Effects of altered gravity induced by clinorotation on the cholinesterase activity of the non-sentient model *Paramecium primaurelia* (Protozoa)

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Abstract

Compounds known as chemical mediators, including acetylcholine, have been found not only in animals and humans, but also in living organisms, like protozoa, which lack nervous system. In *Paramecium primaurelia* has been described a cholinergic system, which is proven to play an important role in cell-cell interactions during its developmental cycle. In our work we investigated the effects of exposure to simulated microgravity (3D Random Positioning Machine, 56 rpm, 10^{-6} g) on the cholinesterase activity of the eukaryote unicellular-organism alternative-model *P. primaurelia*. Our results show that the exposure of *P. primaurelia* to microgravity for 6 h, 24 h, 48 h affects the localization and the amount of cholinesterase activity compared to cells grown under Earth gravity conditions (1 g). However, these effects are transient since *P. primaurelia* restores its normal cholinesterase activity after 72 h under microgravity conditions, as well as cells exposed up to 72 h to microgravity and then placed under terrestrial gravity for 48 h.

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Introduction

Life on Earth evolved in an environment characterized by chemical-physical properties which gradually changed from the earliest times to the present. These slow and progressive shifts led to the affirmation of organisms able to adapt and to survive in a new environment. Therefore, organisms living on our planet grow and develop under specific environmental conditions in terms of atmosphere, magnetic fields, sun radiation and gravity.

Gravity, in particular, has been a pervasive factor throughout evolutionary history. Actually, also the unicellular eukaryote-organisms used this external track to find and to stay in favorable living condition, which offers ecological advantages.[1]

In the last decade there was a reborn interest in *Space*, which raised new questions about the ability of terrestrial organisms to live in an environment different from the original one.

After space flights, for example, astronauts experience neurophysiological problems like alterations in movement/orientation control[2] and dysfunctions in kinematics and postural adaptation.[3]

Neurophysiological structure-function correlation studies on Central Nervous System neurons indicate that these are less active in real and simulated microgravity,[4] by showing possible effects on neurogenesis, neural regeneration and signal transmission.[5]

It is well known that protozoa are a simplified model for functional studies on neurons. In fact, there are numerous similarities between ciliated protozoa and invertebrate and vertebrate nervous cell;[1] ciliates are able to behave as sensory receptors (like sensory cells), transductive-integrative systems (like nerve cells) and effectors (like muscular cells).

It is not coincidence that cholinergic molecules and nitrergic signal transduction pathway, responsible for neurotransmission in Metazoa, have been identified in Protozoa.[6-9] In addition, informations derived from both the *Paramecium* genome sequencing[10] and the experimental studies on *Paramecium*[11-17] have demonstrated that this protozoan is excellent model for studies on humans.

Concerning the space research, attention has been focalized on

the effect of microgravity on *Paramecium*. Authors observed that the growth rate of *Paramecium tetraurelia* increased in microgravity and simulated-microgravity[1,18,19] as well as *Paramecium biaurelia* cells swam faster under altered gravity induced by clinorotation.[20]

However Sawai *et al*.[21] point out that *P. tetraurelia* reduced proliferation rhythm under slow-clinorotation and in this condition the swimming velocity decreased too.

Lastly, Hemmersbach *et al*.[22] showed gravity-dependent decrease in *Paramecium* cAMP level while cGMP concentration was gravity-independent.

On this basis, in our work we investigated the effect of the exposure to simulated microgravity (10^{-6} g), generated by a 3D Random Positioning Machine (56 rmp) on the cholinesterase (ChE) activity of the eukaryote unicellular-organism alternative-model *Paramecium primaurelia.*

Materials and Methods

Clinostat: characteristics and specifications

Sawai *et al*.[21] reported that the experiment on *Paramecium* cells performed by slow-clinorotation (2.5 rpm) with the RT-5 TAITEC rotator (Tokyo) was not connected with those under microgravity and simulated microgravity by fast-rotating clinostat. In addition Russomano *et al*.[23] showed the high reproducible quality of simulated microgravity generated by 3D clinostat on cell and to predict the effect of biological specimens prolonged exposure to space. Therefore, for our experimental purpose, we used a 3D special machinery called Random Positioning Machine (RPM) (Dutch Space, Netherland), which has been kept under fast-continuous rotation at 56 rpm, at the temperature of 24°C, to generate a simulated microgravity of 10^{-6} g.

