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Original

Culture of dental epithelial cells: impact of fetal bovine serum

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ABSTRACT

Objective. Describe the influence of Fetal bovine serum (FBS) on the survival, growth and expression of cellular organelles in rat dental epithelial cells. **Material and methods.** Cell cultures of rat dental epithelial cells were carried out at 37°C in a humid atmosphere, in the absence and at a concentration of 10% FBS. Morphological evaluation was performed during the proliferation and confluence of cell in culture. Double immunofluorescence labels were made using anti-Actin, anti-TOMM20A, and anti-LAMP1 antibodies. **Results.** Circular or ovoid dental epithelial cells with bulky nuclei were evidenced during proliferation and confluences in a similar manner in culturing cells in the presence and absence of FBS. The lack of FBS negatively impacts the proliferation of epithelial cells. No alterations were observed in the localization of the anti-actin, anti-TOMM20 and anti-LAMP1 immunomarkers in both conditions of experimental cultures. **Conclusion.** FBS suppression in rat dental epithelial cells decreased survival, proliferation and suggests not having an impact on the organelles evaluated.

Keywords: Dental enamel; Ameloblasts; Culture media serum-free; Actin; Mitochondria; Lysosomes (*Source: MeSH*).

RESUMEN

Objetivo. Describir la influencia del Suero Fetal Bovino (SFB) en la supervivencia, crecimiento y expresión de organelas celulares en las células epiteliales dentales de rata. **Materiales y métodos**. Cultivos de células epiteliales dentales de rata fueron llevados a cabo a 37°C en una atmosfera húmeda, en ausencia y a una concentración de 10% de SFB. Una evaluación morfológica fue realizada durante la proliferación y confluencia de las células en cultivo. Dobles marcajes por inmunofluorencia fueron efectuados haciendo uso de anticuerpos anti-actina, anti-TOMM20 y anti-LAMP1. **Resultados.** Se evidenciaron células epiteliales dentales circulares u ovoides con núcleos voluminosos durante la proliferación y confluencias de manera similar en las células cultivas en presencia y ausencia de SFB. La carencia de SFB impactó negativamente la proliferación de las células epiteliales. No fueron observadas alteraciones en la localización de los inmunomarcajes anti-actina, anti-TOMM20 y anti-LAMP1 en las dos condiciones de cultivos experimentales. **Conclusiones.** La supresión del SFB en el cultivo de células epiteliales dentales de rata disminuyó la supervivencia, proliferación y sugiere no tener un impacto sobre las organelas evaluadas.

Palabras clave: Esmalte dental; Ameloblastos; Medio de Cultivo Libre de Suero; Actina; Mitocondrias; Lisosomas (*Fuente: DeCS*).

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INTRODUCTIÓN

Teeth are anatomical structures of epithelial and mesenchymal origin, they have focused researcher's attention from different disciplines in recent years because of their ability to reflect a variety of pathological processes. Increased knowledge of cellular, molecular and genetic aspects in tooth development, have demonstrated complexity in processes that, through interaction of cephalic neural crest cells with oral epithelium, make tooth formation possible in an appropriate time, position and correct structure (1). However, teeth can be affected by various syndromic or non-syndromic anomalies, mostly related to rare and/or orphan diseases, alterations that cause changes in teeth number, position, dental crown shape, its composition, dentin structure, cement, and tooth enamel. Therefore, knowledge of cellular and molecular mechanisms that explain normal dental development constitutes a fundamental aspect in understanding the source of these anomalies.

Tooth formation begins at fourth week of embryogenesis, when neural crest cells arrive at epithelium of the first branchial arch, followed by epithelial-mesenchymal interactions between oral epithelium and dental ectomesenchyme (2). Within framework of these interactions, tooth enamel formation develops, a process known as amelogenesis. After experiencing a terminal differentiation, ameloblasts, cells responsible for formation and organization of tooth enamel, suffer changes in their structure and function, finally developing an acellular, avascular and non-innervated structure: tooth enamel. This structure is formed by a mineral phase organized in a network of hydroxyapatite crystals, which constitutes approximately 97% of its composition, another 2.5% formed by water and remaining structure corresponds to an organic phase consisting of proteins, enzymes, and lipids (3.4).

