Sigma). Intracellular protein was measured by Bio-Rad detergent compatible protein assay kit (Bio-Rad laboratories, Hercules, CA). The glucagon levels were adjusted to protein concentration.

**Statistics**

One-way ANOVA was used for the statistical analysis. Significant differences were considered for \( P \) values less than 0.05. Data are expressed as the mean ± SEM.

**Results**

**Effects of a 72h exposure to palmitate on glucagon secretion**

Fig. 1 shows the impact of palmitate (0.25-1.0 mM) on glucagon secretion from alpha TC1-6 cells in the presence of high glucose (18 mM glucose) with or without palmitate (0.25-1.0 mM).

Alpha TC1-6 cells were exposed to 18 mM glucose for 72 h in the absence (Fig. 1A) or presence (Fig. 1B) of 0.5 mM palmitate, followed by 2h incubation with 18 mM glucose and palmitate at concentrations ranging between 0.25 and 1.0 mM. A stimulatory effect on glucagon secretion was found at palmitate concentrations \( \geq 0.25 \) mM (\( P<0.05 \)) (Fig. 1A and 1B) being most pronounced at 1.0 mM palmitate (\( P<0.01 \)) (Fig. 1B).
Alpha TC1-6 cells were also cultured 72h with palmitate concentrations ranging from 0 to 1.0 mM in the presence of 18 mM glucose, followed by 2h incubation without (Fig. 1C) or with 1.0 mM palmitate (Fig. 1D).

As shown in Fig. 1C, a significant stimulation of glucagon release was observed at palmitate concentrations ≥ 0.5 mM (P<0.05). In the presence of 0.5 and 1.0 mM palmitate for 72h, glucagon secretion was increased by 29 % and 44 % (P<0.05), respectively.

Fig. 1D shows that palmitate (0.25-1.0 mM) induces a subsequent stimulation to 1.0 mM palmitate (P<0.05). The percentage increase to 0.25, 0.5 and 1.0 mM palmitate was 24%, 47% and 75% (P<0.05), respectively.

**Effects of stevioside on palmitate-induced changes of glucagon secretion**

As indicated in Fig. 2, the effect of 72h culture with stevioside on glucagon secretion in the presence of 18 mM glucose and without (left part) and with (right part) 0.5 mM palmitate. Stevioside per se (10-8 to 10-6 M) had no effect on glucagon secretion. However, in the presence of 0.5 mM palmitate, stevioside caused a dose-dependent inhibition of glucagon release (P<0.01). Stevioside at concentrations of 10-8, 10-7 and 10-6 M reduced palmitate-induced glucagon release by 22 %, 38 % and 45 % (P<0.01), respectively.

**Influence of palmitate and stevioside on glucagon content**

Although we found a tendency towards reduced glucagon content after 72h culture with palmitate (0.25-1.0 mM), this did not attain statistical significance (Table 2). Stevioside had no impact on glucagon content after 72h culture with 0.5 mM palmitate (Table 3).

**Impact of palmitate and stevioside on TG content**
At Fig. 3, the TG content of alpha cells exposed to palmitate (0.5 mM) for 72h was increased by 73% (P<0.01). Stevioside (72h) did not influence the TG content (P>0.05) or was able to alter the increased TG content seen in the presence of palmitate.

**Gene expression**

Fig. 4 shows the effect of palmitate on the gene expression profile of acetyl-CoA carboxylase-1 (ACC1), carnitine palmitoyl transferase-1 (CPT-1), glucagon, peroxisome proliferator-activated receptor-gamma (PPAR gamma), stearoyl-CoA desaturase (SCD), and sterol regulatory element-binding protein-1c (SREBP-1c) in alpha TC1-6 cells. When compared to control, only 0.5 mM palmitate increased CPT-1 mRNA level 2.1-fold (P<0.05) while palmitate did not affect the expressions of the other genes.

As seen at Fig. 5, stevioside per se did not cause any major change in the expression of the selected genes. However, in the presence of palmitate (0.5 mM), stevioside (10⁻⁷ and 10⁻⁶ mM) caused an upregulation of the CPT-1 expression by 150% and 170%, respectively. The expressions of PPAR gamma and SCD were also enhanced by the higher stevioside concentration (10⁻⁶ mM) in the presence of palmitate, the ratios being increased 2.7- and 1.7-fold, respectively (P<0.05).

**Discussion**

We have used clonal alpha TC1-6 cells to study the impact of palmitate and stevioside on glucagon release and changes in the expression of selected genes. These cells possess the advantage over primary islets of being a homogeneous cellular population
and have been previously used to study glucagon secretion (16; 20; 21) and gene expressions (21-23).