Paramecium primaurelia and culturing methods

The cell cultures of *P. primaurelia* were grown at 24°C in tubes with lettuce infusion, inoculated with *Enterobacter aerogenes* as food.[14,16] The lettuce medium was obtained by drying green leaves from organic farming for 1 h at 180°C. Thirty grams of dried leaves were boiled for 15 min in 1 L of distilled water. The concentrated infusion thus obtained was filtered and divided into tubes (10 mL per tube). The tubes were closed, properly autoclaved at 120°C for 20 min and then stored at 4°C. The concentrated infusion was diluted into 500 mL of distilled water, balanced to pH 7.1 and autoclaved (120°C, 20 min) into 50-mL tubes. Logarithmic growing cells of *P. primaurelia* were transferred onto a depression slide. The cells were starved until they reached autogamy, a process involving a nuclear reorganization that resets the age of *Paramecium* cells.

Autogamy was detected using 4,6-diamidino-2-phenylindole (DAPI), a fluorescent compound that stains the macronucleus in blue. Cell cultures were considered in the autogamy phase when the totality (100%) of 30 cellular samples showed the typical fragmented macronucleus. The autogamous cells were isolated onto a depression slide by using a glass micropipette and they were fed with *E. aerogenes*. It is known that autogamous *Paramecium* cells have a constant fission rate from 10 to 30 days. As consequence, after 15 days from the autogamy process, the monoclonal cells cultures were used for the experimental purposes, as suggested by Amaroli *et al*.[14,16]

Experimental samples

Our studies were carried out on monoclonal cell cultures of *P. primaurelia* transferred into tubes (5 mL) filled completely with bacterized lettuce medium, in order to avoid the formation of fluxes during the clinorotation.[23] The tubes were closed and positioned in the 3D RPM under continuous rotation (simulated microgravity, 10^{-6} g). Ground controls were placed onto the supporting frame of the same machine, in order to subject all cells to the same vibration.[23] The experiment was performed at a temperature of 24°C.

The different samples were named as follows:

Control: sample placed on the non-rotating arm of the clinostat and exposed to the Earth's gravity for 6 h, 24 h, 48 h or 72 h.

Micro (*g*): sample exposed to simulated microgravity for 6 h, 24 h, 48 h or 72 h.

Rec: sample exposed to simulated microgravity for 24 h, 48 h or 72 h and then resubmitted to Earth's gravity for 24 h, 48 h or 72 h.

To detect the ChE activity by cytochemical reaction, cell samples were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS) for 120 min at 24°C. Cells were then transferred onto slides and allowed to air-dry.

To perform the non-denaturing electrophoresis and the immunoblot analysis, cell samples were collected by centrifugation and lysed by freezing at –80°C for 10 min. Proteins were extracted in 0.1% Triton X-100 and the total protein concentration was evaluated using the BIO-RAD protein assay kit.[17]

Cytochemical procedure to detect the cholinesterase activity in *Paramecium primaurelia*

The presence and localization of the ChE activity was investigated by incubating the Control, Micro(*g*) and Rec samples overnight at 4°C with the acetyl-β-methyl thiocholine iodide (AcTChI) substrate according to the Karnovsky and Roots method,[24] modified by Falugi *et al.*[25] Samples were then washed in 0.1 M PBS, pH 7.4. Nuclei were counterstained with DAPI (1:10,000 in PBS for 5 min). Cells were observed under a Leica DMRB Epi-fluorescence microscope equipped with four Leica objectives: N Plan 2.5×/0.07, PL Fluotar 10×/0.30, PL Fluotar 20×/0.50, PL Fluotar 40×/0.70, PL Fluotar 100×/1.3 Oil. Images were acquired with a Leica CCD camera DFC420C equipped with the Leica Application Suite software, Version 3.7.0 (Leica, Germany). Different images of the same cell, acquired using Normanski imaging and epi-fluorescence, were overlapped by using Adobe Photoshop CS6 Extended. We used the ImageJ 1.33J software (National Institute of Health, Bethesda, MD, USA) to evaluate the ChE activity (optical density) in the experimental cell samples (30 cells for sample).