Nowadays, studies of pathophysiological events that give rise to tooth enamel have been explored through animal models. One mostly popularized and used since 1960 is continuously growing rat incisor, a model that allows studying complex processes associated with amelogenesis, thanks to exploration in a single tooth of all development stages of tooth enamel. However, information obtained from said model must be cautiously extrapolated due to notable differences related mainly to a continuous eruption process of rat incisors, as opposed to a limited period of eruption of human dentition. In spite of this, it has been demonstrated with scientific evidence fundamental similarities in basic structure and formation of tooth enamel between rat and human incisors that allows validation of this model (5).

Contrarily, in recent years due to controversy for using animals in research, alternative models have been developed to help to understand pathophysiological aspects in tooth formation. Consequently, different study models have been proposed including cellular models, which are easy to maintain in laboratories compared to animal models. Its use has been important for knowing morphological aspects, cellular division mechanisms and transport of fundamental elements at cellular levels (6). Teeth and their different cells are not distant from these advances. Study of cellular aspects that take place during tooth formation is an aid that will allow us to understand tooth development and some protein's role in this process. Therefore, employing cellular models is now an open thematic for dental research.

Recently, a dental epithelial cell model of a rat incisor, also known as rat ameloblastic cells, has been used. These epithelial cells described by Kawano et al (7) originate in cervical loop epithelium of rat incisors. According to results shown by these authors, such cells had positive expression of amelogenin and ameloblastin, enamel matrix proteins secreted during amelogenesis and involved in tooth mineralization, these proteins belong to Secretory calcium-binding PhosphoProtein family (SCPP) (8). Using dental epithelial cells of rat incisors has made it possible to confirm regulatory role of bone morphogenetic protein-2 (BMP-2) as an inducer of tooth development. Positive regulation of BMP-2 has been found in 63 genes from this cell type during the first 24 hours, including adhesion molecules, regulatory genes and extracellular matrix (ECM) present in tooth enamel (9). Other studies have shown importance of dental differentiation in vitro as a result of interactions between epithelial and mesenchymal cells and have even allowed progress in understanding ion transportation process during amelogenesis (10,11).

Studies carried out using rat ameloblastic cells as a cellular model have adopted very similar culture conditions. There are limited questioning about possible influence of different elements present in a culture medium that may affect these type

of cells. Essential culture medium replacing metabolic and functional cellular needs and vital for its survival and proliferation. Therefore, it is important to initially investigate influence of certain elements present in rat dental epithelial cells culture medium and, consequently, continue to improve a cellular model useful for understanding pathophysiological processes of ameloblasts differentiation and formation of tooth enamel.

A primary constituent in cell culture media is Fetal Bovine Serum (FBS). FBS induces growth, proliferation, and maintenance of cells, including dental cells (12). Although using FBS in cell cultures raises some scientific and ethical concerns regarding its production, this element continues to be used regularly and almost routinely due to its contribution to morphology, physiology and biochemical characteristics of cultured cells. Hence, it is imperative to know if modification of FBS concentration affects morphofunctional characteristics of rat dental epithelial cells and specifically if it has repercussions on cell organelles, such as mitochondria and lysosomes.

According to described aspects and with the purpose of improving understanding of rat dental epithelial cells as an in vitro cell model essential for knowing pathophysiological aspects involved in tooth enamel formation, this study aimed to describe influence of Fetal Bovine Serum on survival, growth, and expression of cellular organelles in rat dental epithelial cells.

MATERIALS AND METHODS

Type of study. A descriptive study was conducted using a continuous cell line of cervical loop epithelial cells from rat incisors, previously described by Kawano et al (7), grown at 37°C in a humid atmosphere containing 5% CO2 in two different culture media. First one contained Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 Gibco™ (DMEM/F12) + 10% of FBS, Gibco™ + 1% penicillin-streptomycin (10.000 U/mL) (Gibco™) and 0.5% of Amphotericin B, Gibco™. On the other hand, second one was constituted by DMEM/F12 Gibco™ FBS, 0, 1 and 0.5% penicillin-streptomycin and Amphotericin B, respectively. Subculturing was performed every one week and culture media were replaced three times per week until a confluence of 80-90% was achieved.

