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Volutin granules of *Eimeria* parasites are acidic compartments and have physiological and structural characteristics similar to acidocalcisomes

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Abstract

The structural organization of parasites has been the subject of investigation by many groups and has lead to the identification of structures and metabolic pathways that may represent targets for anti-parasitic drugs. A specific group of organelles named acidocalcisomes has been identified in a number of organisms, including the apicomplexan parasites such as *Toxoplasma* and *Plasmodium*, where they have been shown to be involved in cation homeostasis, polyphosphate metabolism, and osmoregulation. Their structural counterparts in the apicomplexan parasite *Eimeria* have not been fully characterized. In this work, the ultrastructural and chemical properties of acidocalcisomes in *Eimeria* were characterized. Electron microscopy analysis of *Eimeria* parasites showed the dense organelles called volutin granules similar to acidocalcisomes. Immunolocalization of the vacuolar proton pyrophosphatase, considered as a marker for acidocalcisomes, showed labeling in vesicles of size and distribution similar to the dense organelles seen by electron microscopy. Spectrophotometric measurements of the kinetics of proton uptake showed a vacuolar proton

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pyrophosphatase activity. X-ray mapping revealed significant amounts of Na, Mg, P, K, Ca, and Zn in their matrix. The results suggest that volutin granules of *Eimeria* parasites are acidic, dense organelles and possess structural and chemical properties analogous to those of other acidocalcisomes, suggesting a similar functional role in these parasites.

Keywords

Acidocalcisome; *Eimeria acervulina*; *Eimeria tenella*; elemental mapping; vacuolar proton pyrophosphatase; volutin granule

Eimeria species are intracellular protozoan parasites that infect poultry flocks all over the world, causing major parasitic diseases of great economical importance. Infection by *Eimeria* species is responsible for considerable economical losses in poultry production, estimated to 700 million dollars per year in the USA (Lillehoj et al. 2007).

Several studies on the structural organization of parasitic protists have shown that they contain unique organelles and intracellular structures that are interesting either from the cell biology point of view, because new structures, interaction mechanisms, and metabolic reactions are identified, or from the chemotherapy side, because the identification of novel structures and their physiological roles may reveal potential biochemical targets for the rational development of anti-parasitic drugs (De Souza 2002; Docampo 2008). In this regard, combined structural and biochemical studies on the mechanisms involved in the regulation of ion transport in protist parasites have led to the identification of an organelle named the acidocalcisome (Vercesi et al. 1994; reviewed in Docampo et al. 2005).

The first microscopical observations of acidocalcisomes showed that they share some characteristics with volutin granules, such as staining with basic dyes and a high calcium and phosphate content. For this reason, they have been considered to correspond to volutin or metachromatic granules previously described in some bacteria and protists (Kornberg 1995; Meyer 1904). Acidocalcisomes have been characterized by their acidic nature, high electron density, and high concentration of elements, such as Na, Mg, P, K, Ca, Fe and Zn, which can be detected either by fluorimetric assays using appropriate fluorescent probes (Miranda et al. 2005; Vercesi et al. 1994) or by analytical electron microscopy (LeFurgey et al. 2005; Miranda et al. 2000, 2004a, 2004b, 2004c, 2008; Scott et al. 1997; Vickerman and Tetley 1977). Cation uptake and release and water transport across these organelles are powered by a number of ion pumps (i.e. Ca^{2+} -ATPases, V-H^{+} -ATPases, V-H^{+} -PPases), exchangers (i.e. $\text{Na}^{+}/\text{H}^{+}$ and $\text{Ca}^{2+}/\text{H}^{+}$ exchangers), and channels (i.e. Cl^{-} channel, aquaporin) present in their enclosing membrane (reviewed in Docampo et al. 2005). Due to the presence of these transport mechanisms, these organelles have been regarded to participate in several biological functions, such as control of intracellular pH, calcium homeostasis, ion storage, polyphosphate metabolism, and osmoregulation (Besteiro et al. 2008; Fang et al. 2007; Miranda et al. 2008; Rohlof and Docampo 2008; Schoijet et al. 2008). Little is known about acidocalcisomes in apicomplexan parasites and most of the studies have been done in *Toxoplasma gondii*. The biochemical characterization of acidocalcisomes was carried out mainly using permeabilized cells and cell fractions of tachyzoites, and by electron microscopy. These studies have shown that the physiological and structural properties of *T. gondii* acidocalcisomes are similar to those that have been previously described for trypanosomes, where they are better characterized (Lu et al. 1998; Moreno and Zhong 1996; Rodrigues et al. 2000). The presence of acidocalcisomes in *Plasmodium* species has been reported (Bakar et al. 2009; Marchesini et al. 2000; Ruiz et al. 2004), although little information on their physiological properties has been provided and the characterization of their fine structure in different developmental stages of the parasite is