Non-denaturing electrophoresis to detect the cholinesterase activity in *Paramecium primaurelia*

The amount of the ChE activity was investigated by non-denaturing electrophoresis on the Control, Micro(*g*) and Rec samples. ChE-like molecules were separated on a 8% polyacrylamide running gel. Electrophoresis was carried out at 12 mA, for 16 h at 5°C, according to the procedure described by Falugi *et al.*[25] The ChE activity was detected with the Karnovsky and Roots method,[24] using AcTChI as substrate. *Electrophorus electricus* AChE (Sigma-Aldrich) was used as positive control. The β-actin was used as loading control and detected by immunoblot analysis with a commercial antibody.[17] We used the ImageJ 1.33J software (National Institute of Health, Bethesda, MD, USA) to evaluate the optical density (O.D.) of the experimental samples bands. To quantify the enzyme activity, the data were expressed as O.D. of the sample band normalized with the β-actin.[26]

Statistical analysis

The experiments were carried out at least in triplicate. The significant differences between the controls and the irradiated samples were determined by using one-way ANOVA followed by the Tukey-Kramer multi-comparison test (GraphPad InStat 3).

Results

No difference in the localization and amount of the ChE activity was observed in control cells. Therefore, the imperceptible vibration was not effective on the parameters here considered, so all the observation on control cells are reported and analyzed together, with no distinction among times.

Effects of simulated microgravity on the localization of the cholinesterase activity in *Paramecium primaurelia*

Figure 1 shows the localization of the ChE in cells of *P. primaurelia* exposed to simulated microgravity from 6 h up to 72 h and stained with the Karnovsky and Roots method. After 6 h of exposure to simulated microgravity, the ChE activity was affected. If compared to the control group (Figure 1A), the enzyme activity was increased in the cytoplasmic and perinuclear regions (control=13.1%±20% of the total activity – 6h=73.2%±16% of the total activity; P<0.001), while it was decreased in association to the cell membrane (control=86.9%±18% of the total activity – 6h=26.8%±17% of the total activity; P<0.001) (Figure 1B). The increase or decrease in the ChE activity was more evident in those cells exposed to modeled microgravity for 24h (24h cytoplasmic and perinuclear activity=83.9%±19% of the total activity; P<0.001 - 24h cell membrane activity=16.1%±16% of the total activity; P<0.001) (Figure 1C). Cells exposed for 48 h to microgravity (Figure 1D) showed an increase in the ChE activity associated to the cell membrane (48h=37.5%±18% of the total activity; P<0.001). Furthermore, the activity in the cytoplasm was still more evident than in the control group (48h=62.5%±19% of the total activity; P<0.001).

After 72 h of exposure to microgravity (Figure 1E), the localization of the staining returned similar to the one of the control (72h cell membrane activity=87.0%±19%; P>0.05 - 72h cytoplasmic and perinuclear activity=13.0%±16%; P>0.05).

Figure 2 shows the effect of Earth's gravity conditions (1 g) on cells that were previously exposed to simulated microgravity. *Paramecium* cells submitted for 24h to modeled microgravity and then placed for another 24h under Earth's gravity (Figure 2B') did not show differences in the Karnovsky and Roots staining (24h cell membrane activity=17%±15%; P>0.05 - 24h cytoplasmic and perinuclear activity=83.0%±18%; P>0.05), if compared to those cells that were only exposed to simulated microgravity for 24 h (Figure 2B) (24h cell membrane activity=16.1%±16% - 24h cytoplasmic and perinuclear activity=83.9%±19%). In contrast with the control, the ChE activity was localized mainly in the cytoplasm and only slightly on the cell membrane. However, after 48 h under Earth's gravity conditions (Figure 2B''), the localization of the ChE activity progressively decreased in the cytoplasm (14%±13% of the total activity; P>0.05), while increasing on the membrane (85%±21% of the total activity; P>0.05), becoming similar to the pattern showed by the control (Figure 2A) (control=13.1%±20% of the total activity - 86.9%±18% of the total activity). Conversely, cells exposed for 48h and 72h to simulated microgravity and then restored to Earth's gravity for 24 h, displayed the ChE activity at the perinuclear level, and a decrease in the membrane staining localization (48h cell membrane activity=27.0%±15%; 48h cytoplasmic and perinuclear activity=73.0%±15% - 72h cell membrane activity=67.0%±19%; 72h cytoplasmic and perinuclear activity=33.0%±16%).