Morphological analysis. Morphological aspects of rat dental epithelial cells were observed during proliferation and confluence of cultured cells by a phase-contrast microscope. A nuclear counterstaining of nucleic acid was carried out in both culture media using Hoechst 33342 stain solution (Thermo Fisher Scientific), and following manufacturer's recommendations for each case.

Immunohistochemistry. Once they achieved confluence, cells were fixed for 15 minutes in a 4% paraformaldehyde solution. Before blocking non-specific binding sites for 20 minutes in a 1% (Bovine Serum Albumin) BSA/ 1% glycine solution, cells were permeabilized in 0.5% Triton (Sigma-Aldrich®). Then, primary rabbit antibodies anti-TOMM20 (ABCam) and anti-LAMP1 (ABCam) diluted 1:200, for detection and immunolabeling of mitochondria and lysosomes respectively were in contact with cells overnight at 4°C. Next day, after rinsing in PBS1x + 0.1% Triton solution, incubation was carried out using Alexa Fluor® 488 Donkey Anti-Rabbit secondary antibody (Life Technologies Corporation) for a 2 hours period. After 3 rinses, cells were incubated with Alexa Fluor™ 594 Phalloidin (Thermo Fisher Scientific) (1: 1000) for 15 minutes and following manufacturer's recommendations. Phalloidin immunolabeling allowed detecting actin filaments. Finally, a DAPI (Millipore) staining was performed and immediately mounting was carried out using Immu-Mount™ (Thermo Scientific). A Leica DM2500 LED Fluorescent microscope was used to observe all staining.

RESULTS

Morphologically, dental epithelial cells presented a circular or ovoid appearance, characterized by voluminous nuclei during proliferation and confluence. These aspects were equally observed in both rat dental epithelial cells cultured with DMEM/F12 at 10% and 0% concentration of SFB and verified by nuclear counterstaining (Figure 1, A, B).

It was found that during one week of culture, cells in absence and at 10% concentration of FBS presented a survival and non-quantifiable differential proliferation in both experimental conditions (Figure 2).

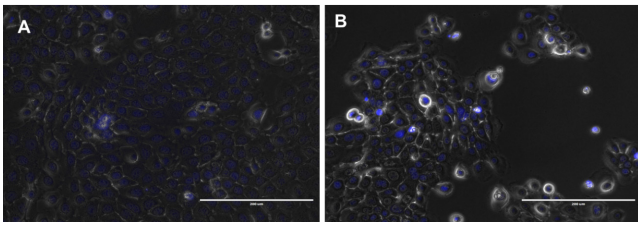

Figure 1. Against nuclear coloration of the cellular DNA in blue of the rat dental epithelial cells cultured with FBS 10% (A) and FBS 0% (B) for 96 hours. White bar: 200 µm

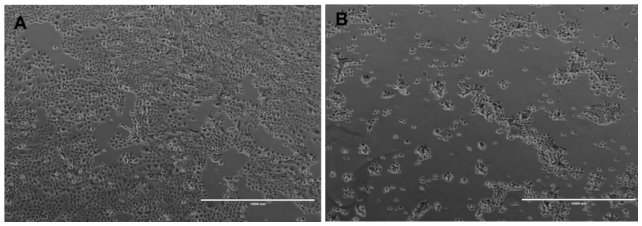

Figure 2. Monitoring of proliferation of rat dental epithelial cells cultured with FBS at a concentration of 10% (A) and 0% of FBS (B) at 96 hours. FBS: Fetal Bovine Serum. White bar: 1000 µm.

A confluence of approximately 20-30% of cultured cells in medium 1 (DMEM/F12+FBS 10%) was observed during the first 24 hours (results not shown). In culture medium 2 (DMEM/F12+FBS 0%), a decreased confluence rate of cultured cells was observed compared to cells that were in contact with medium 1 during same period of time (results not shown). After 24 hours, progressive growth of cultured rat dental epithelial cells was continued. In those cells in contact with culture condition 1, an approximate confluence of 95% could be seen after 96 hours (Figure 2, A). Meanwhile, in cells cultured in absence of FBS, confluence showed an oscillation of 50 to 60% after 96 hours (Figure 2, B). It is important to note that an imprecise number of non-adherent cells were identified in medium 2 during culture period.