lacking. The presence of acidocalcisomes in *Eimeria* parasites has for long been neglected and only a few images suggesting the presence of acidic organelles that resemble in shape acidocalcisomes of *T. gondii* have been shown in *Eimeria acervulina* (Miranda et al. 2008). As with *Plasmodium* species, any detailed structural and physiological characterization of these organelles in *Eimeria* parasites is lacking as well.

Methods

Production of *Eimeria acervulina* and *Eimeria tenella* oocysts and purification of sporozoites

Hy-line chickens were reared from one day of age with ration free of anticoccidial drugs and water *ad libitum* in a pathogen free room at the Avian Coccidiosis Laboratory, Department of Animal Biology, Institute of Biology, Universidade Estadual de Campinas (UNICAMP), Campinas, São Paulo, Brazil. Four week-old chickens were inoculated with 1×10^4 sporulated oocysts of *E. acervulina* “Cu” strain (Kawazoe et al. 2005) or 5×10^3 sporulated oocysts of *E. tenella* “Pa” strain. *Eimeria tenella* “Pa” strain was isolated from chicken feces at “Sitio Palmito”, city of Frutal, state of Minas Gerais, in a backyard without contact with chicken food or anticoccidial drugs and kept in liquid nitrogen in the Laboratory of Avian Coccidiosis, Biology Institute at Universidade Estadual de Campinas, São Paulo, Brazil. Oocysts were isolated from feces and sporulated after one week of inoculation according to the procedure of either Long et al. (1976) and Chapman (1978). Sporozoites, obtained from oocysts after mechanical rupture, enzymatic treatment, and purification by anion-exchange chromatography on DE-52 cellulose columns (Schumatz et al. 1984), were collected and stored at 4 °C.

Visualization of acidic compartments

Sporozoites were incubated for 10 min with 25 µg/ml of acridine orange in phosphate buffered saline (PBS), washed twice, and wet-mounted. Images were obtained in a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Baden-Württemberg) equipped with a 488-nm excitation filter set. Emission signal (above 500 nm) was detected with a Hamamatsu digital CCD Model C5810 camera (Hamamatsu, Hamamatsu City, Shizuoka) and an image analysis system attached to the microscope.

Vacuolar proton pyrophosphatase (V-H⁺-PPase) activity

Acidification of internal compartments was followed by measuring changes in the absorbance of acridine orange at the wavelength pair 493–530 nm in an SLM-Aminco DW2000 dual-wavelength spectrophotometer (SLM-Aminco, Rochester, NY). Sporozoites of *E. tenella* (0.1 mg/ml of protein) were incubated at 28 °C in 2.5 ml standard reaction medium, containing 130 mM KCl, 2 mM MgCl₂, 1 µM oligomycin, 10 mM HEPES buffer, pH 7.2, with 16 µM digitonin before addition of 3 µM acridine orange. PPi-driven proton uptake by the acidocalcisomes was measured with the addition of 100 µM sodium pyrophosphate to the reaction medium containing digitonin permeabilized cells. The results shown are representative of at least three experiments.

Immunofluorescence microscopy

Cells fixed in freshly prepared 4% (v/v) formaldehyde in PBS were allowed to adhere to poly L-lysine-coated coverslips, permeabilized with 0.3% (v/v) Triton X-100 in PBS, washed, incubated with 50 mM ammonium chloride in PBS, and blocked with 3% (v/v) bovine serum albumin in PBS. Polyclonal antibodies raised in rabbits against the putative hydrophobic loop III of *Arabidopsis thaliana* V-H⁺-PPase were kindly provided by Dr. Phillip Rea, University of Pennsylvania (Sarafian et al. 1992).