However, after 48 h under Earth's gravity conditions, the localization of the ChE activity progressively decreased at the cytoplasmic level (48h=16%±22% of the total activity; 72h=14%±16% of the total activity), while it increased on the

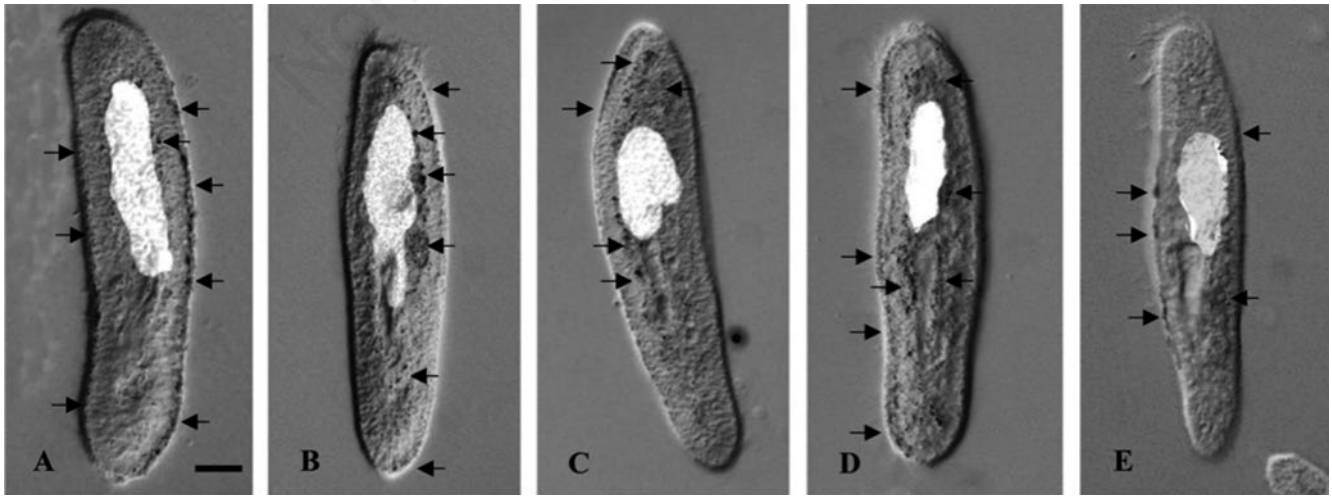

Figure 1. Cytochemical detection (Karnovsky and Roots method, 1964) of the cholinesterase activity in cells of *Paramecium primaurelia* exposed to simulated microgravity for 6 h (B), 24 h (C), 48 h (D) and 72 h (E). The reaction was carried out using acetyl-β-methyl thiocholine iodide as substrate. The enzyme reaction product appears as a black precipitate (arrows). The white fluorescence evidences the macronucleus. A=control. Bar=15 µm.

membrane (48h=84%±18% of the total activity; 72h=86%±12% of the total activity) (Figure 2C'', 2D''), becoming similar to the staining of the control (Figure 2A).

Effects of simulated microgravity on the amount of the cholinesterase activity in *Paramecium primaurelia*

Figure 3 shows differences in the amount of ChE activity in *P. primaurelia* exposed to simulated microgravity for 6 h (B), 24 h (C), 48 h (D), 72 h (E). After 6 h and 24 h there was not a significant increase in the ChE activity respect to the control (A) ($P>0.05$). However, the activity increased after 48 h of exposure to simulated microgravity, becoming significantly ($P<0.001$) higher than both the control and the samples exposed for 6 h and 24 h. In contrast, after 72 h of exposure to modeled microgravity, the ChE activity was not significantly different than that of the control ($P>0.05$).