In this study, we demonstrated that long-term exposure of alpha cells to elevated fatty acids caused glucagon hypersecretion and TG accumulation. Palmitate enhanced glucagon secretion both after 72h culture and after the subsequent 2h incubation in the presence of high glucose to mimic a diabetes-like condition. Whether the hypersecretion of alpha cell reflects “lipotoxicity” or a normal response is unclear. Since palmitate increases glucagon secretion and the net cellular glucagon content appears stable, this indicates glucagon biosynthesis is enhanced. Data presented on a per cell basis rather than normalized to protein content may be a more accurate method.

Lipid overflow causes excessive storage of TG in non-adipocyte cells, leading to impairment of cellular metabolism (3-6; 24). Little is known about the effect of fatty acids on alpha cells. Here, palmitate caused a marked increase in TG content in alpha cells and enhanced the expression of CPT-1, that plays a central role in the partitioning of fatty acids in mitochondrial oxidation (25). The reports of Unger et al indicate that fatty acids raise TG content, upregulate enzymes of oxidation (acyl-CoA oxidase and CPT-1) in normal nonadipocytes, such as islets, and direct fatty acids into oxidative rather than lipogenic pathways (1; 24). This leads to a disposal of unwanted fatty acids and prevents their accumulation as TG (26). Enhancing the expression of CPT-1 by fatty acids with a possible reduction in malonyl-CoA formation, could result in elevated fatty acid oxidation (1; 13; 27). Thus we propose that an increase in beta-oxidation via up-regulation of the expression of CPT-1 gene in alpha cells protects against fatty acid induced alpha cell hypersecretion. Causative relationships
between these alterations in gene expression and secretory function will need to be substantiated in future experiments.

In the diabetic rat, stevioside significantly lowers blood glucose, increases insulin release and suppresses glucagon levels (17; 18). A stimulation of insulin secretion and insulin content was observed in a clonal beta cell line (17; 28). In rat liver, stevioside also slows down gluconeogenesis by decreasing phosphoenol pyruvate carboxykinase (PEPCK) gene expression (29). The direct role of stevioside on alpha-cells is still not clarified. Interestingly, we demonstrated that stevioside is able to reduce the exaggerated glucagon secretion caused by fatty acids and enhance expressions of CPT-1, PPAR gamma and SCD in the presence of palmitate. Stevioside per se did not cause any changes in glucagon levels or in the expression of selected genes. This indicates that stevioside may not influence alpha cell metabolism under normal metabolic conditions. CPT-1 gene is an early response gene induced by fatty acids in beta-cell (27). Up-regulation of CPT-1 can improve the insulin response and is protective against fatty acid-induced insulin resistance (30). This may also be the case for alpha cells. Parton LE et al (31) showed that enhanced activation of PPAR gamma in islet leads to the stimulation of multiple metabolic pathways that favor the disposal of fatty acids (mitochondrial and peroxisomal oxidation, cellular export, and incorporation into diglycerides) rather than the synthesis of fatty acids. In the presence of palmitate, stevioside also enhanced the gene expression of SCD, a central enzyme catalyzing the synthesis of mono-unsaturated fatty acids, mainly oleate (C18:1) and palmitoleate (C16:1) (32). This conversion of palmitate to palmitoleate, would represent a beneficial adaptation to elevated palmitate, because palmitoleate protects against beta-cell apoptosis induced by saturated fatty acids (33). The
transcription factor SREBP-1c was associated with control to the regulation of enzymes implicated in fatty acid synthesis (14; 34).

Taken together, the beneficial effects of stevioside on alpha cell function are likely to relate to enhance the expression of genes involved in fatty acid oxidation (CPT-1), fatty acid disposal (PPAR gamma) and fatty acid conversion (SCD). The genes encoding lipogenesis were unchanged, including ACC1 and SREBP-1c. Therefore, stevioside seems not to be associated with genes that regulated fatty acid synthesis. Our work only analysed a few selected genes related to fuel metabolism. This does not exclude other pathways being involved simultaneously. In future studies, it will be important to clarify possible changes of enzyme activities in lipid metabolism and elucidate the complex gene regulatory networks to understand the precise mechanisms of the action of stevioside.

In conclusion, we have demonstrated that long-term exposure to palmitate causes a hypersecretion of glucagon and an accumulation of TG in clonal alpha TC1-6 cells similar to the diabetic state. Stevioside was able to counteract the alpha cell hypersecretion to palmitate and enhanced the expression of genes involved in fatty acid metabolism, but did not reduce the TG content. This indicates that stevioside may be a promising antidiabetic agent in treatment of type 2 diabetes via counteraction alpha cell dysfunction.

**Acknowledgments**

We thank Dorthe Rasmussen, Kirsten Eriksen, Lene Trudsø and Tove Skrumsager Hansen for excellent technical assistance and D. Jianzhong Xiao and Dr. Yingping Li for helpful instruction. We are grateful to Hans Tornqvist at Novo Nordisk for advice regarding TG content measurement. We are also deeply grateful to Professor Shimon
Efrat, Department of Human Genetics and Molecular Medicine, Tel Aviv University, Israel for providing the alpha TC1-6 cell line.