Immunofluorescence was carried out by incubating adhered permeabilized cells in a 1:100 dilution of the antibody for 1 hour. After washing, a 1:100 dilution of fluorescein isothiocyanate-coupled goat anti-rabbit IgG was used as a secondary antibody. Images were obtained in Zeiss Axioplan fluorescence microscope at the same conditions used for imaging acridine orange stained cells. Images were deconvolved using a no neighbor method and a deblur algorithm.

Sequence alignments

Multiple alignments between a conserved domain of *Arabidopsis thaliana* vacuolar proton pyrophosphatase (326-peptide sequence-Loop XII, 38 aminoacids GPISDNAGGIAEMAGMSHRIRERTDALDAAGNTTAAIG), *Trypanosoma cruzi*, *Toxoplasma gondii* and *Plasmodium falciparum* (<http://www.ncbi.nlm.nih.gov/blast>), and sequences from *Eimeria* database deposited at the Sanger Institute (<http://www.sanger.ac.uk>) were conducted using the NCBI BLAST server (<http://www.ncbi.nlm.nih.gov/blast>) and the CLUSTALW2 program using default parameters (Larkin et al., 2007).

Electron microscopy of ultrathin sections

Sporozoites of both species were washed in PBS, pH 7.2, fixed in 2.5% (v/v) glutaraldehyde plus 4% formaldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% (w/v) OsO₄, and embedded in Polybed 812 epoxide resin. Sections were stained for 30 min in uranyl acetate, 5 min in lead citrate, and observed in a JEOL 1200EX electron microscope (JEOL, Akishima, Tokyo) operating at 80 kV.

Imaging of unfixed whole cells

Cells were washed in PBS and resuspended droplets were applied to 100-mesh formvar-coated copper grids, allowed to adhere for 10 min, carefully blotted dry, and observed in an energy-filtering LEO EM 912 electron microscope (Zeiss, Oberkochen, Baden-Württemberg) operating at 80 kV. Electron spectroscopic images were recorded at an energy loss of ~ 60 eV with spectrometer slit width of 20 eV.

Morphometric analysis

Volumetric density of the acidocalcisomes was determined in thin sections. Randomly selected cells were observed in a Zeiss EM 900 transmission electron microscope equipped with a Megaview III camera and an iTEM image analysis system. Profiles of 73 cells of each *Eimeria* species were acquired and measured. Number and absolute volume of acidocalcisomes were estimated by using whole cells prepared as described above. Absolute volume of acidocalcisomes was determined by measuring the diameters of ~50 organelles assuming they are spheres. Statistical significance was determined by Student's *t* test (*P* < 0.05).

Electron probe X-ray microanalysis and elemental mapping

Energy dispersive X-ray spectra were recorded from the dense vesicles of whole cells dried onto Formvar-coated grids. Control spectra were collected from regions adjacent to the acidocalcisomes and from the Formvar film. Specimens were analyzed in a LEO 912 Omega scanning transmission electron microscope (Zeiss, Oberkochen, Baden-Württemberg). X rays were collected for 200 sec using a Li-drifted Si-detector (front area 30 mm²) equipped with an ATW atmospheric window. The microscope was operated at 80 kV using a tungsten filament, in the scanning transmission (STEM) imaging mode with spot size set to 40 nm. Analyses were performed using a Link multichannel energy analyzer and Link ISIS 3.00 software (Oxford Instruments, Wiesbaden, Germany).

Localization of Polyphosphate (poly P)

Sporocysts of *E. acervulina* and *E. tenella* were fixed with 4% (v/v) formaldehyde in PBS for 10 min, permeabilized with 0.3% (v/v) Triton X-100 for 5 min and incubated at room temperature with 50 µg/mL of the fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) for 30 min. Samples were mounted on glass slides and observed in a Zeiss Axioplan fluorescence microscope at an excitation wavelength of 370 nm and emission filtered lower than 500 nm. Images were deconvolved using a no neighbor method and a deblur algorithm.