Figure 4 shows the effect of restored Earth's gravity on *P.*

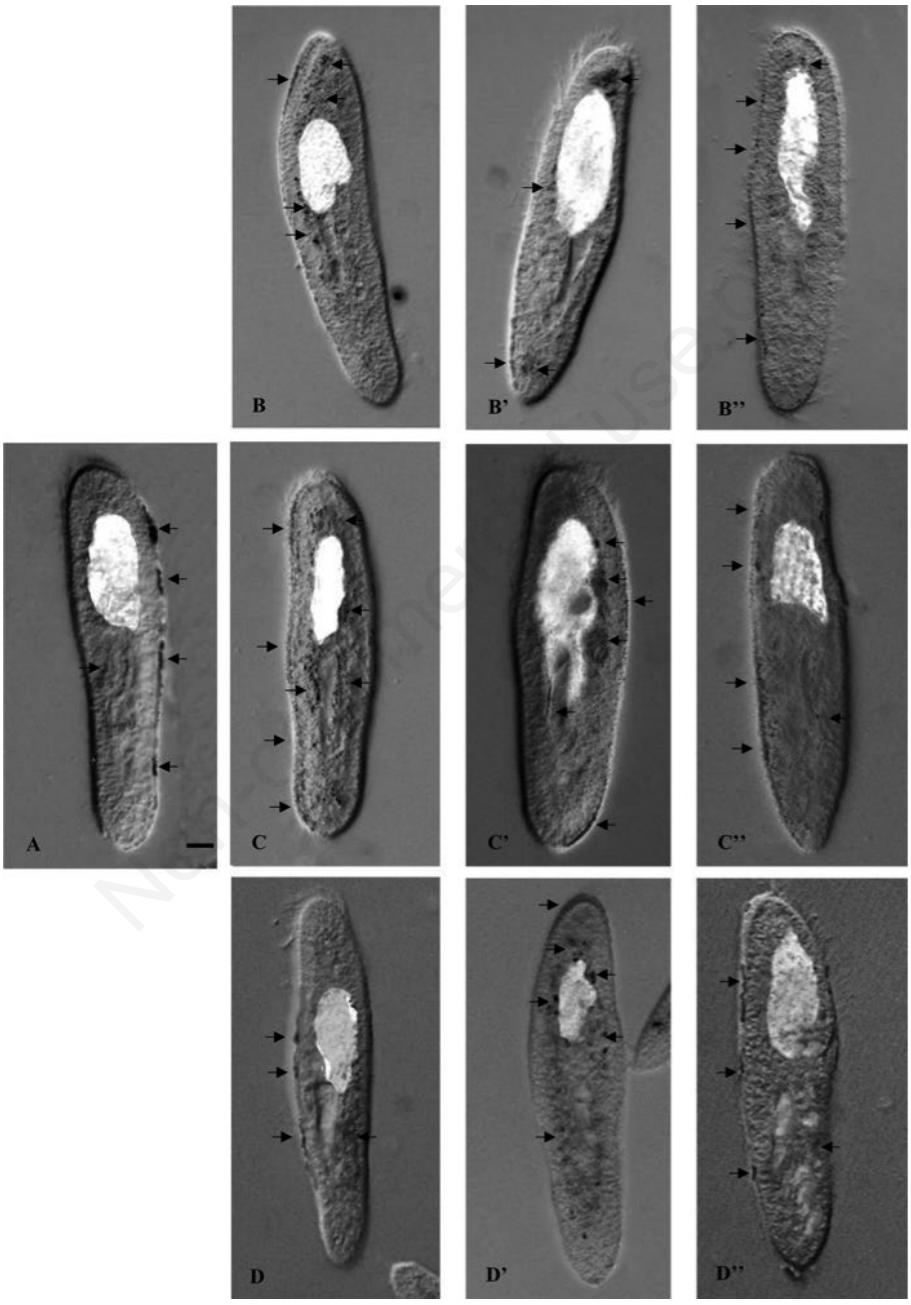

Figure 2. Cytochemical detection (Karnovsky and Roots method, 1964) of the cholinesterase activity in cells of *Paramecium primaurelia*. The reaction was carried out using acetyl-β-methyl thiocholine iodide as substrate. Cells were exposed to simulated microgravity for 24 h (B), 48 h (C) and 72 h (D). After exposure to microgravity, the cells were restored to Earth's gravity for 24 h (B', C', D') and 48 h (B'', C'', D''). The enzyme reaction product appears as a black precipitate (arrows). The white fluorescence evidences the macronucleus. A=control. Bar=15 µm.

primaurelia cells previously exposed from 24 h up to 72 h to simulated microgravity. Cells exposed for 24 h to simulated microgravity and then replaced under terrestrial gravity for 24 h (Figure B) or 48 h (Figure 4B''), maintained the same level of the enzyme activity, showing a value similar to that of the control (Figure 4A) ($P>0.05$). Cells exposed for 48 h to simulated microgravity and then kept under Earth's gravity, mantained the same high level ($P<0.001$) of ChE activity during the first 24 h (C'), then it decreased and after 48 h at 1 g (C'') the enzyme activity returned to the control value ($P>0.001$). Finally, *Paramecium* cells exposed for 72 h to microgravity and submitted to Earth gravity for another 24 h (D'), increased the ChE activity ($P<0.05$). The enzyme activity displayed a decrease after 48 h at 1 g (D''), showing a value similar to the one of the control ($P>0.05$).