**Grants**

Financial support was obtained from the Danish Medical Research Council, Novo Nordisk Foundation, Poul and Erna Sehested-Hansens Foundation, and the Danish Diabetes Association.

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Itakura M and Yoshimoto K.** Differentiation phenotypes of pancreatic islet 
beta- and alpha-cells are closely related with homeotic genes and a group of 


Fig. 1
Fig. 2
Fig. 3
Fig. 4
Fig. 5
Table 1. PCR primers for alpha TC1-6 cells

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense Primer (5’-3’)</th>
<th>Antisense Primer (5’-3’)</th>
<th>Base Pairs</th>
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<tr>
<td>ACC1</td>
<td>ACAATCCCGCACCTTCTTCTACTTG</td>
<td>CCTCACGCGCCCTTCTACTAAG</td>
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<tr>
<td>CPT1</td>
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<td>AGGCCAGGCGCGAGGACAGG</td>
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<tr>
<td>GG</td>
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<tr>
<td>PPARr</td>
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<tr>
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<td>TGGAGCAGGTTGGCAGGTAGCTTC</td>
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<tr>
<td>GAPDH</td>
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<td>GGGCATCAGTCTCTTG</td>
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Table 2. Effect of palmitate on glucagon content in alpha TC1-6 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucagon content (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (18 mM glucose)</td>
<td>297.9±33.2</td>
</tr>
<tr>
<td>18 mM glucose + 0.25 mM palmitate</td>
<td>248.4±62.2</td>
</tr>
<tr>
<td>18 mM glucose + 0.5 mM palmitate</td>
<td>255.2±16.9</td>
</tr>
<tr>
<td>18 mM glucose + 1.0 mM palmitate</td>
<td>227.4±43.8</td>
</tr>
</tbody>
</table>

Alpha TC1-6 cells were cultured for 72h with palmitate (0-1.0 mM) (N=6). Values are mean ± SEM.
Table 3. Effect of stevioside on glucagon content in alpha TC1-6 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucagon content (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (18 mM glucose)</td>
<td>304.3±37.5</td>
</tr>
<tr>
<td>18 mM glucose + 10^{-7}M stevioside</td>
<td>280.8±29.8</td>
</tr>
<tr>
<td>18 mM glucose + 10^{-6}M stevioside</td>
<td>266.4±25.4</td>
</tr>
<tr>
<td>18 mM glucose + 0.5 mM palmitate</td>
<td>236.1±6.5</td>
</tr>
<tr>
<td>18 mM glucose + 0.5 mM palmitate + 10^{-7}M stevioside</td>
<td>229.5±16.7</td>
</tr>
<tr>
<td>18 mM glucose + 0.5 mM palmitate + 10^{-6}M stevioside</td>
<td>235.1±16.1</td>
</tr>
</tbody>
</table>

Cells were cultured for 72h with palmitate (0.5 mM) and/or stevioside (10^{-7} to 10^{-6} M) (N=8). Values are mean ± SEM.
Legends to figures

Fig. 1. Dose dependence of palmitate-induced glucagon secretion

Alpha TC1-6 cells were cultured with palmitate (P) (0-1.0 mM) (Fig. C and D) or 0.5 mM palmitate and 18 mM glucose (G) (Fig. A and B). After 72h culture, the cells were incubated 2h with KRB containing palmitate (0-1.0 mM) (Fig. A and B) or 1.0 mM palmitate (Fig. A and B) (N=18). Data are given as mean ± SEM. § denotes a significant difference (P<0.05) compared to control.

Fig. 2. Effect of stevioside on glucagon secretion from alpha TC1-6 cells exposed to 0.5 mM palmitate (P) (grey bars: without palmitate, black bars: with palmitate) in the presence or absence of stevioside (S) (10^{-8} to 10^{-6} M) for 72h (N=18). Values are mean ± SEM. # denotes a significant difference (P<0.05) between the group with palmitate and the group without palmitate. § represents a significant difference (P<0.05) between the group with palmitate and the group with palmitate as well as stevioside.

Fig. 3. Impact of palmitate and stevioside on TG content in alpha TC1-6 cells cultured with (black bars) or without (grey bars) 0.5 mM palmitate (P) as well as stevioside (S) (10^{-7} or 10^{-6} M) for 72h (N=16). Values are mean ± SEM. § denotes a significant difference (P<0.05) compared to control.

Fig. 4. Gene expression (ratios compared with GAPDH) in alpha TC1-6 cells culture for 72h with palmitate (p) (0-1.0 mM) (N=3). All data are expressed relative to control. § denotes a significant difference (P<0.05) compared to control.
Fig. 5. Gene expression (ratios compared with GAPDH) in alpha TC1-6 cells culture for 72h with 0.5 mM palmitate (P) as well as stevioside (S) (10^{-7} to 10^{-6} M) (N=3). All data are expressed relative to control. § denotes a significant difference (P<0.05) compared to control.