Results

Volutin granules of *Eimeria* parasites are acidic

Sporozoites of *E. acervulina* and *E. tenella* incubated in the presence of acridine orange showed orange labeling in a number of vesicles and intracellular structures (Fig. 1, 2, respectively). Although green labeling also occurred in the nucleus and in the refractile bodies, due to intercalation of the dye with components of these organelles, the number and distribution of orange-labeled vesicles, presumably corresponding to volutin granules, was similar to that observed for these organelles using other techniques (see below).

Immunolocalization of the vacuolar proton pyrophosphatase and proton pyrophosphatase (V-H⁺-PPase) activity

Antibodies raised against a conserved domain (326 peptide) of *A. thaliana* vacuolar proton pyrophosphatase (V-H⁺ PPase) have been shown to recognize acidocalcisomes in a number of parasites (Soares Medeiros et al., 2005, Miranda et al., 2004a). To confirm that the 326 domain was conserved in *Eimeria* parasites, sequence alignments of the 326 peptide from *A. thaliana* V-H⁺-PPase with *Eimeria* sequences were carried out. Results showed that this domain is highly conserved in *Eimeria* parasites, as occurs with other parasites such as *Toxoplasma* and *Plasmodium* and *Trypanosoma*, where the presence of acidocalcisomes has been extensively demonstrated (Fig. 3). Immunofluorescence results revealed labeling in intracellular organelles in both *Eimeria* species (Fig. 4A, B, 5A, B) with size and distribution similar to what was previously seen after acridine orange labeling (Fig. 1, 2). Measurement of the pyrophosphate-driven H⁺ uptake (i.e. vacuolar proton-pyrophosphatase activity), carried out in permeabilized cells showed that acridine orange uptake (indicating acidification) was triggered by addition of 0.1 mM sodium pyrophosphate. Addition of 1 µM nigericin abolished acridine orange uptake (Fig. 6), suggesting an intracellular PPi-dependent acidification process. Accordingly, cells incubated in the absence of Na-PPi, did not show acidification (data not shown).

Ultrastructural analysis of acidocalcisome-like organelles

In thin sections, the volutin granules of both *Eimeria* species appeared as vesicles with a dense material apposed to the inner face of the membrane (Fig. 7–12). Sporozoites showed volutin granules with different degrees of preservation of the dense material, from an almost totally empty profile to vacuoles containing a considerable amount of dense material (Fig. 10, 12, white arrows). Fusion between these organelles was occasionally observed (Fig. 8).

Elemental composition and morphometric analysis of volutin granules

The size, distribution, and ultrastructural characteristics of the volutin granules seen by electron microscopy together with their acidic nature and presence of a V-H⁺-PPase activity strongly indicated that they might correspond to acidocalcisome-like structures. One efficient technique that has been applied to the observation of acidocalcisomes or organelles with similar electron scattering properties is the preparation of whole cells directly dried on Formvar-coated grids followed by observation of contrast tuned images in an energy-filtered

TEM (EFTEM). In these preparations, spherical dense organelles randomly spread throughout the cell body could be readily observed (Fig. 13). Approximately 40 acidocalcisomes, with diameters between 100–400 nm, were observed per cell. Morphometric analysis showed that they occupied ~1.52 % of the total volume of the cell (Table 1). X-ray microanalysis (Fig. 14) revealed considerable amounts of oxygen, magnesium, phosphorus, calcium, and zinc concentrated in these compartments. Elemental mapping showed that these elements were mainly concentrated in the matrix of the dense organelles (Fig. 14, 15). Although very weak, the signal of zinc is present both in the spectrum and the map (Fig. 14, inset).

Polyphosphate localization

Polyphosphate was localized by fluorescence microscopy in DAPI-stained samples according to the procedure described in Methods. Fluorescence emission of DAPI shifts to a longer wavelength (to 525 nm) when it binds to poly P, giving a green-yellow emission color in poly P containing structures. Poly P was present in small vesicles present inside the sporocysts of *E. acervulina* and *E. tenella* with size and distribution similar to what was seen in sporozoites of both *Eimeria* species after acridine orange labeling and immunolocalization with antibodies against the H⁺-PPase (Fig. 16, 17).