Discussion

The relationship among living organisms occurs via what is known as irritation events.[9] The mechanism of irritability appears to have a common base in the form of chemical signals.[9] Compounds like acetylcholine have been found not only in humans and animals, but also in living organisms which lack a nervous system,[9] including *Paramecium*.[8] In particular, in *P. primaurelia* has been described a cholinergic-like system and its role in the cell-cell interaction during the developmental cycle has been proven.[8,27] This function, similar to the one observed in nerve cells, shows that the role of cholinergic system evolved from bacterial chemotaxis (nutrition, growth, attraction, defense, repellency) to cell-cell interactions among organisms of the same species, as described in protozoa, animals and humans.[9]

In our study, the exposure of *P. primaurelia* to simulated microgravity influenced the localization and the amount of ChE, compared to cells grown under Earth's gravity conditions (1 g). In particular, after 6 h and 24 h of simulated microgravity, the ChE was mainly localized in the perinuclear and cytoplasmic regions rather than on the cellular membrane. It is well known that in *P. primaurelia*, as well as in nerve cells, the ChE is synthesized in the perinuclear compartment and then the enzyme is transferred to the cell membrane, where it plays an important role in the modulation of cellular interaction. Therefore, a change in its location induces alterations in the cell-cell communications. In our previous work on NTera2 cells, pluripotent cells that are able to differentiate into cholinergic cells, we showed that the process of differentiation into neuronal phenotype is altered by environmental perturbations, as a consequence of an unsuccessful transfer from the perinuclear compartment, typical of undifferentiated cells, to the cell membrane, typical of excitable cells.[15]

In an attempt to restore the cellular homeostasis and the proper cellular communication, *P. primaurelia* cells showed an increase in the production of ChE, which seemed to reach its maximum after 48 hours of exposure to simulated microgravity. This finding is in agreement with a study on the protozoan *Dictyostelium discoideum*, which showed an increase in the amount of ChE after the exposure to an electromagnetic field of low intensity and frequency.[28] *Paramecium* cells, as observed in *Dictyostelium* cells,[28] increased the ChE in the attempt to restore the proper homeostasis, through a

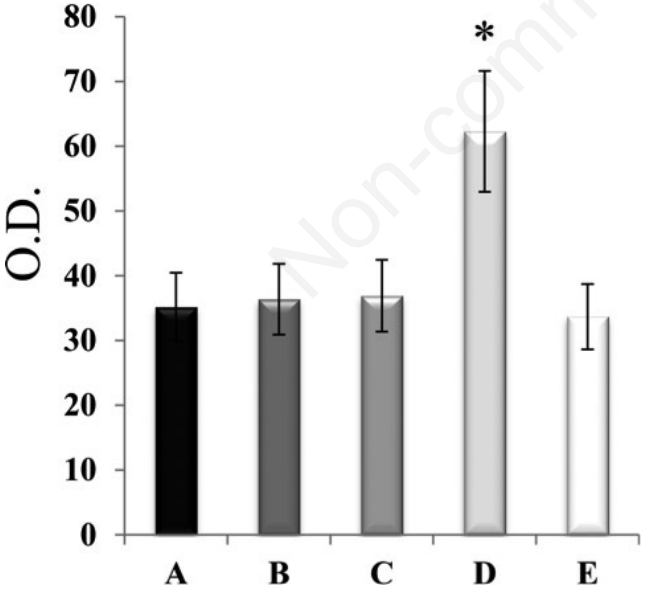

Figure 3. Image analysis of the electrophoretic pattern of the cholinesterase (ChE) activity detected with the Karnovsky and Roots method (1964). The figure shows the effect of simulated microgravity on the amount of the ChE activity in *Paramecium primaurelia*. Cells were placed in the clinostat and exposed to simulated microgravity for 6 h (B), 24 h (C), 48h (D) or 72h (E). A=control. The values that are statistically different from the control are marked with a star symbol (*). O.D., Optical density.