Cytoskeleton and cellular organelles. Actin expression, a cytoskeleton fundamental component, was detected by phalloidin in this study. Actin immunodetection in evaluated cells was present in both culture conditions, DMEM/F12 in absence and presence of FBS in a 10% concentration (Figure 3). Observation of cytoskeleton protein complex as a result of actin expression revealed a compact filament system in those cells grown in DMEM/F12 culture medium with a 10% concentration of FBS (Figure 3, A). This same complex, although in a smaller proportion was observed in cells cultured with DMEM/F12 and in absence of FBS (Figure 3, D).

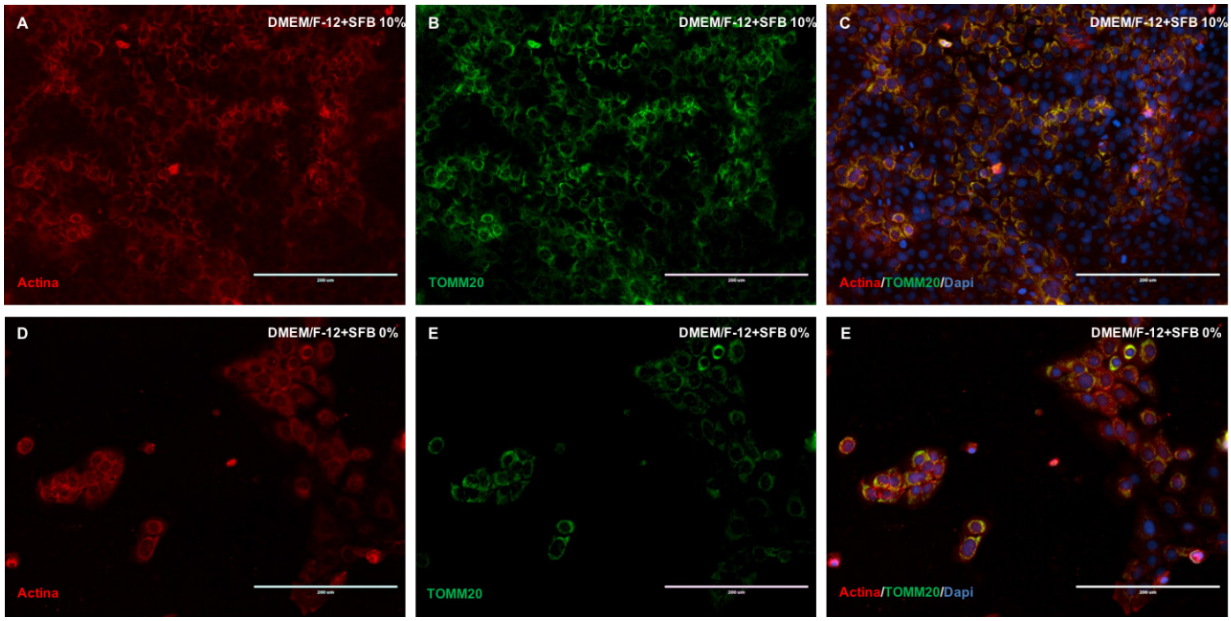

Figure 3. Immunomarking of actin and mitochondrial filaments in rat dental epithelial cells in DMEM / F12 culture media at 10% and 0% FBS. Double marking: actin (red) / mitochondria (green) grown with a concentration of 10% of FBS (A, B, C) and in the absence of FBS (D, E, F). Nucleus colored in blue. DMEM / F12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12. FBS: Fetal Bovine Serum. White bar: 200 µm

Immunolabeling of mitochondria by anti-TOMM20 allowed to verify presence of these organelles in cell cultures (Figure 3. B, E). An expression of anti-TOMM20 was present without differential patterns in dental epithelial cells cultured with medium 1 (Figure 3, B) and also in cells treated with culture medium 2 (Figure 3, E). According to microscopic observation, there were no evident changes in detection of mitochondrial labeling by anti-TOMM20 under neither of both evaluated conditions.

Lysosomal immunolabeling of rat dental epithelial cells was performed by anti-LAMP1 (Figure 4. B, E). Results revealed presence of labeled lysosomes with differential absence between rat dental epithelial cells cultured with DMEM/F12 culture medium at 10% concentration of FBS (Figure 4. B) And cells treated with DMEM/F12 culture medium in absence of FBS (Figure 4.E).