Discussion

Apicomplexans are natural invaders with a complex life cycle, usually involving more than one host. Therefore, they must be prepared to face different environmental conditions, which require extreme changes in metabolism. Acidocalcisomes have been associated with a number of functions in environmental adaptation of several parasites, such as osmoregulation and pH homeostasis. In trypanosomatids, the participation of acidocalcisomes in the regulatory volume decrease mechanism, polyphosphate metabolism, and calcium storage has been demonstrated (Docampo et al. 2005). In *T. gondii*, acidocalcisomes are the largest store of Ca²⁺ (Bouchot et al. 1999; Luo et al. 2001; Moreno and Zhong 1996), indicating a possible role of this organelle in cell signaling during host cell invasion and egress, processes that are crucial for maintaining their life cycle. Large amounts of short and long chain polyphosphate were detected in different blood stages of *P. falciparum* whose concentrations increase during differentiation of the blood stages, reaching maximal concentrations in the trophozoite stages and then decreasing in the schizonts (Ruiz et al. 2004). This would be compatible with a role for acidocalcisomes in the adaptation of apicomplexan parasites to environmental stress (Miranda et al. 2010), and suggest that the same functions may be present in *Eimeria* parasites. Fusion between these organelles was occasional (Fig. 8), as observed before in *T. gondii* (Miranda et al. 2008) and *Leishmania* parasites (Miranda et al. 2004a), where polymorphic acidocalcisomes have been seen. These fusion and budding events indicate that their participation in the physiology of the parasite is dynamic.

Here, we have shown that the ultrastructural, chemical, and physiological properties of volutin granules of *E. tenella* and *E. acervulina* resemble in several aspects acidocalcisomes of *Toxoplasma*, *Plasmodium*, and trypanosomatid species. Fluorescence microscopy of acridine orange stained cells, spectrophotometric measurements of the kinetics of pyrophosphate-driven proton uptake, and polyphosphate localization suggest that both *Eimeria* species have organelles with physiological properties analogous to those of acidocalcisomes so far characterized (Docampo et al. 2005; Miranda et al. 2008). In addition, thin sections and energy-filtered transmission electron microscopy (EFTEM) images of whole intact sporozoites showed the presence of a number of dense organelles, 100–300 nm in diameter. The advantage of this type of preparation is the observation of the whole parasite (and whole organelles) without the possible side effects of fixatives and other

chemicals used in the routine processing necessary for transmission electron microscopy. This reduces significantly the extraction of the mineral content from the acidocalcisomes and, therefore, allows the qualitative detection of several cations within the organelle by analytical TEM (Miranda et al. 2000, 2004a, b, c, 2008; Moreira et al. 2005; Soares Medeiros et al. 2005). In addition, as cells are imaged as a whole mount, several morphometric parameters such as the number of acidocalcisomes per cell, their shape and mean volume is carried out with higher precision. Furthermore, X-ray microanalysis revealed considerable amounts of oxygen, magnesium, phosphorus, calcium and zinc concentrated in these compartments, showing that the chemical properties of *Eimeria* dense granules are similarly to what has been reported previously in the acidocalcisomes of trypanosomatid and apicomplexan parasites (Miranda et al. 2000, 2004, 2010; Ruiz et al. 2004; Scott et al. 1997; Soares Medeiros et al. 2005).

Due to their enzyme repertoire, which in some aspects differ from mammalian cells, and due to their acidic characteristics, which allow them to accumulate basic drugs, it has been postulated that acidocalcisomes are potential targets for anti-parasitic drugs in a number of protozoa, including apicomplexan parasites (reviewed in Docampo & Moreno 2008). Anti-coccidial compounds such as monensin (Na^+/H^+ exchanger) have been suggested to interfere with acidocalcisome physiology (Docampo & Moreno, 2001), whereas bisphosphonates, pyrophosphate analogs used to treat a variety of bone resorption diseases (Rodan 1998), have also been shown to have activity against different protozoan parasites *in vitro* and *in vivo*. Accumulation of these compounds in the acidocalcisomes has also been suggested (Docampo & Moreno, 2008).

The identification of acidocalcisomes in different species of *Eimeria* strongly suggests that these organelles are conserved among the different members of the *Eimeria* genus. The identification of acidocalcisomes in *Eimeria* parasites, therefore, represents a step towards further understanding and controlling the biology of *Eimeria* parasitism, and may also provide interesting targets for the future development of anti-coccidial drugs against parasites of veterinary importance.