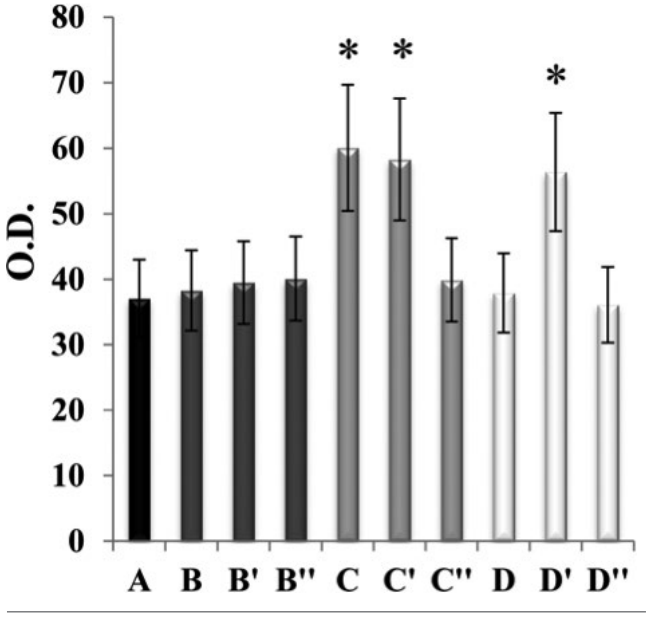

Figure 4. Image analysis of the electrophoretic pattern of the cholinesterase (ChE) activity detected with the Karnovsky and Roots method (1964). The figure shows the effects of Earth's gravity (1g) on the amount of the ChE activity in *Paramecium primaurelia* previously exposed to simulated microgravity. Cells were placed in the clinostat for 24 h (B), 48 h (C) or 72 h (D) and then recovered to Earth's gravity for 24 h (B', C', D') or 48 h (B'', C'', D'') A=control. The values that are statistically different from the control are marked with a star symbol (*). O.D., Optical density.

process of hormesis similar to the one shown by the cells of vertebrates.

Paramecium cells seemed to adapt themselves to the new conditions of simulated microgravity after 72 h, when the ChE activity returned to the control value. After 24 h and 48 h, there was an initial alteration in the pattern of ChE. However, the effect of microgravity seemed to be reversible, since cells restored to Earth's gravity showed, after 48 h, a localization and amount of cholinesterase similar to the ones of the control. These results suggest that *Paramecium* cells are more able to adapt themselves to Earth's gravity after exposure to microgravity, rather than the opposite situation. However, this hypothesis needs more insights to be confirmed.

Li *et al*.[29] showed that modeled microgravity induced disorganization of cytoskeleton filaments (microfilaments and microtubules) as well as Janmaleki *et al*.[30] described that simulated microgravity was able to disorganize actin filaments, microtubules and to reduce main cytoskeleton components. Corydon *et al*.[31] proved by life-cell-imaging that significant alterations of the cytoskeleton occurred during microgravity exposure.

However, studies on cell cultures exposed to microgravity showed that the cytoskeleton, after an initial alteration, was able to adapt to the new conditions of gravity.[32,33] In order to move from the perinuclear level to the cell membrane, ChE needs cytoskeletal filaments working properly, so we can assume that also in *P. primaurelia*, the different pattern of the ChE activity presence is correlated to initial alteration of cytoskeletal structure followed by a recovery in 48 h or 72 h.

Conclusions

Our results show that *P. primaurelia* cells are able to cope with gravity variation. Alteration in the localization and amount of ChE for 24 h and 48 h have not irreversible effects on the cells of *P. primaurelia* during the phase of asexual reproduction. However, this result raises questions about the possible effects during the phase of sexual reproduction (conjugation). The conjugation is a delicate and peculiar phase of the reproductive cycle of *Paramecium* and it requires accurate and timely cellular interactions in which the ChE plays an important role.[27] The same question could be raised for neuronal development in humans, since this process involves ChE and requires specific steps that, if altered, would lead to redundancy and disorders in the formation of synapses.[15]

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