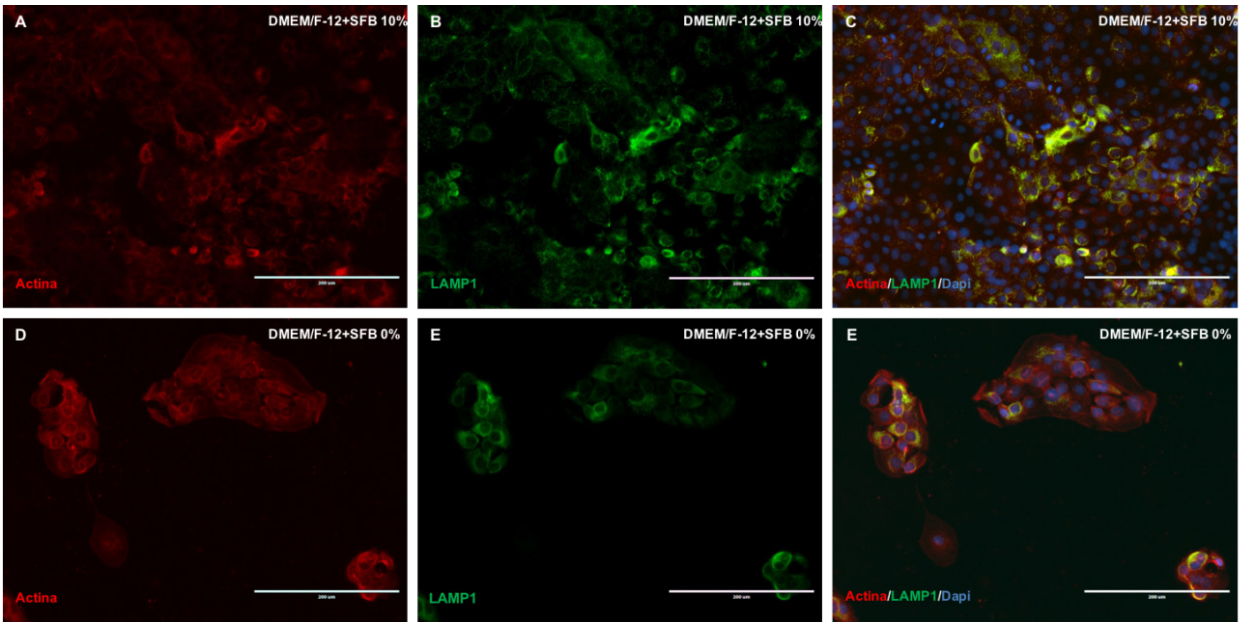

Figure 4. Expression of actin and lysosome filaments in rat dental epithelial cells in DMEM / F12 culture media at 10% and 0% FBS. Double marking: actin (red) / lysosomes (green) grown at a concentration of 10% of FBS (A, B, C) and in the absence of FBS (D, E, F). Nucleus colored in blue. DMEM / F12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12. FBS: Fetal Bovine Serum. White bar: 200 µm.

DISCUSSION

Although cell biology and biotechnology research is constantly advancing, for many decades culture media have been supplemented with FBS, a supplement used in multiple eukaryotic cell cultures. Despite that promising new synthetic media have been developed, many researchers continue to use basal culture media with SFB supplements. However, given its frequent use and critical role in cell cultures, there are a limited number of reports that indicate biological repercussions related to survival and cell markers expression of main organelles and FBS concentration in culture media. FBS is known for stimulating growth, maintenance, and cell differentiation, due to its richness in growth factors, hormones, proteins and other essential nutrients useful for cell proliferation in cultures (13).

To conduct this study, DMEM/F12 culture medium was employed due to its low immunoglobulin content and complement factors (14). Election of a null FBS concentration in culture of evaluated epithelial cells in comparison with a standard concentration widely reported in literature (10% of FBS), responded to a critical needing for knowing biological effects of FBS absence and eventually, explore their replacement in future works (7,15,16). As a relevant aspect, infrequently described in literature, we chose to study FBS influence on survival, growth and immunohistochemical expression of mitochondria

and lysosomes on rat dental epithelial cells in culture, using DMEM/F12 culture medium. Consequently, we proposed to describe FBS absence effects related to behavior, proliferation and survival of rat dental epithelial cells.