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Fig. 1, 2.

Fluorescence detection of acidic compartments of *Eimeria acervulina* and *Eimeria tenella* sporozoites. Several acidic organelles (stained in orange) are observed in the cytoplasm of *E. acervulina* (1) and *E. tenella* (2). Note binding of acridine orange to the nucleus and the refractile body (green). Scale bars: 3 μ m.

Fig. 3–6.

Antibodies raised against a plant vacuolar proton pyrophosphatase (H^+ -PPase) recognize *Eimeria* sp. structures that are associated with the *Eimeria* acidocalcisomes. **3.** Sequence alignment of the 326 peptide from *Arabidopsis thaliana* with sequences from *Eimeria* and other parasites shows the conservation of the “326” domain. **4, 5.** Immunofluorescence microscopy using antibodies raised against *A. thaliana* vacuolar proton pyrophosphatase ($V-H^+$ -PPase) shows labeling of large spherical intracellular structures of *Eimeria acervulina* (4A, B) and *Eimeria tenella* (5A, B), in a disposition similar to the acidocalcisomes observed in whole cells images. Scale bars: 10 μ m. **6.** Pyrophosphate (PPI)-driven proton uptake in digitonin-permeabilized sporozoites of *E. tenella*. Traces are representative of three different experiments. Trace before PPI addition represents the control.

Fig. 7–12.

Thin sections of sporozoites of *Eimeria acervulina* (7–10) and *Eimeria tenella* (11, 12) after conventional glutaraldehyde-osmium fixation. Cells display circular vacuoles with a dense material associated with the inner face of their membranes similar to that observed in the acidocalcisomes. Acidocalcisomes have single membrane (9, white head arrow) with different degrees of preservation of the dense material, from an almost totally empty profile to vacuoles containing a considerable amount of dense material (10, 12, white arrows). Apparent fusion between these organelles was seen (8) resulting in a polymorphic organization. Scale bars: A = 1 μ m; B = 400 nm; C, D = 200 nm; E, F = 1 μ m.

Fig. 13–15.

Whole cells show spherical acidocalcisomes that are the main site for cation storage in *Eimeria* parasites. **13.** Electron spectroscopic imaging of whole unfixed *Eimeria acervulina* (ΔE between 60–80 kV). **14.** Corresponding X-ray spectrum of the acidocalcisomes in the inset to Fig. 13 using energy-filtered scanning transmission electron microscopy (EFSTEM). Nickel peaks in the spectrum came from the grid and silicon from the detector itself. Carbon and sulfur signals were similar in the acidocalcisomes and control regions (cytoplasm, data not shown). Inset shows an increased scale of the peak representing Zn. **15.** An EFSTEM electron spectroscopic image of a portion of the cell displayed in Fig. 13, inset (ΔE between 60–80 kV). Elemental images of the cell displayed correspond to (P) phosphorus, (O) oxygen, (Ca) calcium, (Mg) magnesium, and (Zn) zinc. Scale bars: A = 3 μm ; EFSTEM = 500 nm; P, O, Ca, Mg and Zn = 500 nm.

Fig. 16, 17.

Localization of polyphosphate in sporocysts of *Eimeria acervulina* (16) and *Eimeria tenella* (17). To localize poly P, sporocysts of both *Eimeria* species were incubated with DAPI. Differential interferential contrast microscopy of sporocysts of *E. acervulina* (16A) and *E. tenella* (17A). 16B, 17B. Fluorescence images of DAPI staining, showing several organelles containing poly P, similar in size and distribution to what corresponds to the acidocalcisomes in sporozoites. (16C, 17C) Merge. Scale bars: 3 μ m.

Table 1Morphometric analysis of acidocalcisomes of *Eimeria tenella*.

	Number per cell	% Volume occupied	Mean Diameter (nm)
Acidocalcisomes	40 ± 8	1.52 ± 0.3	364.07 ± 14
Refractile body	1 ± 0	30.5 ± 3.7	ND

n = 73; Values are expressed as mean values ± standard error of the mean (SEM); ND = not determined; Statistical significance was determined by Student's *t* test ($P < 0.05$).

Values are expressed as means ± SEM; (n = 73).