Use of FBS has been mostly questioned because of scare knowledge regarding the exact composition of its elements, variation from one batch to another and even structure of regulatory RNAs and protein-coding genes that can be absorbed by cells and generate changes in certain genetic expressions (17,18). Additionally, utilization of FBS has generated serious ethical concerns in relation to animal abuse (19). Therefore, in this last decade implementation of FBS-free culture media has been encouraged in order to eliminate problems related to reproducibility of results and animal experimentation. Many research teams have encouraged using FBS substitutes by employing them, mainly when it comes to human cell culture. Among said substitutes are some platelet derivatives: Platelet-rich plasma, platelet concentrates and human serum. As being derived from humans, they provide a culture medium free of animal components and guarantees a low percentage of protein content (20).

According to regulatory agencies such as FDA (Food and Drug Administration), employing FBS as a culture media element has not been definitively dismissed, mostly because of its importance in aspects related to cell growth and proliferation. Findings from this work, allowed us to verify that rat dental epithelial cells grown in culture medium 2, in opposition to cells grown in culture medium 1, exhibited a considerable decrease in cell proliferation and confluence. These results correlate with those presented by Wu et al (21), who analyzed viability and proliferation of corneal epithelial cells supplemented with FBS concentrations between 5% and 30%. Although these researchers did not evaluate FBS absence, they reported how cell migration was slower in cultured cells at a lower concentration (FBS 5%), compared to those that were in contact with higher concentrations (22,23).

Cytoskeleton's main component is actin, which is essential for cellular growth, proliferation, communication, degradation and renewal of its structures (24). Present work made possible to verify by actin immunostaining, a cytoskeletal framework present in rat dental epithelial cells when cultured in presence or absence of FBS, however, in those cells cultured with 10% FBS a more compact and dense filamentous system was notorious compared to cells grown without FBS. This phenomenon leads us to suppose that in cells cultured with FBS deprivation less adhesion and proliferation occurred, possibly due to decreased integrins and not because of pH stabilizing elements (25).

PH stability in cell culture is given by media ability to directly or indirectly inhibit proteases. Direct inhibition is generated by blocking α1-Antitrypsin and α2-Macroglobulin, and indirect inhibition is done by nonspecific blocking of proteases (25,26). Maintenance of pH can also be established through buffer systems, including a natural one, offered by a CO2 content between 5-10% (used in this study), and another system generated by using a zwitterion, known as a chemical buffer (27). DMEM/F12 culture medium, used this study, incorporated 15 mm of HEPES, a zwitterionic buffer containing a 7.3 pKa at 37 °C, allowing maintenance of a 7.00-7.60 pH in cell culture.

Immunohistochemical expression of mitochondria using translocase of the outer membrane subunit 20 (TOMM20), allowed to verify that absence of FBS apparently does not have an impact on structure and function executed by these organelles, among them are, generation of energy, metabolism of amino acids and nucleotides, as well as regulation of intercellular communication (28). Regarding lysosomal labeling, results indicate that there was no alteration in immunolabelling in rat epithelial cells cultured in absence of FBS. This causes us to think that absence of FBS in culture of these cells does not affect cell homeostasis and macromolecules degradation processes, functions performed by lysosomes. Likewise, results indicated that there was probably no disturbance of lysosomes, consequently avoiding an indiscriminate release of proteolytic enzymes that could induce autophagy or apoptotic processes in rat epithelial cells.

Results of this study showed that absence of FBS does not impact organelles functions involved in cell homeostasis. It is possible to consider that rat dental epithelial cells are an in vitro cellular model that can be cultured even in absence of FBS without risk of phenotypic alterations other than their proliferation rate. However, it is pertinent to continue evaluating cell changes resulting from rat dental epithelial cells when concentration of FBS varies, such evaluations can be performed by proteomic, electron microscopy and scanning electron microscopy studies.

In conclusion, absence of FBS in culture of rat dental epithelial cells reduced survival and proliferation, but it suggests not having a repercussion on mitochondria and lysosomes of evaluated cells.

Conflict of interests

None, declared by the authors.

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